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# Embryonic Stem Cells: Prospects for Developmental Biology and Cell Therapy

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**Wobus, Anna M., and Kenneth R. Boheler.** Embryonic Stem Cells: Prospects for Developmental Biology and Cell Therapy. *Physiol Rev* 85: 635–678, 2005; doi:10.1152/physrev.00054.2003.—Stem cells represent natural units of embryonic development and tissue regeneration. Embryonic stem (ES) cells, in particular, possess a nearly unlimited self-renewal capacity and developmental potential to differentiate into virtually any cell type of an organism. Mouse ES cells, which are established as permanent cell lines from early embryos, can be regarded as a versatile biological system that has led to major advances in cell and developmental biology. Human ES cell lines, which have recently been derived, may additionally serve as an unlimited source of cells for regenerative medicine. Before therapeutic applications can be realized, important problems must be resolved. Ethical issues surround the derivation of human ES cells from in vitro fertilized blastocysts. Current techniques for directed differentiation into somatic cell populations remain inefficient and yield heterogeneous cell populations. Transplanted ES cell progeny may not function normally in organs, might retain tumorigenic potential, and could be rejected immunologically. The

number of human ES cell lines available for research may also be insufficient to adequately determine their therapeutic potential. Recent molecular and cellular advances with mouse ES cells, however, portend the successful use of these cells in therapeutics. This review therefore focuses both on mouse and human ES cells with respect to in vitro propagation and differentiation as well as their use in basic cell and developmental biology and toxicology and presents prospects for human ES cells in tissue regeneration and transplantation.

## I. INTRODUCTION

Several seminal discoveries during the past 25 years can be regarded not only as major breakthroughs for cell and developmental biology, but also as pivotal events that have substantially influenced our view of life: *1*) the establishment of embryonic stem (ES) cell lines derived from mouse (108, 221) and human (362) embryos, *2*) the creation of genetic mouse models of disease through homologous recombination in ES cells (360), *3*) the reprogramming of somatic cells after nuclear transfer into enucleated eggs (392), and *4*) the demonstration of germ-line development of ES cells in vitro (136, 164, 365). Because of these breakthroughs, cell therapies based on an unlimited, renewable source of cells have become an attractive concept of regenerative medicine.

Many of these advances are based on developmental studies of mouse embryogenesis. The first entity of life, the fertilized egg, has the ability to generate an entire organism. This capacity, defined as totipotency, is retained by early progeny of the zygote up to the eight-cell stage of the morula. Subsequently, cell differentiation results in the formation of a blastocyst composed of outer trophoblast cells and undifferentiated inner cells, commonly referred to as the "inner cell mass" (ICM). Cells of the ICM are no longer totipotent but retain the ability to develop into all cell types of the embryo proper (pluripotency; Fig. 1). The embryonic origin of mouse and human ES cells is the major reason that research in this field is a topic of great scientific interest and vigorous public debate, influenced by both ethical and legal positions.

ES cell research dates back to the early 1970s, when embryonic carcinoma (EC) cells, the stem cells of germ line tumors called teratocarcinomas (344), were established as cell lines (135, 173, 180; see Fig. 2). After transplantation to extrauterine sites of appropriate mouse strains, these "funny little tumors" produced benign teratomas or malignant teratocarcinomas (107, 345). Clonally isolated EC cells retained the capacity for differentiation and could produce derivatives of all three primary germ layers: ectoderm, mesoderm, and endoderm. More importantly, EC cells demonstrated an ability to participate in embryonic development, when introduced into the ICM of early embryos to generate chimeric mice (232). EC cells, however, showed chromosomal aberrations (261), lost their ability to differentiate (29), or differentiated in vitro

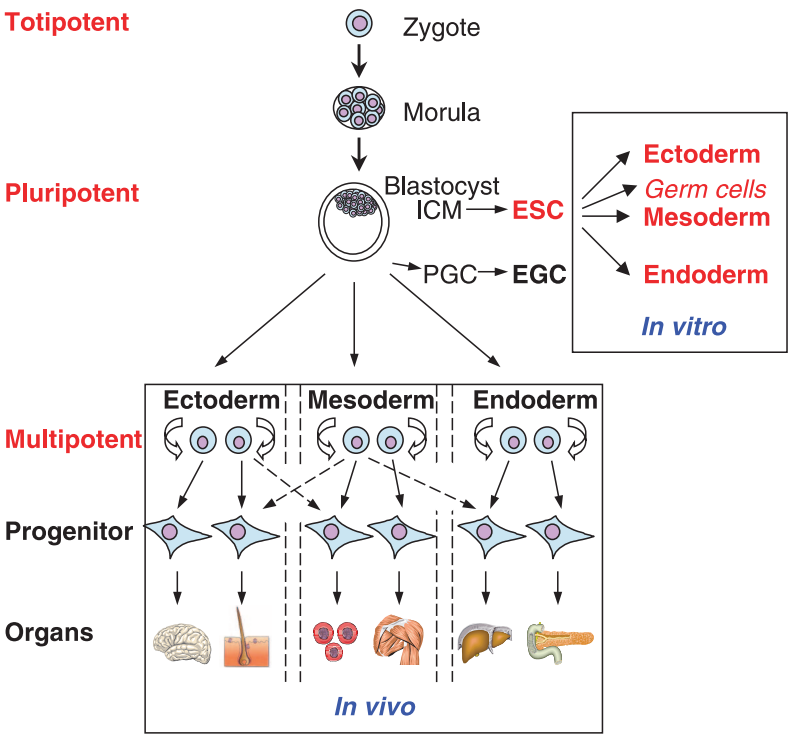


FIG. 1. Stem cell hierarchy. Zygote and early cell division stages (blastomeres) to the morula stage are defined as totipotent, because they can generate a complex organism. At the blastocyst stage, only the cells of the inner cell mass (ICM) retain the capacity to build up all three primary germ layers, the endoderm, mesoderm, and ectoderm as well as the primordial germ cells (PGC), the founder cells of male and female gametes. In adult tissues, multipotent stem and progenitor cells exist in tissues and organs to replace lost or injured cells. At present, it is not known to what extent adult stem cells may also develop (transdifferentiate) into cells of other lineages or what factors could enhance their differentiation capability (dashed lines). Embryonic stem (ES) cells, derived from the ICM, have the developmental capacity to differentiate in vitro into cells of all somatic cell lineages as well as into male and female germ cells.

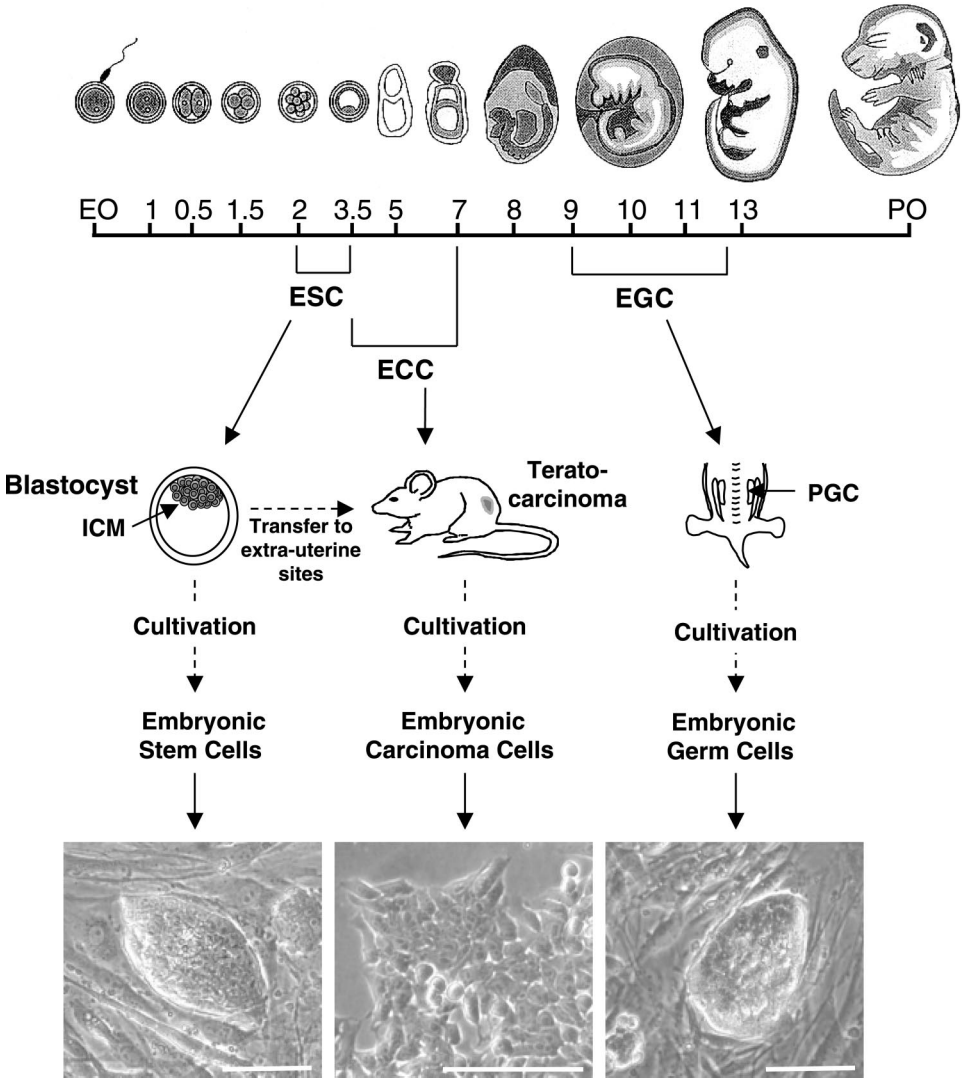


FIG. 2. Developmental origin of pluripotent embryonic stem cell lines of the mouse. The scheme demonstrates the derivation of embryonic stem cells (ESC), embryonic carcinoma cells (ECC), and embryonic germ cells (EGC) from different embryonic stages of the mouse. ECC are derived from malignant teratocarcinomas that originate from embryos (blastocysts or egg cylinder stages) transplanted to extrauterine sites. EGC are cultured from primordial germ cells (PGC) isolated from the genital ridges between embryonic day *9* to *12.5*. Bar = 100 $\mu$m. [From Boheler et al. (40).]

only under specialized conditions (248) and with chemical inducers (224). Maintenance of the undifferentiated state relied on cultivation with feeder cells (222), and after transfer into early blastocysts, EC cells only sporadically colonized the germ line (232). These data suggested that the EC cells did not retain the pluripotent capacities of early embryonic cells and had undergone cellular changes during the transient tumorigenic state in vivo (for review, see Ref. 7).

To avoid potential alterations connected with the growth of teratocarcinomas, a logical step was the direct in vitro culture of embryonic cells of the mouse. In 1981, two groups succeeded in cultivating pluripotent cell lines from mouse blastocysts. Evans and Kaufman employed a feeder layer of mouse embryonic fibroblasts (108), while Martin used EC cell-conditioned medium (221). These cell lines, termed ES cells, originate from the ICM or epiblast and could be maintained in vitro (Fig. 2) without any apparent loss of differentiation potential. The "pluripotency" of these cells was demonstrated in vivo by the introduction of ES cells into blastocysts. The resulting mouse chimeras demonstrated that ES cells could contribute to all cell lineages including the germ line (46). In vitro, mouse ES cells showed the capacity to reproduce the various somatic cell types (98, 108, 396) and, only recently, were found to develop into cells of the germ line (136, 164, 365). The establishment of human ES cell lines from in vitro fertilized embryos (362) (Fig. 3) and the demonstration of their developmental potential in vitro (322, 362) have evoked widespread discussions concerning future applications of human ES cells in regenerative medicine.

Primordial germ (PG) cells, which form normally within the developing genital ridges, represent a third embryonic cell type with pluripotent capabilities. Isolation and cultivation of mouse PG cells on feeder cells led to the establishment of mouse embryonic germ (EG) cell lines (198, 291, 347; Fig. 2). In most respects,

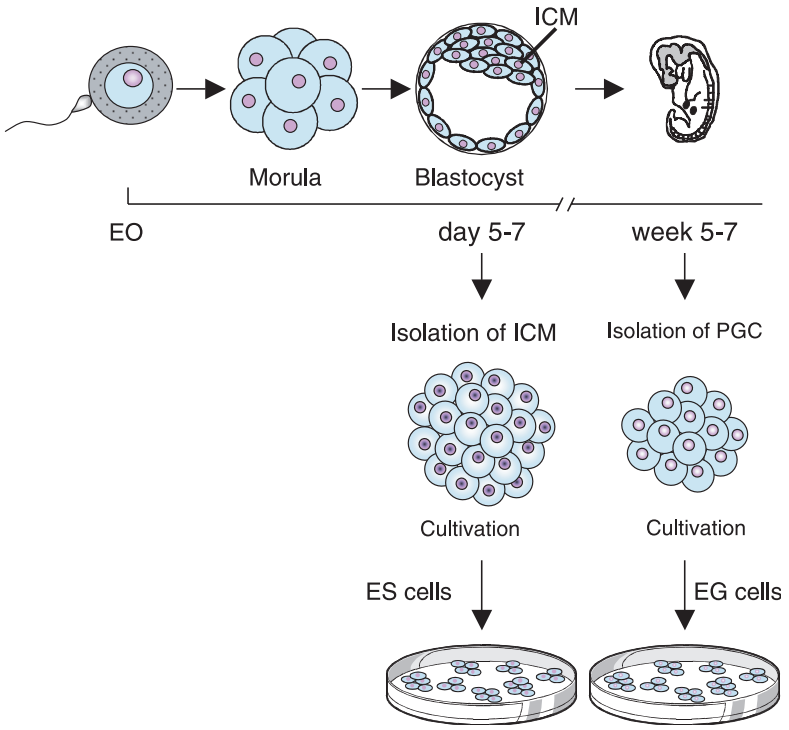


FIG. 3. Human pluripotent embryonic stem (ES) and embryonic germ (EG) cells have been derived from in vitro cultured ICM cells of blastocysts (after in vitro fertilization) and from primordial germ cells (PGC) isolated from aborted fetuses, respectively.

these cells are indistinguishable from blastocyst-derived ES cells and are characterized by high proliferative and differentiation capacities in vitro (310), and the presence of stem cell markers typical of other embryonic stem cell lines (see sect. II). Once transferred into blastocysts, EG cells can contribute to somatic and germ cell lineages in chimeric animals (197, 223, 347); however, EG cells, unlike ES cells, retain the capacity to erase gene imprints. The in vitro culture of PG cells from 5- to 7-wk-old human fetuses led to the establishment of human EG cell lines (326) (Fig. 3). These cell lines showed multilineage development in vitro but have a limited proliferation capacity, and currently can only be propagated as embryoid body (EB) derivatives (325). Following transplantation into an animal model for neurorepair, human EG cell derivatives, however, show some regenerative capacity, suggesting that these cells could be useful therapeutically (190). Although pluripotent EG and EC cells represent important in vitro models for cell and developmental biology, this review focuses mainly on fundamental properties and potential applications of mouse and human ES cells for stem cell research.

## II. PROPERTIES OF UNDIFFERENTIATED EMBRYONIC STEM CELLS

### A. Mouse ES Cell Lines

Mouse ES (mES) cell lines were first established in the early 1980s (17, 98, 108, 221, 396). Initially, this required the isolation and cultivation of preimplantation embryos (blastocysts) on mouse embryonic fibroblasts (MEFs), followed by the expansion of primary ES cell outgrowths through careful enzymatic dissociation (trypsin/EDTA) and subculture regimes (see Ref. 301). The efficiency of ES cell derivation proved strain dependent, and inbred mice, like the 129 mouse strain, demonstrated the highest rates of success for the generation of ES cells (321). Once established, murine ES cell lines displayed an almost unlimited proliferation capacity in vitro (review in Ref. 333) and retained the ability to contribute to all cell lineages. In vitro, mES cells maintained a relatively normal and stable karyotype, even with continued passaging. ES cells were also characterized by a relatively short generation time of ~12–15 h with a short $G_1$ cell cycle phase (310).

Because the generation of ES cell lines initially required a monolayer of inactivated MEFs, it was reasoned that fibroblasts provided some critical factor(s) either to promote self-renewal or to suppress differentiation. Two groups independently identified leukemia inhibitory factor [LIF (391); identical to the "differentiation inhibitory activity" DIA (334)] as the trophic factor responsible for this activity. LIF is a soluble glycoprotein of the interleukin (IL)-6 family of cytokines, which acts via a membrane-bound gp130 signaling complex to regulate a variety of cell functions through signal transduction and activation of transcription (STAT) signaling (review in Ref. 59). These cytokines, including IL-6, IL-11, oncostatin M (OSM), ciliary neurotrophic factor (CNTF) and cardiotrophin-1 (CT-1), all show similar properties with respect to

the maintenance of pluripotency of mES cells (57, 250). The absence of IL-6 family members, the removal of MEFs, or the inactivation of STAT3, a downstream signaling molecule of the gp130 signaling complex, promote ES cells to spontaneously differentiate in vitro (39).

Studies on hematopoietic stem cell expansion had suggested that ligand-receptor complex thresholds of soluble cytokines could be maintained by high concentrations of soluble cytokines or by cytokine presentation on the cell surface. According to this model, when a relevant ligand-receptor interaction falls below a certain threshold, the probability of differentiation is increased; otherwise, self-renewal is favored. Examination of ES cells over a range of LIF concentrations demonstrated that LIF supplementation had little effect on growth rates, but it significantly altered the probability of cells undergoing self-renewal versus differentiation (414). To further address this question, a designer cytokine (a fusion protein of sIL6/sIL-6R linked to a flexible peptide chain) called Hyper-IL-6 (HIL-6) (118) together with LIF were employed to experimentally and computationally test their capacity to sustain ES cell self-renewal. Quantitative measurements of ES cell phenotypic markers, functional assays (EB formation), and transcription factor (Oct-3/4) expression over a range of LIF and HIL-6 concentrations demonstrated a superior ability of LIF to maintain ES cell pluripotentiality at higher concentrations ($\geq$500 pM). These results supported a ligand/receptor signaling threshold model of ES cell fate modulation that requires appropriate types and levels of cytokine stimulation to maintain self-renewal (375).

Identification of cell surface and molecular markers has proven critical to define the molecular basis of stem cell identity or "stemness." It is now well established that undifferentiated mES cells express specific cell surface antigens (SSEA-1; Ref. 336) and membrane-bound receptors (gp130; Refs. 57, 250) and possess enzyme activities for alkaline phosphatase (ALP; Ref. 396) and telomerase (review in Refs. 11, 277; see Table 1). ES cells also contain the epiblast/germ cell-restricted transcription factor Oct-3/4 (268, 318). In vivo, zygotic expression of this POU domain containing transcription factor is essential for the initial development of pluripotentiality in the ICM (247). In ES cells, continuous Oct-3/4 function at appropriate levels is necessary to maintain pluripotency. A less than twofold increase in expression causes differentiation into primitive endoderm and mesoderm, whereas loss of Oct-3/4 induces the formation of trophectoderm concomitant with a loss of pluripotency (251; see Fig. 4).

Recently, two groups identified the homeodomain protein Nanog as another key regulator of pluripotentiality (73, 233). In preimplantation embryos, its expression is restricted to and required in epiblast cells from which ES cells can be derived. The dosage of Nanog is a critical determinant of cytokine-independent colony formation, and forced expression of this protein confers constitutive self-renewal in ES cells without gp130 stimulation. Nanog may therefore act to restrict the differentiation-inducing potential of Oct-3/4.

Both Nanog and Oct-3/4 are essential to maintain ES cell identity, but STAT3, following LIF activation, plays an accessory role. LIF, when applied to serum-free ES cell cultures, is insufficient to maintain pluripotency or block (neural) differentiation. In combination with bone morphogenetic protein (BMP), LIF sustains self-renewal, multilineage differentiation, chimera colonization, and germ-

TABLE 1. *Comparison of some properties of mouse and human embryonic stem cells*

| Marker | Mouse ES Cells | Human ES Cells | Reference Nos. |
|---|---|---|---|
| SSEA-1 | + | − | 336 |
| SSEA-3/-4 | − | + | 151, 293, 362, 401 |
| TRA-1-60/81 | − | + | 151, 293, 362, 401 |
| TRA-2-54 | − | + | 151 |
| GCTM-2 | − | + | 265, 293 |
| TG 343 | ? | + | 151 |
| TG 30 | ? | + | 265 |
| CD 9 | + | + | 265 |
| CD133/prominin | + | + | 70, 183 |
| Alkaline phosphatase | + | + | 362, 396 |
| Oct-4 | + | + | 268, 362 |
| Nanog | + | + | 73, 233 |
| Sox-2 | + | + | 16, 138 |
| FGF4 | + | − | 138 |
| LIF receptor | + | +/− | 296 |
| Telomerase activity | + | + | 11, 362 |
| Regulation of self-renewal | Via gp 130 receptors, MEF feeder layer, Nanog, BMP-4 | Feeder cells (MEF or human cells), serum, bFGF, Matrigel | 73, 250, 362, 401, 410 |
| Growth characteristics in vitro | Tight, rounded, multilayer clusters | Flat, loose aggregates | 362 |
| EB formation | Simple and cystic EBs | Cystic EBs | 98, 168, 362 |
| Teratoma formation in vivo | + | + | 362, 396 |

MEF, mouse embryonic fibroblasts; EB, embryoid body.

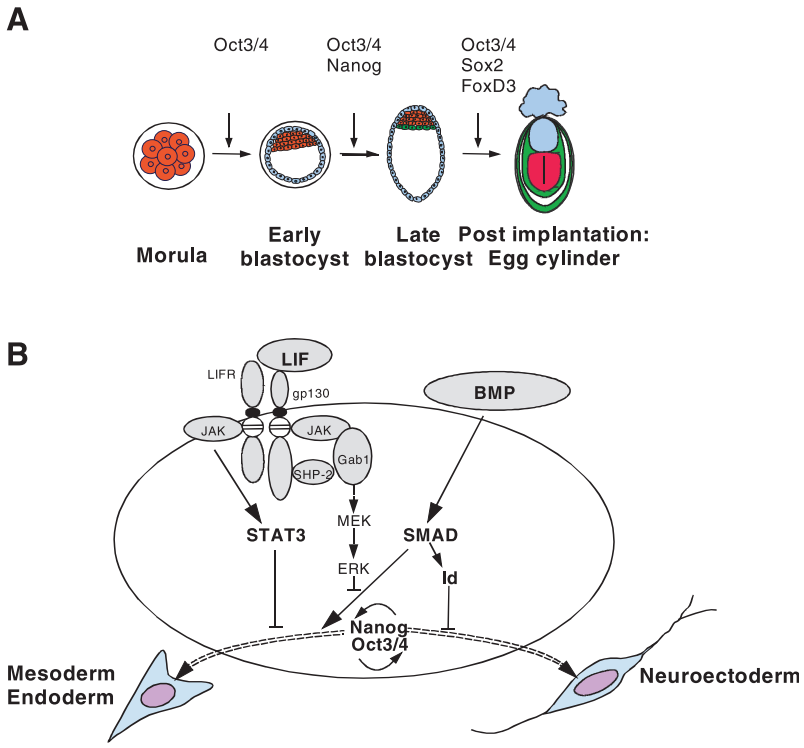


FIG. 4. Regulation of self-renewal in mouse ES cells by Oct3/4, Nanog, BMP-dependent SMAD, and LIF-dependent JAK/STAT3 signaling pathways. *A*: transcription factors, such as Oct3/4, Nanog, Sox2, and FoxD3, control early developmental stages from totipotent to pluripotent developmental stages. *B*: self-renewal (proliferation) of undifferentiated mouse ES cells is regulated by Nanog, Oct-3/4, and tightly regulated interactions between LIF-dependent JAK/STAT3 pathway(s) and BMP-dependent activation of Id target genes. A MEK-ERK signaling mechanism prevents ES cell self-renewal. Oct-3/4 and Nanog expression prevents differentiation into trophectoderm, primitive endoderm, and mesoderm cells. *C*: the relative expression level of Oct-3/4 determines the fate of ES cells. [Adapted from Cavaleri and Schöler (71), Ying et al. (410), and Niwa et al. (251).]

line transmission properties. The critical contribution of BMP is to induce expression of *Id* ("inhibitor of differentiation") genes via the Smad pathway. Forced expression of *Id* genes liberates ES cells from BMP or serum dependence and allows self-renewal in LIF alone. Blockade of lineage-specific transcription factors by Id proteins enables the self-renewal response to LIF/STAT3 signaling (410). MEK/ERK signaling is also involved in ES cell self-renewal and differentiation. Inhibition of MEK/ERK by the MEK inhibitor PD098059 inhibits differentiation and maintains ES cell self-renewal in culture, and the expression of ERK and SHP-2 is thought to counteract the proliferative effects of STAT3 and promote differentiation (review in Refs. 58, 59). It however remains currently unclear how this pathway interacts with Nanog, Oct-3/4, and LIF signaling to regulate pluripotentiality (see Fig. 4).

Finally, a recent study has implicated Wnt-signaling pathways in the maintenance of ES cell pluripotency. Wnt pathway activation by a specific pharmacological inhibitor (BIO; 6-bromoindirubin-3′-oxime) of glycogen synthase kinase-3 (GSK-3) maintains the undifferentiated phenotype in both mouse and human ES cells and sustains expression of the pluripotent stage-specific transcription factors Oct-3/4 and Nanog (314). The reversibility of the BIO-mediated Wnt-activation in hES cells also suggests a practical application of GSK-3-specific inhibitors to regulate early steps of differentiation, which may prove valuable for the derivation of cells suitable for regenerative medicine.

The ES cell property of self-renewal therefore depends on a stoichiometric balance among various signaling molecules, and an imbalance in any one can cause ES cell identity to be lost. Other molecular markers potentially defining pluripotentiality include Rex-1 (163, 304), Sox2 (16), Genesis (353), GBX2 (75), UTF1 (254), Pem (112), and L17 (303). All of these have been shown to be

expressed in the ICM of blastocysts and are downregulated upon differentiation; however, they are not exclusively expressed by pluripotent embryonic stem cells and can be found in other cell types in the soma. Their potential role in maintaining pluripotentiality or self-renewal remains to be determined.

### B. Human ES Cell Lines

The techniques used to isolate and culture murine ES cells proved critical to the generation of human (h) ES cell lines from preimplantation embryos produced by in vitro fertilization (265, 293, 362) and after in vitro culture of blastocysts (349) (see Fig. 3). The resulting hES cells shared some fundamental characteristics of murine lines, such as Oct-3/4 expression, telomerase activity, and the formation of teratomas containing derivatives of all three primary germ layers in immunodeficient mice (295, 362). Similar to mES cells, hES cells maintained proliferative potential for prolonged periods of culture and retained a normal karyotype even in clonal derivatives (4). In contrast to mES cells, hES cells formed mainly cystic EBs (168) and displayed proteoglycans (TRA-1–60, TRA-1–81, GCTM-2) and different subtypes of stage-specific antigens (SSEA-3, SSEA-4), which were absent from mouse ES cell lines (Table 1).

Several potentially important differences exist between mouse and human ES cells. hES cells show a longer average population doubling time than mES cells [~30–35 h vs. 12–15 h (4)]. With murine cells, it is possible to substitute the feeder layer of embryonic fibroblasts with recombinant LIF, which signals through the gp130 receptor subunit to activate STAT3 (see above and Fig. 4). In contrast, LIF is insufficient to inhibit the differentiation of hES cells (293, 362), which continue to be cultured routinely on feeder layers of MEFs or feeder cells from human tissues. The identity of the essential self-renewal signal(s) provided to ES cells by MEF feeder cells remains ill defined. Despite the recent finding of a functional activation of the LIF/STAT3 signaling pathways in hES cells, LIF is unable to maintain the pluripotent state of hES cells (91). The cultivation of hES cells on extracellular matrix proteins, such as Matrigel (a complex mixture of ECM proteins isolated from Engelbreth-Holm-Swarm tumor) and laminin with MEF-conditioned media (401), causes hES cells to express high levels of $\alpha_6$- and $\beta_1$-integrins, which are involved in cell interactions with laminin (401). These results show that the application of extracellular matrix-associated factors can be employed to improve the culture and maintenance of pluripotent hES cells.

At the end of 2001, ~70 hES lines had been established using feeder layers of mouse embryonic fibroblasts. This panel of cells, however, suffers from significant limitations, including possible murine retrovirus infections (from the feeder cells) that have rendered them inappropriate for therapeutic applications. As of December 2004, only 22 of the cell lines listed in the NIH register have been successfully propagated in vitro [see update of December 10, 2004 in (http://escr.nih.gov/)], and although 17 karyologically normal (euploid) hES cell lines derived from human blastocysts were recently generated that could be subcultured by enzymatic dissociation (87), these cells were also established on MEFs. Importantly, hES cell lines have now been cultivated both on human feeder cells to avoid xenogenic contamination (5, 295) and in the absence of feeder cells under serum-free conditions (205) as had been previously done for mES cells (411). These technological advances suggest that new hES cell lines free from potential retroviral infections will be prepared and that these cells, unlike most of those currently available, might be suitable for eventual therapeutic applications.

Although the principle techniques necessary to culture (up to 80 and more passages) and manipulate hES cells have been established [cell cloning (4), cryo-preservation (294), transfection (104), and gene targeting by homologous recombination (419)], other methods (single-cell dissociation and proliferation) are still not yet optimal. Because of the variabilities among human ES cell lines (growth characteristics, differentiation potential, and culturing techniques), it will be important to define a reliable set of molecular and cellular markers that characterize the undifferentiated pluripotent (stemness) or differentiated state of hES cells. Recent attempts to define molecular markers of undifferentiated cells, however, indicate a high degree of variability among four hES cell lines maintained in a feeder-free culture system (70) and examined after long-term culture (312).

Several properties and molecular markers of hES cells are listed in Tables 1 and 2, but it is evident that the present data do not allow an unambiguous molecular definition of pluripotent stem cell properties. The application of transcriptome profiling with proteomic analyses to ES cell lines may prove useful to define which lines and growth conditions are optimal for human ES cells in vitro (see sect. VI). This information will also be necessary to set standards for hES cell research (see Ref. 52) and to answer the question, how many ES cells are necessary for research and medical applications (for further information on properties of specific hES cell lines, their cultivation, and differentiation abilities, see Ref. 79).

### C. ES Cells of Other Species

Pluripotent stem cell lines have been generated from livestock (review in Ref. 277) and model organisms, such as chicken (74, 258), hamster (97), rabbit (142, 320), and rat (51, 56, 166, 372); however, only mouse and chicken

TABLE 2. *Molecular markers of human ES cells*

| GenBank | Unigene | Gene | Reference Nos. |
|---|---|---|---|
| NM_002701 | Hs.2860 | Oct-3/4 | 82, 151, 293, 314, 362, 401 |
| NM_003212 | Hs.75561 | Tdgf1 (Cripto) | 52 |
| L07335 | Hs.816 | Sox2 | 151 |
| NM_003240 | Hs.25195 | LeftyA | 52 |
| AL558479 | Hs.125359 | Thy-1 cell surface antigen | 151 |
| BF510715 | Hs.1755 | FGF4 | 151 |
| NM_009556 | Hs.335787 | Rex-1 (Zfp-42) | 151 |
| NM_001001553.1 | Hs.528118 | Stellar | 82 |
| NM_001351 | Hs.1618 | Dazl | 82 |
| NM_024865 | Hs.79923 | Nanog | 82 |
| NM_199461 | Hs.340719 | Nanos | 82 |
| NM_014676 | Hs.9698 | Pum1 | 82 |
| NM_015317 | Hs.23369 | Pum2 | 82 |
| NM_020634 | Hs.9573 | Gdf3 | 82 |

Dazl, deleted in azoospermia like; Stellar, Stella-related; Pum, Pumilio homolog (*Drosophila*); Gdf, growth and differentiation factor.

ES cells have proven capable of colonizing the germ line. Of special importance for human stem cell research is the establishment of ES cell lines from nonhuman primates [rhesus monkey (263, 363), common marmoset (*Callithrix jacchus*, Ref. 364), and cynomolgus monkey (*Macaca fascicularis*, Ref. 352)]. Monkey ES cells, characterized by typical markers of human ES and EC cells (Oct-4, SSEA-4, TRA-1–60, TRA-1–81), retain a normal karyotype and have a high differentiation capacity in vitro (187, 363). These properties may qualify these cell lines as alternative and substitute model systems for hES cell lines. Moreover, after in vivo parthenogenetic development of *Macaca fascicularis* eggs to blastocyst-stage embryos, a pluripotent monkey stem cell line (Cyno-1) has been established that showed all the properties of hES cells, such as high telomerase and ALP activity; expression of Oct-3/4, SSEA-4, TRA 1–60, and TRA 1–81; and the ability to differentiate into various cell lineages (377). Specifically parthenogenesis is the process whereby a single egg can develop without the presence of the male counterpart.

These results suggest that stem cells derived from parthenogenetically activated eggs may also provide a potential source for autologous therapy (in the female), thus bypassing the need for creating embryos. However, aberrant expression of imprinted genes, either increased expression of maternally imprinted genes or reduced expression of paternally imprinted genes, may limit the usefulness of parthenogenetic lines and their derivatives due to their abnormal or diminished proliferative capabilities (152).

## III. GENETIC MANIPULATION OF EMBRYONIC STEM CELLS

Cell biology-based techniques have proven critical to the early isolation of ES cells and the subsequent delineation of differentiation protocols (see sect. IV). Except for neurogenesis, in vitro differentiation has required an initial aggregation step with formation of EBs before specialized cell types form in vitro. Two impediments initially prevented the full potential of the in vitro ES cell model from being realized. *1*) We knew relatively little about differentiation pathways in culture and how these pathways compared with those in the developing embryo, and *2*) differentiation protocols resulted in the simultaneous production of heterogeneous cell populations, thus constraining studies on selected subsets of cells. To overcome these limitations, genetic tools have proven indispensable to the study of ES cells and their progeny, both in vitro and in vivo. The capacity of ES cells to be clonally expanded permits the identification of independent and stable integration events (301), and a number of technologies have been developed to rapidly generate stably transfected ES cell clones and transgenic mouse models.

DNA can be introduced into ES cells by conventional infection, transfection, or electroporation protocols (66, 67). Random insertion events have been employed to overexpress, mutate, and tag genes in phenotype-driven screens, and the discovery that DNA (cloned or genomic) introduced into ES cell lines can undergo homologous recombination at specific chromosomal loci has revolutionized our ability to study gene function. The ability to introduce virtually any mutation into the genome following gene targeting in mouse ES cells provides a powerful approach for elucidating gene function both in vitro and in the whole animal. ES cell progeny can therefore be biased into a desired cell lineage by exposure to appropriate differentiation factors and by genetic manipulations of key developmental genes. Recent advances have shown that hES cells are also amenable to genetic manipulation, thus opening the door to genetic analysis of human development and disease in vitro (104, 202, 419).

### A. Random Transgenesis

Random transgenesis results in the indiscriminate incorporation of DNA within the genome. The use of sequences that confer antibiotic resistance (e.g., neomycin, puromycin, hygromycin, and herpes simplex virus thymidine kinase) for clonal selection or of reporter genes [e.g., green fluorescent protein (GFP/EGFP), LacZ ($\beta$−galactosidase)] to identify specific cell lineages has greatly facilitated this approach both in vitro and in vivo (140). Additional constructs have been designed to overexpress transcription factors (e.g., GATA4, Twist), signaling molecules (e.g., insulin-like growth factor II, Cripto), or cellular proteins in differentiated phenotypes of myogenic (95, 278, 308), erythroid (150), pancreatic (38), and cardiomyocytic (262) cell lineages. Promoters of either viral or mammalian origin have, however, often proven incon-

sistent in the formation of stably expressing ES cell clones.

Retroviral vectors have been used for the delivery of genetic material into cells for over 20 years. The advantage of a retroviral system is that genetic sequences can easily, efficiently, and permanently be introduced into target cells. In fact, the first successful reports of genetic manipulation of ES cells involved retroviral vectors. These early experiments demonstrated that integrated viruses (provirus) could be transmitted through the germ line (300, 348); however, sustained transgene expression from integrated proviruses proved difficult to achieve. ES cells have high de novo cytosine methylation at CpG dinucleotides, which effectively represses gene expression regulated from viral long-terminal repeats (LTRs) (28, 171, 348). In addition, provirus gene silencing is mediated by *trans*-acting factors that bind to the LTRs of some viral promoters (76, 260). The lack of significant provirus transcription in ES cells and ES cell progeny have effectively limited the use of simple retroviral vectors in experiments of random transgenesis (300).

The development of more complex lentiviral vectors, based on the human, feline, equine, or simian immunodeficiency viruses (246, 255, 274, 317), offer several advantages over other retroviruses (for review of vectors, see Ref. 282). Lentiviruses infect both dividing and nondividing cells, and transgene expression is not silenced in ES cells. Pfeifer et al. (271) furthermore demonstrated that lentiviral vectors could efficiently transduce human ES cells, and subsequent analyses have shown that lentivirus infections are highly effective for the delivery of functional transgenes into human ES cells (143, 214). Importantly, transgene expression is not "shut off" during differentiation in vitro (EBs) or in vivo (teratomas), and functional transgenes can be successfully passed through the germ line without loss of expression (271). These proof-of-principle experiments, with reporter constructs, demonstrate that lentiviruses are capable of foreign gene transfer to hES cells. This is particularly important, because electroporation, which has served as the main method for the introduction of foreign DNA into murine ES cells (331, 360), adversely affects the survival of hES cells (104). Lipofection-based transfection techniques, similarly, show transfer efficiency rates in hES cells that are generally $<$10% (104). Lentiviral delivery of foreign DNA to hES cells therefore has significant relevance for the isolation of stably transfected hES cell clones and for the future development of gene- and cell-based therapies.

Random integration of DNA plasmid constructs containing tissue-restricted promoters has been used extensively to purify or mark cells, including neurons (210), pancreatic $\beta$-cells (338), cardiomyocytes (192), and endothelial cells (220, 281); however, data from these studies should be interpreted with care. In vitro expression is not always consistent with in vivo analyses. For example, vimentin, which is usually restricted to mesenchymal cells in vivo (84, 125), is expressed in most cell types in vitro (126). The myosin light chain 2v (Mlc2v) promoter has also been used to identify ventricular chamber myocytes derived from differentiating ES cells in vitro (230), but this "specific" expression is only apt for adult rodent heart. During development, this gene is expressed in the anterior (atrial and atrio-ventricular) portions of the heart tube, and at later stages, in the caval myocardium (81, 123, 124). Since ES cell-derived cardiomyocytes are not typical of adult myocardium, the Mlc2v promoter probably cannot be used to identify purely ventricular myocytes. It is therefore essential that in vitro results be analyzed in conjunction with developmental models before deciding which ES cell progeny are most useful for cellular therapeutics. Finally, integration-dependent events can adversely affect gene expression in ES cells. As with pronuclear injection, the location of integration and the number of copies of integrated DNA can affect transgene expression. In particular, transgenes randomly introduced into ES cell lines tend to be progressively silenced, resulting in mosaic expression, heterogeneous phenotypes, or complete silencing. These limitations have restricted the use of random transgenesis in functional studies of ES cells and their progeny.

### B. Gene Targeting

Targeting approaches that selectively modify endogenous genes have generally proven more powerful than random transgenesis in generating mutations in endogenous mouse genes. In 1987, Thomas and Capecchi (360) first showed that transfected DNA could integrate into the mES cell genome via homologous recombination. In 1989, the first report of germ-line transmission of a targeted allele was published (361), demonstrating that genetically modified ES cells could contribute in the developing mouse embryo to produce viable chimeras. Today the production of germ-line chimeras is a standard procedure for many laboratories, and the topic has been extensively reviewed in the literature (47, 179).

The ability to produce mice that carry altered genomic DNA has greatly facilitated the study of many biological processes; however, not all biological processes can be studied by gene inactivation. Gene-targeting that results in developmental arrest or embryonic lethality in vivo reflects the earliest nonredundant role of a gene and precludes analysis of function at later stages. Additionally, some genes have functions during embryogenesis that may differ from those in the adult [e.g., LIF (18, 19) and vimentin (84)]. Inactivation of these genes may lead to adaptations that preclude their functional analysis at later stages. To address these problems, a number of modifications to the original gene-targeting strategies have been developed.

Embryonic lethality can be overcome by generating conditional knock-out or knock-in ES cells and mice, which can be used to activate or inactivate a gene both spatially and temporally (243). Typically, a conditionally targeted allele is made by inserting loxP or frt sites into two introns or at the opposite ends of a gene. Expression of P1 bacteriophage-derived Cre or yeast-derived Flp recombinases in mice carrying the conditional allele catalyzes recombination (insertions, deletions, inversions, duplications) between the loxP/frt sites, respectively, to inactivate (or activate) the gene (209). By expressing Cre recombinase from an endogenous or tissue-specific promoter, the conditional allele can be recombined in a restricted lineage or cell type. The timing of recombinase expression can also be controlled using inducible expression systems (313) or viral delivery systems such as adenovirus or lentivirus (270, 328), which makes it possible to inactivate a gene in a temporal-specific fashion. This technique has been widely used in the analysis of mice, and its use in adult mice overcomes a major limitation associated with standard transgenics, i.e., the developmental consequences of inactivated genes (209). The system has also been adapted for ES cell lines, both for in vitro studies and the generation of new mouse models [e.g., allele replacement by double loxP recombination (2, 395); Fig. 5]. The use of site-specific recombination events (insertions, deletions, inversions, or duplications) can also be extended to the engineering of long-range modifications in the ES cell genome (416).

## IV. IN VITRO DIFFERENTIATION POTENTIAL OF EMBRYONIC STEM CELLS

During mouse embryogenesis, the primitive ectoderm of the epiblast forms three primary germ layers: the ectoderm, the mesoderm, and the definitive endoderm. These germ layers interact to form all tissues and organs of the developing embryo. The complex interactions that control the transition of ectoderm to visceral and parietal endoderm in the postimplantation embryo, followed by the formation of mesoderm at the gastrulation stage (*days 3* to *7* post coitum), are only beginning to be defined. The in vitro differentiation potential of mES cells has facilitated the examination of these processes.

Differentiation is induced by culturing ES cells as aggregates (EBs; Fig. 6) in the absence of the self-renewal signals provided by feeder layers or LIF, either in hanging drops (40, 394, 395, 398), in liquid "mass culture" (98), or in methylcellulose (390). Moreover, coculture with stromal cell line activity (i.e., of PA6 cells, Ref. 186), and recently, even adherent monolayer cultures in the absence of LIF (411) have been used to differentiate mES cells in vitro. Scaleable production of ES-derived cells can furthermore be achieved through the use of stirred suspension bioreactors with encapsulation techniques (92).

Once differentiation has begun, cells representing primary germ layers spontaneously develop in vitro. Initially, an outer layer of endoderm-like cells forms within the EB, followed over a period of a few days by the development of an ectodermal "rim" and subsequent specification of mesodermal cells. Transfer of these EBs to tissue culture plates allows continued differentiation into a variety of specialized cell types including cardiac, smooth, and skeletal muscle as well as hematopoietic, pancreatic, hepatic, lipid, cartilage, or neuronal and glial cells (see Table 3 and Fig. 6). The temporal expression of tissue-specific genes and proteins in ES-derived cells during in vitro differentiation indicates that early processes of in vivo development into ectoderm, mesoderm, and endoderm lineages are recapitulated in vitro (204, review in Ref. 306).

Both the pattern and the efficiency of differentiation are affected by parameters like ES cell density, media components (high glucose concentration, i.e., at least 4.5 g glucose/l is required) and amino acids, growth factors and extracellular matrix (ECM) proteins, pH and osmolarity, and the quality of the fetal calf serum (FCS). Because the differentiation efficiency depends on the presence of FCS, and even the "batch" of serum used, many efforts have been taken to avoid these uncertainties: dextran-coated charcoal (DCC)-treated FCS [to remove ECM and growth factor activity from FCS (397)], chemically defined medium (176, 279), and recently by substitution of FCS with BSA fraction V (411). Furthermore, different ES cell lines display unique developmental properties in vitro (see Ref. 395).

Another model to study early events of differentiation are "early primitive ectoderm-like" (EPL) cells derived from mES cells by adherent culture in medium conditioned by human hepatocellularcarcinoma HepG2 cells (MEDII-CM) (288, 289). EPL cells exhibit many properties consistent with embryonic primitive ectoderm, but are distinct from ICM and ES cells (compare Tables 1 and 2 with Fig. 1 of Ref. 302). The cells do not participate in embryogenesis following blastocyst injection. But, EPL cells allow modeling of early differentiation events without genetic modification. The aggregation of EPL cells into EBs results in a loss of visceral endoderm and neuroectoderm differentiation, whereas late primitive ectodermal, parietal endodermal, and mesodermal cells develop (302). This pattern suggests that the EPL-EB differentiation model may be suitable for studying mesoderm development in vitro and that failure to appropriately form visceral endoderm in EPL-derived EBs is responsible for the lack of ectoderm lineage formation. The defect in ectoderm differentiation, however, can be achieved by culture of EPL-EBs in the presence of MEDII-CM, which results in the formation of neuroectoderm (primitive ectoderm, neural plate, and neural tube) and an almost

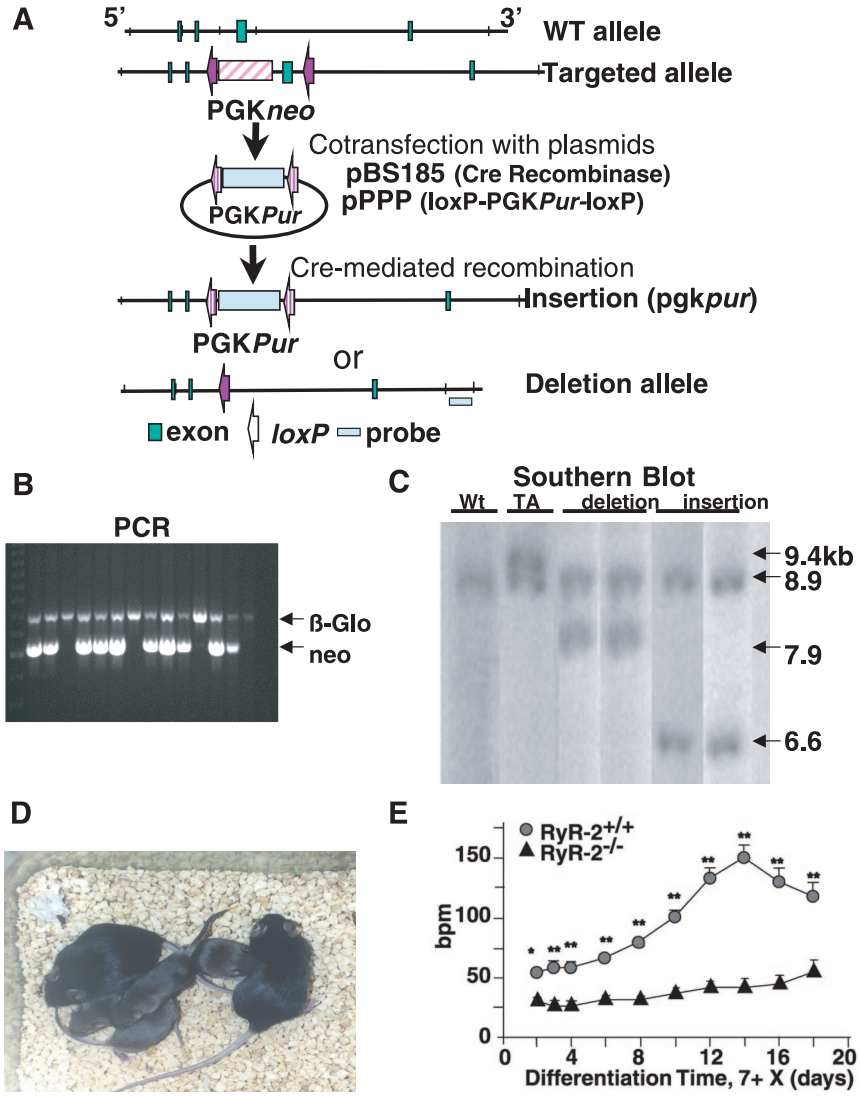


FIG. 5. Gene targeting, conditional expression, and ES cell-derived models in vivo and in vitro. *A*: site-specific insertion and excision events in ES cells can be mediated by Cre recombinase-*lox*P recombination. In this example, a gene locus in ES cells has been targeted by homologous recombination to insert a PGK-*neo*$^R$ cassette flanked by two *lox*P sites. Following selection with G418, a clonal ES cell line containing one wild-type (WT) allele and one targeted allele (TA) was isolated and transiently transfected with pBS185 (CMV promoter-driven Cre recombinase) and pPPP (PGK-*Pac*$^R$ cassette flanked by two *lox*P sites). After puromycin selection, the ES cells were clonally expanded to identify independent and stable integration events. Possible Cre recombinase-mediated insertion or deletion events are indicated in the diagram. *B*: genotyping by PCR was performed to identify clonal ES cell lines that had lost the neomycin resistance cassette. An internal control ($\beta$-globin, $\beta$-Glo) was included for each DNA preparation to ensure against false negatives. Similar protocols are employed to genotype transgenic mice. *C*: clonal ES cell lines can be tested by Southern analysis to identify which cell clones had undergone deletion or insertion events. In this example, four distinct bands could be identified: *1*) an 8.9-kb band corresponding to the WT allele; *2*) a 9.4-kb band of the original targeted allele containing the neomycin resistance cassette; *3*) a 7.9-kb band where the neomycin resistance cassette has been lost and the flanking *lox*P sites have recombined (deletion); and *4*) a 6.6-kb band generated by digestion of the newly inserted Cre recombinase targeted allele. *D*: targeted ES cell lines can be injected into blastocysts and used to generate chimeric mice that can be bred to generate homozygous animal models. *E*: in some instances, gene targeting can lead to embryonic lethality, but targeted chromosomal pairs coupled with in vitro differentiation can be used to elucidate the underlying mechanisms of embryonic lethality in mice. Loss of functional ryanodine receptor (RyR2), for example, leads to embryonic lethality at ~E10.5, but following in vitro differentiation of ES cells, we found that RyR2 regulated the spontaneous rate of beating (beats per minute, bpm) in ES cell-derived cardiomyocytes (408), and this effect on rate resulted in inadequate blood perfusion and embryonic lethality in mice.

complete inhibition of endodermal and mesodermal differentiation (287) (see also sect. IV*A*).

hES cells differentiate when removed mechanically ("cut and paste") or by enzymatic dissociation from feeder layers and cultured as aggregates in suspension. Cystic EBs formed under these conditions are heterogeneous and express markers of various cell types, including those of neuronal, cardiac, and pancreatic lineages (168, 293, 323; Table 4). However, none of the factors known to influence mES cell differentiation directs hES cells exclusively into a single cell type (323). For instance, activin-A and transforming growth factor (TGF)-$\beta$ were found to induce mainly mesoderm; retinoic acid (RA), epidermal growth factor (EGF), BMP-4, and basic fibro-

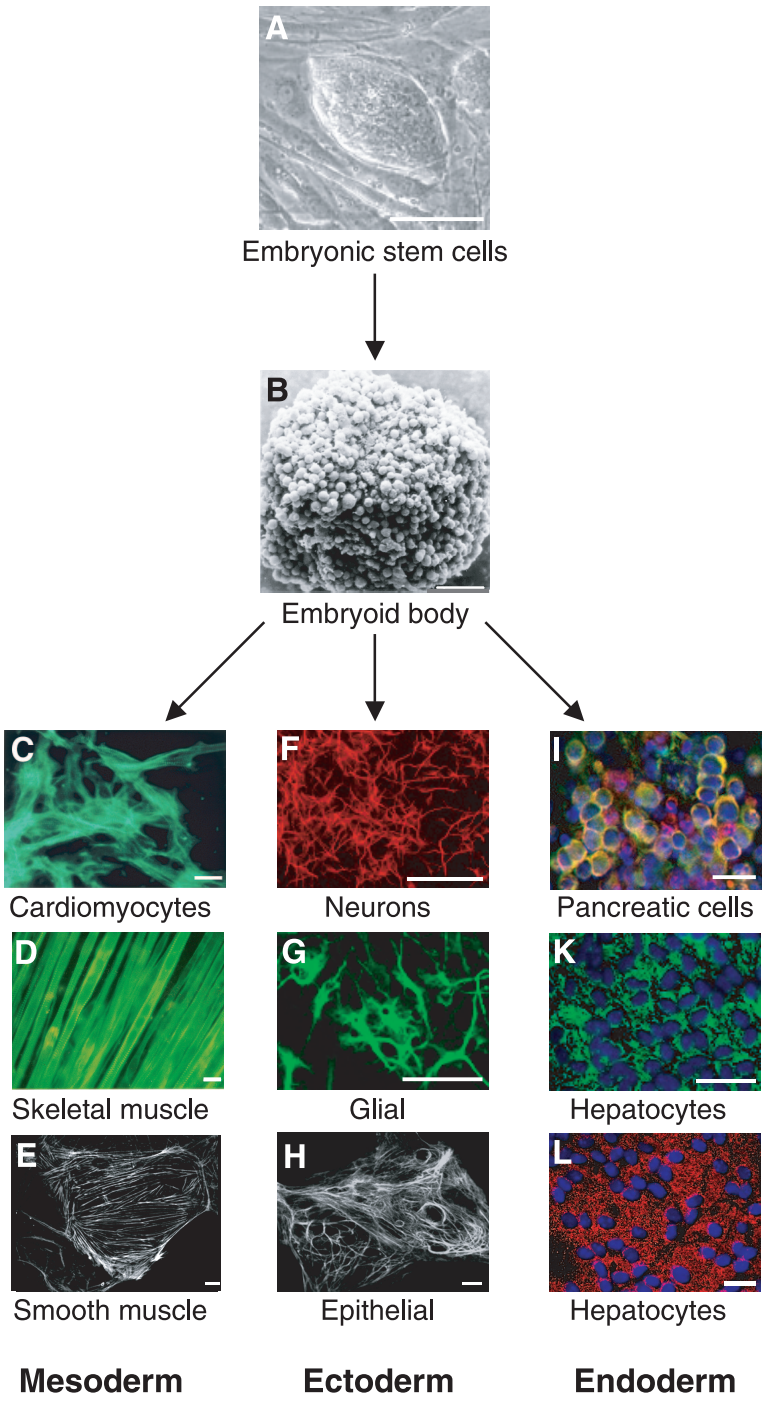


FIG. 6. In vitro differentiation of ES cells. Undifferentiated mouse ES cells (*A*) develop in vitro via three-dimensional aggregates (embryoid body, *B*) into differentiated cell types of all three primary germ layers. Shown are differentiated cell types labeled by tissue-specific antibodies (in parentheses). *C*: cardiomyocytes (titin Z-band epitope). *D*: skeletal muscle (titin Z-band epitope). *E*: smooth muscle (smooth muscle $\alpha$-actin). *F*: neuronal ($\beta$III tubulin). *G*: glial (glial fibrillary acidic protein, GFAP). *H*: epithelial cells (cytokeratin 8). *I*: pancreatic endocrine cells [insulin (red), C-peptide (green), insulin and C-peptide colabeling (yellow)]. *K* and *L*: hepatocytes (*K*, albumin; *L*, $\alpha$1-antitrypsin). Bars = 0.5 $\mu$m (*H*), 20 $\mu$m (*I*), 25 $\mu$m (*C*, *D*, *E*), 30 $\mu$m (*K*, *L*), 50 $\mu$m (*B*, *G*), and 100 $\mu$m (*A*, *F*).

blast growth factor (bFGF) elicited both ectodermal and mesodermal differentiation; whereas nerve growth factor (NGF) and hepatocyte growth factor (HGF) promoted differentiation of hES cells into all three primary germ layers. Interestingly, BMP-4 induced hES cells to develop into extraembryonic, trophoblast-like cells (403), a property clearly different from mES cells.

In section IV, *A–D*, we describe principal pathways and properties of differentiating mouse and human ES cells into derivatives of the three primary somatic and germ cell lineages. For methodical details such as differentiation protocols and differentiation factors, we refer the reader to the recent publications (79, 369, 382).

### A. Ectodermal Differentiation

Among the various lineages produced by the embryonic ectoderm during normal mouse development, the neuroectodermal lineage gives rise to the peripheral and

TABLE 3. *Examples for the in vitro differentiation capacity of mouse ES cells*

| Cell Type | Reference Nos. |
|---|---|
| Adipocytes | 93 |
| Astrocytes | 6, 122, 311, 357 |
| Cardiomyocytes | 98, 217, 218, 227 |
| Chondrocytes | 194 |
| Definitive hematopoietic cells | 245, 249, 390 |
| Primitive hematopoietic cells | 98, 245 |
| Dendritic cells | 111 |
| Endothelial cells | 299, 406 |
| Hepatocytes | 149, 177, 182 |
| Keratinocytes | 20 |
| Lymphoid precursors | 275 |
| Mast cells | 368 |
| Neurons | 386 |
| Dopaminergic neurons | 186, 206, 311 |
| Serotonergic neurons | 206 |
| GABAergic neurons | 22, 350 |
| Cholinergic neurons | 122 |
| Glutamatergic neurons | 116, 350 |
| Motor neurons | 389 |
| Oligodendrocytes | 8, 32, 54, 211, 366 |
| Osteoblasts | 61 |
| Pancreatic cells | 38, 162, 213 |
| Smooth muscle cells | 99, 406 |
| Skeletal muscle cells | 309 |
| Yolk sac | 98 |

central nervous systems (review in Ref. 212) and to the epithelial lineage, which is committed to becoming epidermal tissue (review in Ref. 130). Vascular smooth muscles are also partially derived from embryonic ectoderm.

Epithelial cell differentiation from ES cells can be identified by the presence of cytokeratin intermediate filaments and keratinocyte-specific involucrin (20, 367). After in vitro differentiation of mES cells, enrichment of keratinocytes and seeding onto various ECM proteins in the presence of BMP-4 and/or ascorbate promotes formation of an epidermal equivalent, which is composed of stratified epithelium (when cultured at the air-liquid interface on a collagen-coated acellular substratum). The resulting tissue displays morphological patterns similar to normal embryonic skin. The cells express late differentiation markers of epidermis and markers of fibroblasts, consistent with those found in native skin. The data suggest that ES cells have the capacity to reconstitute in vitro fully differentiated skin (86).

Of specific importance with regard to cell therapies of neurodegenerative disorders are neuronal and glial cells. The differentiation of mES cells into neuronal cells was published independently by three groups in 1995 (22, 122, 350). The spontaneous differentiation of ES cells into neuronal cells was rather limited (see Ref. 350) but has improved significantly by a number of strategies, involving the use of RA (review in Ref. 306), lineage selection (210, 411), and stromal cell-derived inducing activity (for review, see Refs. 141, 186). Whereas high concentrations of RA originally promoted efficient neuronal differentiation, characterized by the expression of tissue-specific genes, proteins, ion channels, and receptors in a developmentally controlled manner (122, 350), the survival and development of neurons derived in response to RA is limited. Furthermore, the teratogenicity of RA (see Ref. 306) makes it unsuitable for therapeutic applications. For these reasons, alternative protocols, involving multiple steps of differentiation and selection of neural progenitor cells, have been established. Following EB formation, serum is withdrawn to inhibit mesodermal differentiation. The proliferation of neural precursor cells is then induced by the addition of bFGF and EGF. Thereafter, neuronal cell differentiation can be supported by the addition of neuronal differentiation factors (22, 253) and maintained in vitro by neurotrophic differentiation (206) and survival-promoting factors. These include the glial cell line-derived neurotrophic factor (GDNF), neurturin (NT), TGF-$\beta$3, and IL-1$\beta$ (311). Significant improvements in the generation and in vitro survival of dopaminergic neurons have been achieved using these factors. Neurons can also be generated from mES cells by RA treatment combined with the genetic selection of lineage-restricted precursors (see Ref. 210), by using EPL-derived EBs in the presence of

TABLE 4. *Examples demonstrating the developmental potential of human ES cells in vitro*

| Cell Types Developed | Reference Nos. |
|---|---|
| Ectoderm, endoderm, mesoderm, and neural precursors | |
| Cardiomyocytes | 188, 239, 240, 402 |
| Cardiomyocytes, endodermal, hematopoietic, and neuronal cells | 168 |
| Neuronal, epithelial, pancreatic, urogenital, hematopoietic, muscle, bone, kidney, and heart cells | 323 |
| Neural epithelium, embryonic ganglia, stratified squamous epithelium, gut epithelium, cartilage, bone, smooth and striated muscle cells | 362 |
| Cells with properties of pancreatic $\beta$-like cells | 13, 324 |
| Cardiomyocytes, pigmented and nonpigmented epithelial cells, neural cells, mesenchymal cells, erythroid, macrophage, granulocyte, and megakaryocyte cells | 252 |
| Myeloid, erythroid, megakaryocyte colony-forming cells | 185 |
| Neural precursors, glial and neuronal cells: incorporation into the brain (H1, H9, H9.2 lines) | 415 |
| Neural precursors, glial and neuronal cells: incorporation into the brain (HES-1 line) | 292 |
| Neural progenitor, dopaminergic, GABAergic, glutamatergic, glycinergic neurons, astrocytes | 69 |
| Neural progenitor, neuronal cells | 322 |
| Trophoblast | 403 |
| Hepatocytes | 285 |

MEDII-CM (287), or by the cocultivation of ES cells with PA6 stromal cells in serum-free medium (186). In the latter case, the stromal cells produce an inducing activity, which efficiently activates neuronal differentiation, including dopaminergic cells.

Gene expression and electrophysiological studies of cell derivatives indicate the presence of all three major cell types of the brain: neurons [dopaminergic, GABAergic, serotonergic, glutamatergic and cholinergic neurons (22, 116, 122, 186, 206, 311)], astrocytes, and oligodendrocytes (8, 366; see Table 3). An elegant genetic approach to identify and validate ES cell neural regulatory genes was recently described (14). In these experiments, the earliest known specific marker of mouse neuroectoderm (early neural plate and neural tube), Sox1, was targeted with a construct containing GFP. In Sox1-GFP positive ES cell progeny, fluorescence was observed only in early neural precursors. This strategy provided a robust quantitative assay for early steps in neural differentiation. By then using an episomal expression system (see sect. v*D*) for uniform expression of candidate cDNAs in RA-induced ES cell derivatives, the authors identified one gene, *sfrp2*, that could strongly stimulate the production of neural progenitors. SFRP2 is an extracellular antagonist of the Wnt family of signaling proteins. Transfection of ES cells with Sfrp2 resulted in enhanced neural differentiation in response to RA (and neural differentiation was obtained even in the absence of RA). Overexpression of Wnt-1 in ES cells inhibited neural differentiation, thus confirming a role of Wnt signaling in ES-derived neuronal differentiation (90, see also Ref. 307). Recently, the authors went on to show that for efficient differentiation into the neural lineage, neither multicellular aggregation nor coculture is necessary. In these experiments, targeted Sox1-GFP ES cells cultured in adherent monolayers, following an efficient neural differentiation regime (N2/B27 medium) and sorting by FACS, differentiated into a highly enriched Sox1-GFP fraction of neural progenitor cells. These selected cells were further differentiated into specific neuronal, glial, and oligodendrocytic cell types (15).

The ability of human ES cells to generate derivatives of the neural epithelium was demonstrated soon after their isolation (362); however, the selective derivation of a given neuron subtype (e.g., dopamine neuron fate) had, until recently, been unsuccessful. Neural progenitor cells derived from hES cells (292) may be specifically enriched (69) and directed to differentiate into mature neurons (e.g., dopaminergic, GABAergic, serotoninergic), astrocytes, and oligodendrocytes (69; see Table 4). Growth factors, mitogens (such as RA, NGF, bFGF, and EGF) (322), ECM proteins (Matrigel, laminin; Ref. 401), and stromal cell lines (MS5, S2) as well as Wnt1-expressing stromal cells (MS5-Wnt1; Ref. 266) all serve as potent enhancers of neuronal differentiation from hES cells. Coculture of hES cells on MS5 stroma and exposure to differentiation factors, such as FGF8, SHH, and BDNF, leads to efficient differentiation of neuroepithelial structures termed "neural rosettes." Replating of these rosettes followed by terminal differentiation produces midbrain dopaminergic neurons that express the neuronal transcription factors Pax2, Pax5, and engrailed-1; release dopamine; and show characteristic properties of dopaminergic neurons by electrophysiological and electron microscopical methods. High-yield dopaminergic neuron derivation was confirmed for both human and monkey ES cell lines (266). The availability of unlimited numbers of midbrain dopaminergic neurons represents a first step towards exploring the potential of hES cells in animal models of Parkinson's disease.

### B. Mesodermal Differentiation

Mesoderm is the germ layer that develops into muscle, bone, cartilage, blood, and connective tissue. Blood and endothelial cells are among the first differentiated mesodermal cell types to form in the developing vertebrate embryo at around *day E6.5*, leading to the formation of yolk sac, an extraembryonic membrane composed of adjacent mesodermal and primitive (visceral) endodermal cell layers, which give rise to blood and endothelial cells (review in Ref. 26). Hematopoietic cells and blood vessels are believed to arise from a common progenitor cell, the "hemangioblast." As with ectodermal lineages, cultured ES cells have been successfully used to recapitulate these mesodermal developmental processes in vitro. Differentiation of ES cells in complex cystic EBs permits the generation of blood islands containing erythrocytes and macrophages (98), whereas differentiation in semisolid medium is efficient for the formation of neutrophils, mast cells, macrophages, and erythroid lineages (390). Application of FCS and cytokines such as IL-3, IL-1, and granulocyte-macrophage colony stimulating factor (GM-CSF) to ES cells generates early hematopoietic precursor cells expressing both, embryonic z globin ($\beta$H1) and adult $\beta$ major globin RNAs. Use of OP9 cells, which secrete an inducing activity, also leads to the development of all hematopoietic cell types of the erythroid, myeloid, and lymphoid lineages (244) and of natural killer (Nk) cells (review in Ref. 159). Experiments to identify potential inducers of the hematopoietic lineage furthermore indicate that Wnt3 is an important signaling molecule that plays a significant role to enhance hematopoietic commitment during in vitro differentiation of ES cells (199).

The use of endothelial cell restricted promoters illustrates how in vitro analyses of EBs can be used to define complex mesodermal-derived cells. Quinn et al. (281) used the *flt*-1 promoter to regulate EGFP in PECAM-1 positive ES-derived endothelial cells. The expression of this transgene, at least temporally, coincided with the

expression of endogenous flt-1. Further analyses of EGFP expression relative to Sca-1 positive cells suggested that the *flt-1* promoter is active in ES-derived endothelial cells, but that it is downregulated during hemangioblast differentiation to the hematopoietic lineage (281). Similarly, Marchetti et al. (220) employed the vascular endothelium-specific promoter *tie-1* to drive both EGFP and $pac^R$ expression to isolate endothelial cells from genetically modified ES cells. Puromycin ($pac^R$)-resistant cells were positive for the endothelial cell surface markers, but release from puromycin selection resulted in the appearance of $\alpha$-smooth muscle actin positive cells, showing that endothelial cells or their progenitors could also differentiate towards smooth muscle. Finally, the expression of vascular endothelial growth factor receptor 2 (VEGF-2, known in the mouse as fetal liver kinase 1, Flk1) in early mesodermal progenitor cells also enabled the isolation of a $Flk1^+$ cell population that includes endothelial and hematopoietic precursors (127, 249).

A similar strategy was used to study the specification of ES cells into the "hemangioblast." ES cell lines were created that express GFP targeted to the mesodermal gene *brachyury* (114), a transiently expressed mesoderm-specific transcription factor (176). Analysis of brachyury-GFP targeted cells permitted discrimination between mesoderm and neuroectoderm progenitors. Coexpression analysis of GFP with FLK1, furthermore, revealed three distinct mesodermal cell populations: premesoderm (GFP-/Flk1-), prehemangioblast mesoderm (GFP+/Flk1-), and the "hemangioblast" (GFP+/Flk1+) population, the precursor cells of primitive and definitive hematopoiesis and endothelium (114).

The cellular phenotypes of ES-derived hematopoietic cells have been characterized by specific gene expression patterns and by cell surface antigens (380, 390); however, the most important definition for these cells is functional. ES cell derivatives must demonstrate long-term multilineage hematopoietic repopulating properties to be considered true hematopoietic stem cells. Early reports suggested that the repopulating ability of ES-derived hematopoietic progenitors may be restricted to the lymphoid system (236), but subsequent studies showed a long-term multilineage hematopoietic repopulating potential of ES-derived cells (160, 259).

Another mesodermal cell type that has been extensively analyzed is ES cell-derived cardiomyocytes. These cells readily differentiate from aggregates composed of initially 400–800 starting cells that form in the presence of high FCS (20%) and display properties similar to those observed in cardiomyocytes in vivo or in primary cultures. These cells *1*) express cardiac gene products in a developmentally controlled manner (40, 113, 230), *2*) show characteristic sarcomeric structures (146, 228), and *3*) demonstrate contractility triggered by cardiac-specific ion currents and the expression of membrane-bound ion channels (40, 154, 216–218, 394). The cardiomyocytes develop spontaneously (review in Ref. 43; see Ref. 395) or could be induced by differentiation factors including dimethyl sulfoxide (DMSO) and RA (394) and small molecules, such as Dynorphin B (374) and cardiogenol derivatives (399).

Electrophysiological analyses indicate that early differentiated cardiomyocytes are typical of primary myocardium (216), which subsequently differentiate to atrial-, ventricle-, Purkinje-, and pacemaker-like cardiomyocytes (review in Ref. 154). Importantly, patch-clamp and $Ca^{2+}$ imaging techniques have permitted a thorough temporal-dependent analysis of electrical activity and the dynamics of ion channel expression and signaling cascades during cardiomyogenesis (1, 167, 174, 227). Microelectrode arrays (MEA) have furthermore been employed to temporally analyze excitation generation within ES-derived cardiac clusters. When EBs are plated onto MEAs, the electrical signals of the field potentials can be recorded over a period of several days from a multitude of electrodes beneath the spontaneously contracting cardiac clusters (24).

Cardiomyocytes differentiated from hES cells show similar properties to those derived from mES cells. Cardiac clusters have been identified on the basis of spontaneous contractions. The cell clusters are composed initially of small mononuclear cells with round or rod-shaped morphology that progress to form highly organized sarcomeric structures at later stages. The cardiac-specific gene expression pattern, electrophysiological properties, and chronotropic responses to adrenergic and muscarinic agonists are also typical of cardiomyocytes (188, 239, 240, 402). Cardiomyocytes differentiated from mouse and human ES cells show similar responses to $\beta$-adrenergic and muscarinic modulation (290). The differentiation protocols with hES cells, however, yield an insufficient quantity of cardiac cells for experimental analyses. In this context, the recent discovery of cardiac-inducing signals from the endoderm (239) represents a step forward to the generation of cardiomyocytes from hES cells in vitro. The authors cocultured nonbeating EBs of hES cells on a monolayer of END-2 cells, an endodermal derivative generated from P19 embryonic carcinoma cells (241). This procedure resulted in the development of functional cardiomyocytes from hES cells. The continued identification of the molecular nature of the endoderm-derived factors and the application of efficient lineage selection strategies are requirements for the derivation of cardiac tissue from hES cells.

mES cells efficiently differentiate into several other mesodermal cell types, including mesenchymal cell-derived adipogenic (93), chondrogenic (194), osteoblast (61), and myogenic (309) cells (see Table 3). In all cases, the derivation of these cell types was induced by specific differentiation factors. Although all the protocols differ,

they involve the successive treatment with specific growth and matrix factors, followed by a coordinated pattern of successive steps of differentiation. A sophisticated spinner culture system has also been established to generate vascular endothelial cells useful as a murine in vitro model for blood vessel development (381). Differentiation induction of mES cells by RA and dibutyryl cAMP resulted in the development of functional vascular smooth muscle cells typical of cells found in large arteries (99). These data show that complex vascular structures, as part of the cardiovascular system, originating in vivo from both mesoderm and neural-crest lineages, can be generated from ES cells in vitro.

### C. Endodermal Differentiation

Pancreas and liver cells are derivatives of the definitive endoderm. During embryogenesis, the pancreas develops from dorsal and ventral regions of the foregut, whereas the liver originates from the foregut adjacent to the ventral pancreas compartment. Pancreatic and hepatic cells are of special therapeutic interest for the treatment of hepatic failure (147) and diabetes mellitus (337), and both pancreatic endocrine and hepatic cells develop in vitro from ES cells.

ES-derived hepatic cells show hepatic-restricted transcripts and proteins (149, 177) and can successfully integrate and function in a host liver following transplantation (78, 80, 404, 405). These data suggest that mES cells differentiate into all three lineages of the liver (hepatocytes as well as bile duct epithelial and oval cells). Differentiation strategies have begun to identify specific progenitor cells in the ES cell progeny, which may be of further use to isolate hepatic precursor cells of the liver (181, 182).

Hepatocyte-like endodermal markers were also detected in hES cell derivatives (285, 323). The successful differentiation and isolation of hepatic-like cells from hES cells has been demonstrated by using hES cells stably transfected with the reporter gene EGFP fused to an albumin promoter (203).

The generation of ES-derived insulin-producing pancreatic endocrine cells may be critical to the treatment of diabetes. The first successful induction of pancreatic differentiation from ES cells was obtained by stable transfection with a vector containing a neomycin-resistance gene under the control of the insulin promoter. This enabled lineage selection and maturation of insulin-expressing cells which, upon transplantation, resulted in the normalization of glycemia in streptozotocin-induced diabetic mice (338). In contrast, the spontaneous differentiation of mES cells in vitro generated only a small fraction of cells (0.1%) with characteristics of insulin-producing β-like cells (329). This percentage has been increased by the selection of nestin-positive progenitor cells, the products of which showed regulated insulin release in vitro. The insulin-positive clusters, however, failed to normalize high blood glucose levels in transplantation experiments (213). Indeed, subsequent analyses revealed that these insulin-positive cells may be partially committed to a neural fate (330) and are characterized by small, condensed nuclei and are apoptotic. Rather than producing insulin themselves, most of the cells took up this hormone from the culture medium (283).

By modifying the differentiation protocols and using genetically modified mES cells, two groups successfully generated insulin-producing cells (38, 207). Blyszczuk et al. (33) showed that constitutive expression of the pancreatic developmental control gene *Pax4* and histotypic differentiation were essential for the formation of insulin-expressing cells, which were found to contain secretory granules typical of both embryonal and adult β-cells. Importantly, these cells coexpressed C-peptide and normalized blood glucose levels after transplantation into diabetic mice (37, 38). Similarly, lineage selection using mES cells transfected with a plasmid containing the Nkx6.1 promoter upstream of a neomycin-resistance gene could be used to generate insulin-producing cells that normalized glycemia after transplantation into diabetic animals (207).

Also, the treatment of mES cells with a phosphoinositide 3-kinase (PI 3-K) inhibitor during terminal stages of differentiation generated ES cell progeny expressing various β-cell-specific markers. Following engraftment into diabetic mice, these cells also improved the glycemic status and enhanced animal survival (162).

Initial experiments with hES cells indicate that in vitro differentiation generates ~1% insulin-secreting cells that show at least some characteristics of pancreatic endocrine cells (13). Treatment of hES cells with NGF results in upregulation of the Pdx-1 gene, the product of which controls insulin transcription and regulates insulin release (323). A modification of the differentiation protocol (see Refs. 213, 283) allowed the generation of insulin-producing clusters from hES cells (324), but further improvements are necessary for generating functional islet-like cells.

### D. Germ Cell Differentiation

Only recently has the use of a suitable reporter system allowed the visualization of germ cell formation in vitro. Hübner et al. (164) used regulatory elements (CR2 and CR3) within the germ-line specific (gc) *Oct4* gene to visualize initial steps of germ cell formation. To restrict expression of an Oct-3/4-based reporter to germ cells, a genomic *gcOct-3/4-GFP* construct was introduced into ES cells and cultured at high density. Colonies of variable

size formed after 12 days, and GFP-positive cells that expressed *Vasa* (a marker of postmigratory germ cells) formed small aggregates in the supernatant. The isolation and further culture of these aggregates resulted in well-organized structures, morphologically similar to early ovarian follicles. The formation of these oocyte-like structures was paralleled by estradiol synthesis providing evidence for functional activity of somatic (granulosa) cells in the cultures. A detailed analysis of the oocyte-like cells showed that they were fragile and expressed the zona pellucida proteins (ZP) 2 and 3. The loss of ZP1 expression may account for the fragile zona of in vitro-derived oocyte-like cells, because loss of ZP1 has been shown to perturb folliculogenesis. Continued cultivation of oocyte-like cells, until *day 43*, revealed structures similar to mouse preimplantation embryos. It is likely that these blastocyst-like structures represent parthenotes, as suggested by the similarity of the follicle outgrowths (164).

Two other reports describe the formation of male germ cells that have the capacity to participate in spermatogenesis in vivo after engraftment (365) and to fertilize oocytes (136). In the latter study, EBs supported the maturation of primordial germ cells into haploid male gametes, which when injected into oocytes restored the somatic diploid chromosome complement and developed into blastocysts. EG cells show erasure of the methylation markers (imprints) of *igf2r* and *H19* genes, a property characteristic of the germ line. Because these data would essentially close the developmental circle that connects ES cells with the germ line, it may be necessary to redefine the ES cell potential in vitro (totipotency versus pluripotency). Moreover, this property of ES cells provides an accessible in vitro model system for studies of germ-line epigenetic modifications and mammalian gametogenesis and should reveal whether the in vitro generated oocytes may be used as starting material to reprogram somatic cell nuclei. If similar processes can be induced in hES cells, this would open a new perspective to the generation of therapeutically relevant tissues by the "therapeutic cloning" approach (see sect. IX*B*). Indeed, hES cells are able to spontaneously develop into cells representative of meiotic and postmeiotic germ cell development. The in vitro differentiation of hES cell lines as EBs resulted in the formation of VASA-positive cells and the upregulation of transcripts of the meiotic markers *SCP1* and *SCP3* (synaptonemal complex protein) and the postmeiotic markers *GDF9* (growth and differentiation factor) and *TEKT1* (tektin). In contrast to mES cells, in vitro differentiated hES cells express both the male and female genetic programs regardless of whether they were karyotypically XX or XY: both GDF9 (oocyte-specific) and TEKT1 (spermatid-specific) expression was detected with differentiation of hES cells (82).

## V. EMBRYONIC STEM CELLS AS CELLULAR MODELS IN DEVELOPMENTAL BIOLOGY AND PATHOLOGY

Experiments designed to understand gene function in the context of an organism require genetic strategies. Enhancer and promoter traps (129), gene traps, random activation of gene expression (RAGE), and genome-wide cell-based knockout (GECKO) represent genome-wide strategies to identify, isolate, or determine gene function (for information on RAGE and GECKO go to http://www.athersys.com/). Because of gene-targeting techniques, transgenic mice have also proven critical to the creation and evaluation of some models of human disease.

### A. Gene Trapping

Gene trapping is the most commonly employed insertional mutagenesis strategy, and it has been extensively reviewed elsewhere (102, 140, 264); however, this technique is likely to prove very important for the study of human development, i.e., through the exploitation of hES cells in vitro. Essentially, when gene traps are introduced into ES cells, they integrate randomly in the genome (102, 331, 332, 376). Antibiotic-resistant ES cell colonies are easily selected and expanded in vitro, and clonal cells can be isolated for injection into mouse blastocysts or differentiation in vitro. Expression of the gene trap is assayed for reporter gene expression (e.g., β-galactosidase activity), and staining is indicative of an insertion event. The transgene is only activated when it integrates correctly within an active transcriptional unit; however, some translational fusions (frame shifts) inactivate the reporter activity or may target the translated proteins into subcellular locations where reporter activity is not easily detectable. Gene trapping therefore selects for integration events in functional genes, and it is especially useful for the analysis of mammalian cells that have complex genomic organizations that consist of promoters and exons that are separated by introns (Fig. 7).

In vivo gene trap screens in mice have permitted the identification of many developmentally regulated genes that are expressed within specific tissues in a spatiotemporal pattern, including novel RA responsive (120), neuronal, glial, chondrocytic, myocytic (23), and hematopoietic (65, 157, 342) genes. A disadvantage of this approach is that it requires the production of a large number of mice from ES cell clones to identify a limited number of developmental genes. To limit the number of noninformative mice, Bonaldo et al. (45) employed gene trapping together with the differentiation potential of ES cells. By selecting for the activation of the reporter gene in tissue culture, the rate of gene disruption in recovered clones ap-

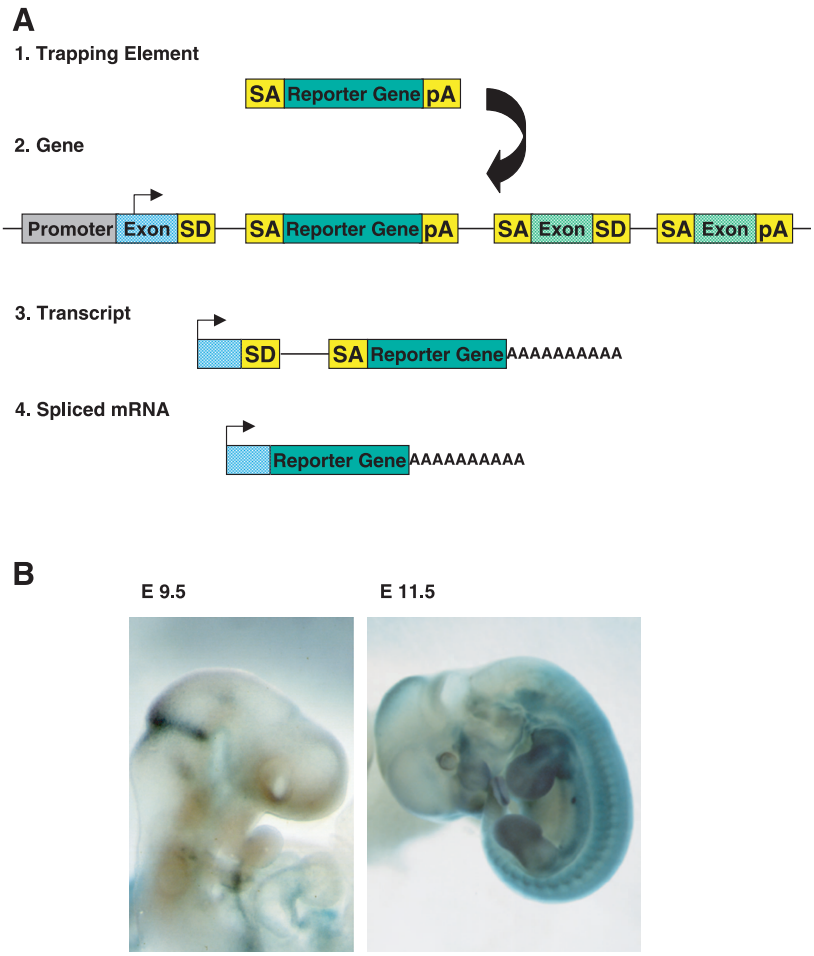


FIG. 7. Schematic overview of gene trapping. *A*: endogenous wild-type genes usually produce heterogeneous nuclear RNA transcripts that are spliced to form mature mRNAs. One approach to gene trapping employs constructs that contain a reporter gene sequence between a splice acceptor (SA) and a polyadenylation signal (pA). When inserted into a functional gene, the endogenous splice donor (SD) and gene trap splice acceptor are processed to form a fusion transcript to activate the reporter gene contained in the gene trap construct. The transgene is only activated when it integrates correctly within an active transcriptional unit. Some translational fusions (frame shifts) may inactivate the reporter activity or may target the translated proteins into subcellular locations where reporter activity is not easily detectable. Gene trapping therefore selects for integration events in functional genes, and it is especially useful for the analysis of mammalian cells that have complex genomic organizations that consist of promoters and exons that are separated by introns. *B*: expression of the gene trap is assayed for reporter gene expression (e.g., β-galactosidase activity), and staining is indicative of an insertion event. In this figure, we show a gene trap construct incorporated within *jumonji* and expressing *LacZ*. Embryos (E9.5 and E11.5) were stained with X-gal. (Figure kindly provided by G. Lyons.)

proaches 100%, and the random insertion of exogenous DNA into single sites in the mammalian genome (gene trapping) provides a genome-wide strategy for functional genomics. ES cell cultures thus provide a simple model system for studying the genetic pathways that regulate embryonic tissue development and permit high-throughput screening of clones for tissue-restricted gene trap expression (45).

In the postgenomic era, bioinformatic-based approaches have accelerated the evaluation of mutant clones (originating from gene traps, RAGE, and GECKO) leading to the rapid identification of informative cell lines on an unprecedented scale. When combined with computational approaches, expression profiling with DNA microarrays, and in situ hybridization analyses, the results often suggest an association with a specific biological process or disease state, which must be tested. For example, a sequence-tagged gene-trap library of >270,000 mouse ES cell clones has recently been developed that has been employed together with a functional screen of knock-out mice to identify genes regulating blood pressure (412). Efforts are also underway to make ES cell lines with gene traps freely available for researchers so that transgenic mice containing a potential gene of interest can be made to further understand the role of specific genes in development and disease (e.g., http://baygenomics.ucsf.edu/overview/welcome.html).

Finally, a new reporter system has been described that permits real-time monitoring of live cells. In this system, a β-lactamase tagged library can be used to clone genes (387). Use of the nontoxic fluorescent substrate of β-lactamase, CCF2-AM, enables real-time and sensitive monitoring of transcription in live cells (417). In theory, the monitoring of live cells can be used to identify cell clones with genes that are induced or repressed by different agents, including receptor ligands, drug candidates, or viruses (286).

### B. In Vitro Models to Study Embryonic Lethality

As pointed out earlier, genetic modifications of ES cells can lead to embryonic lethality, certain aspects of which can be overcome through the use of conditional targeting. Alternatively, embryonic lethal models can also be studied in vitro with ES cells containing targeted mu-

tations on chromosome pairs. In the case of X-linked genes like HPRT or GATA-1, a functional knockout of a gene in ES cells can be generated from a single targeting event (36). In the majority of cases, however, a knock-out ES cell line must be generated either by sequential targeting of chromosomal pairs in vitro or through an intermediate step involving the generation of homozygous mice lacking a functional allele. The generation (either from knock-out mice or by sequential targeting of chromosomal pairs) and analysis of knockout ES cell lines can be labor intensive and is neither practical nor useful for many studies. At times, the use of targeted chromosomal pairs in ES cells has, however, proven indispensable to the elucidation of gene function. Mitsui et al. (233), for example, targeted Nanog on chromosomal pairs to show that it was required for the maintenance of embryonic stem cell pluripotency and identity (see sect. II). When knockout cells have been coupled with random transgenesis, it has also been possible to rescue phenotypes (36), determine how the timing or duration of signaling determines cell fate (262), and develop new developmental paradigms (35, 36).

Targeted chromosomal pairs coupled with in vitro differentiation have also been used to elucidate the underlying mechanisms of embryonic lethality in mice. For example, the ryanodine receptor (RyR2), which serves as the major sarcoplasmic reticulum calcium release channel in heart to mediate a rapid rise of cytosolic free $Ca^{2+}$, is normally expressed early in developing myocardium. A functional knockout of this gene causes the developing mice to die at approximately E10 day post coitum, but the mechanism responsible for this embryonic lethality was unclear (355). Examination of cardiomyocytes derived from RyR2-deficient ES cells showed that RyR2 was essential to increase the spontaneous beating rate in immature cardiomyocytes (408) (Fig. 5). When the heart rate slowed in the transgenic mouse model due to the absence of RyR2, the sphincter mechanism normally utilized in valveless embryonic heart was lost, and blood perfusion proved inadequate. Embryonic lethality in the $RyR2^{-/-}$ mice was therefore postulated to be due to functional incompetence of the contracting myocardium, a finding that was achieved only through complementary studies between in vivo and in vitro systems. Similarly, the use of ES cells homozygously deficient for $\beta_1$-integrin (see Refs. 113, 145, 307) and desmin (385) genes allowed a detailed loss-of-function analysis and description of affected cell types in vitro, because animals deficient for these genes died early during embryogenesis.

### C. Developmental and Disease Models

As described in section III*A*, the production of heterogeneous cell populations in vitro has constrained the analysis of ES cell-derived progeny. The use of transgenesis and gene targeting has overcome many of these limitations, and relatively pure homogeneous populations of ES cell-derived progeny have now been isolated. Genetic approaches involving transgenic mice have also greatly advanced our knowledge of development and disease. This has been accomplished primarily through *1*) the isolation and cultivation of ES cells, which retain the ability to colonize all tissues of a host embryo including its germ line; *2*) the resiliency of mammalian embryos/blastocysts to tolerate the addition or loss of embryonic cells; and *3*) gene inactivation by homologous recombination or overexpression of transgenes to assess gene function and genetic labeling of precursor cells to determine cell lineages.

The earliest use of targeted animal models for gene therapy emphasized mouse models that simulated inherited disease, but these often proved disappointing (393). Subsequent studies have identified many useful mouse models for the study of human disease; however, the utility of these transgenic models frequently depends on the impact of environment and genetic background. A good example is seen with mouse models of cystic fibrosis (CF), where the CF transmembrane conductance regulator (CTFR) gene was interrupted or mutated. The initial CFTR-deficient mice did not develop pulmonary pathologies before death; however, subsequent genetic and environmental modifications have increased its usefulness to model CF (393).

Currently over 1,200 papers with transgenic mice can be found on-line (PubMed), and some 7,000 mutant mice have been described. While not all of these models have proven useful, some have been critical for determining promoter and gene function, functional gene redundancy, spatial distributions of expression, and lineage tracing. Numerous papers have also documented the use of gene targeting for mouse models of development and disease. Some mouse and ES cell studies have shed light on factors indispensable for hematopoiesis (256), while a number of knockout mouse models have been employed to reveal the critical roles for transcription factors (Ets family members) in guiding hematopoiesis, vasculo/angiogenesis, and other cellular differentiation processes (27). Many reviews have already been published showing how gene targeting has been employed to study cardiovascular (77), pancreatic (153), or renal (172) systems, while still others have employed Cre/loxP systems for conditional regulation (313).

Animal models of human diseases are critical to the early development and evaluation of gene- and cell-based therapies; therefore, studies with mouse ES cells in the context of transgenic models form the foundation for current and future work with human ES cells and their derivatives for studies in human. Experimentally, it remains unclear whether human ES cells will be as versatile

as mouse ES cells with respect to self-renewal, genetic manipulation, or developmental capacity, but the ability to test these cells in disease models, transgenic or otherwise, will be critical to this evaluation.

### D. Recent Advances

#### *1. Extrachromosomal expression*

As stated earlier, integration-dependent events can adversely affect gene expression in ES cells. Similar to retroviral sequences, transgenes randomly introduced into ES cell lines tend to be progressively silenced, resulting in mosaic expression, heterogeneous phenotypes, or complete silencing. Extrachromosomal plasmid replication avoids the problem of gene silencing and represents a powerful technique to overexpress genes without disrupting the pluripotentiality or differentiation capacity of ES cells.

One system of extrachromosomal replication exploits the replicative biology of polyoma virus (63, 134). Polyoma virus mutants, which either lack the entire large T intron or lack the splice sites employed to form properly processed middle T and small T mRNAs, are unable to transform cells. As long as large T transcripts are present, mutant polyoma virus DNA can replicate as free, unintegrated mini-chromosomes in infected mouse cells (189), and ES cells that stably express the polyoma large T protein efficiently support the episomal maintenance of plasmids containing the polyoma origin of replication. Gassmann et al. (134) developed a self-replicating vector system (pMGD20neo) for ES cells that contains the polyoma origin of replication with a mutated enhancer, a modified polyoma early region that encodes the large T antigen, and a neomycin resistance cassette (134). The utility of this system was recently demonstrated by Aubert et al. (14), who employed a variant of this extrachromosomal replication system to uniformly express a secreted frizzled related protein (SFRP2) transgene in ES cells (see sect. IV*A* and Ref. 14). They showed in puromycin-resistant cells that both undifferentiated ES cells and their descendents express transgenes more uniformly and stably than that normally achieved with transgenes inserted randomly into the genome. Stable transfectants were established at a frequency of 1–5% compared with $<$0.1% for stable integration events. Importantly, expression of polyoma large T protein at levels sufficient to support episomal replication appears to have no effect on ES cell self-renewal or pluripotency. The use of extrachromosomal vectors thus overcomes some of the major technical problems associated with random integration events: silencing, mosaicism, and/or interference with endogenous genes.

#### *2. Recombineering*

Both random transgenesis and homologous recombination have been limited by the time and site limitations associated with DNA engineering in *Escherichia coli*, particularly if conditional knockout models are being developed. The construction of targeting vectors often utilizes large regions of genomic DNA, and their construction can be labor intensive and complex. For example, restriction enzyme sites are not always conveniently located, and mutations must be made in the genomic sequences to introduce selection cassettes or loxP sites. Recent innovations use homologous recombination to construct targeting vectors in a process termed recombineering (85, 242). This form of chromosome engineering greatly shortens the time it takes to make a targeting vector and makes it possible to introduce selectable markers anywhere in a gene. An example of the approach was the generation of knock-in constructs for Olig-2, a transcription factor first expressed in ventral progenitor cells that gives rise to oligodendrocytes and motor neurons, but in spinal cord is only present in oligodendrocytes (400). With the use of a mouse genomic BAC library, the Olig-2 gene was isolated and a targeting construct was generated by homologous recombination in yeast. Following recombination, the construct was shuttled back into *E. coli*, modified, and introduced into ES cells. G418-resistant colonies were selected and differentiated in vitro. GFP-positive cells were found to be consistent with cells of the oligodendrocyte lineage that could be separated by fluorescence-activated cell sorting and cultured as pure populations. Although originally pioneered in yeast, recombineering explicitly refers to the use of homologous recombination in *E. coli* to manipulate genomic sequences. Specifically homologous recombination in *E. coli* is facilitated by the use of bacteriophage-based homologous recombination systems, which permit linear double-stranded DNA fragments (i.e., those carrying loxP sites and selection markers) that have short regions of homology with the target sequences at their ends to be inserted into virtually any large target DNA (plasmids, BACS, or PACs). The utility of this system in ES cells was recently described by Testa et al. (359), but it has yet to be shown applicable for use with hES cells.

#### *3. RNA interference*

RNA interference (RNAi) is a process whereby double stranded (ds) RNA induces targeted degradation of RNA molecules with homologous sequences. It has become a valuable tool for the analysis of gene function through suppresssion of specific gene products, and it has been extensively employed in *Caenorhabditis elegans* and plants (117). More recently, RNAi has proven useful in the study of mammalian systems (21). The major obstacle for the use of short interfering (si) RNA has been in

the efficient and sustained delivery of dsRNA to mammalian cells; however, when introduced into these cells, the hallmark of RNAi is its specificity; dsRNA triggers specific degradation of homologous mRNA only within the region of identity of the dsRNA (413).

Sequence-specific RNAi has been demonstrated in the preimplantation mouse embryo and in oocytes by direct injection of dsRNA (354, 388). When introduced into mouse zygotes, dsRNAi proved effective at repressing GFP throughout the blastocyst stage up until E6.5. Recent results demonstrate that ES cells maintained in an undifferentiated or in a differentiated state can also respond to dsRNA for gene silencing (409, 418). In the latter case, the authors employed dsRNA to suppress the expression of PU.1 and C/EBP$\alpha$ in $CD34^+$ EB cells. As a consequence, the level of expression of the M-CSF receptor (CD115), a downstream target of PU.1, and C/EBP$\alpha$ were both decreased within 2–3 days after transient transfection. With the success of this approach to knock-down genes in ES cells and recent improvements to the delivery of siRNAs to mammalian cells, RNAi may be an effective approach to the study of ES cell differentiation and as a gene therapy approach (68).

## VI. EXPRESSION PROFILING OF EMBRYONIC STEM CELLS

It is generally assumed that ES cell biology is regulated through transcriptional mechanisms, but the definition of a stem cell remains largely functional (see sects. II and IV). The developmental capacity of ES cell lines requires a set of genes that are not expressed in other cell types, and knowledge of the intricate mechanisms regulating ES cell pluripotentiality and differentiation potential is currently limited to a few signaling pathways (e.g., LIF, BMP, Wnt) and regulatory factors (e.g., Oct-3/4, Nanog). Theoretically, a comprehensive analysis of a cellular transcriptome (i.e., all the RNAs present in a cell type) should be sufficient to define the molecular phenotype of stem cells and establish the determinants of ES cell choice. The underlying hypothesis behind these assumptions suggests that some mRNAs will be uniquely or more abundantly expressed in embryonic and/or adult stem cells than in any other cell type and that comparisons among cell populations will reveal these differences. Although several transcriptome-based (microarrays or SAGE) studies have now been published, which claim to have identified potential stemness-associated factors, a closer inspection of the data indicates that the identification of "stemness" factors has proved elusive (109). This is true for both mouse and human ES cells. The reasons most frequently cited for variations among studies include cell lines, culturing conditions, array and hybridization protocols, data analysis, and potentially contaminating cells. Additionally, many of the studies in mice focused on comparisons among ES cells with adult stem cells, because of earlier studies suggesting a broader potential or plasticity of adult stem cells than previously believed (34); however, this broader plasticity of primary isolates of many adult stem cells has recently been called into question (see review in Ref. 379). The identification of "stemness" genes by these approaches, therefore, remains the topic of lively debate and much conjecture. Finally, the phenotype of ES cells must also involve complex processes that alter protein abundance both as a consequence of gene activation and processing (transcription, splicing, etc.), as well as regulatory events associated with translation and posttranslational modifications (PTM). Proteomic approaches are therefore required to visualize and interpret the phenotype of undifferentiated ES cells.

### A. Microarrays

Ramalho-Santos et al. (284) and Ivanova et al. (169) were the first to employ microarrays to compare mouse ES cells with hematopoietic (HSCs) and neuronal (NPCs) stem/progenitor cells. They identified 216 and 283 transcripts, respectively, that were enriched in all three stem cell libraries. Remarkably only six genes overlapped between the two lists, but when the stemness-associated transcripts were grouped, a common theme emerged. Stem cells expressed a large number of transcripts that could be described as signaling factors, transcription/translation factors, and proteins associated with DNA repair, protein degradation, and protein folding. The stem cells also expressed a prominent set of gene transcripts with unknown function, suggesting that many unique transcripts, either from novel genes or in the form of splicing variants, remain to be identified from embryos (42). Furthermore, some of the stemness-associated factors clustered to chromosome 17, suggesting that characterization of the genomic regions that regulate stem cell-associated factors will further promote our understanding of the regulatory networks required to maintain undifferentiated stem cell populations. About the same time, Tanaka et al. (356) compared ES and trophoblastic stem cells to identify Esg-1 (Dppa5) as an ES cell-restricted transcript that is exclusively associated with pluripotency.

Fortunel et al. (121) subsequently identified 385 transcripts that were highly expressed in mES cells, neural progenitor, and retinal stem/progenitor cells. From this list, only one transcript ($\alpha_6$-integrin) was present in the lists of stemness-associated transcripts published by Ramalho-Santos et al. (284) or Ivanova et al. (169). Most of the commonly enriched transcripts that were identified were not exclusively expressed in stem cells, suggesting

that stem cell abundant transcripts may only be elevated relative to differentiated cells (60), and further analyses comparing stem cell lines with tissues seemed warranted. In 2003, Sharov et al. (327) compared transcript abundances among mouse oocytes, blastocysts, stem cells, postimplantation embryos, and newborn tissue. This comparison led to the identification of groups of genes expressed in preimplantation embryos and various stem cell lines (i.e., ES, EG, trophoblastic stem cells, mesenchymal stem cells, neural stem cells, osteoblasts, and hematopoietic stem cells). Importantly, the ES and EG cells were shown to have a distinct genetic program relative to the other cell types, and one set of 88 genes was identified that showed a decrease in expression with a loss of developmental potential, i.e., more differentiated cell types. These results were consistent with the notion that adult stem cells acquire or retain pluripotency with characteristics of less defined cell types and that ES and EG cells contain a limited but unique set of transcripts that differ from signature molecules in adult stem cells. Because development is often considered to involve a sequential activation and repression of genes, it is likely that differences in transcript abundance were indicative of defined differentiation or developmental stages.

Global expression profiles for hES cells have now been published by several groups (31, 48, 103, 138, 315, 339). A common finding among these studies is the existence of gene transcripts that are present at significantly higher levels in undifferentiated cells than in fully differentiated cells; however, many of the findings, like those for mouse, vary widely among studies. Carpenter et al. (70) had previously shown from FACS analysis that hES cell lines, which had been derived in the same laboratory using similar techniques, consisted of heterogeneous population of cells that make it difficult to quantify their transcriptomes under standard cultivation conditions. Of the cell lines accessible for study, many may also have been isolated at slightly different stages of blastocyst maturation and under different conditions. For these reasons, transcriptome comparisons among hES cell lines are open to interpretation.

Sato et al. (315) published the first analysis of differentiated and undifferentiated human ES cells (Line H1). A set of 918 genes was enriched in undifferentiated cells, including numerous ligand/receptor pairs and secreted inhibitors of the FGF, TGF-$\beta$/BMP, and Wnt pathways, which they suggested to be important for the regulation of hES cells. Two hundred twenty-seven transcripts were shared by the list of mES cell enriched transcripts reported by Ramalho-Santos et al. (284). This is noteworthy because these findings suggested that the molecular programs, which underlie ES cell identity, at least partially, seem evolutionarily conserved at a molecular level. Subsequent analyses, however, suggested that genes implicated in "stemness" of mouse embryonic and adult stem cells differ from those gene sets identified in hES cells (103). Sperger et al. (339) compared the expression profiles of hES cell lines with human germ cell tumor cell lines, tumor samples, somatic cell lines, and testicular tissue samples. The goal of this study was to identify genes specifically expressed at a higher level in pluripotent cell types. Based on the microarray data, the five ES cell lines examined clustered together and secondarily clustered as a branch of EC cell lines, suggesting that their expression patterns were more similar to each other than to any of the other cell types used in this analysis. They furthermore suggested that EC cells most closely resemble transformed ICM or primitive ectoderm cells.

A few general findings were consistent among the studies. These included the presence of transcripts to Oct-3/4, Nanog, Tdgf1, Utf1, and lin-28 in undifferentiated hES cells, but remarkably, Sox2, Dnmt3B, gp130 and Rex-1 (ZFP42) were inconsistently or poorly expressed among several lines (31, 103, 138). Among differentially regulated gene transcripts were several components associated with signaling pathways (48), several of which have been suggested to play key roles in hES cell growth and/or differentiation. These included Wnt, BMP, FGF receptor, and Nodal (Lefty A and B, Nodal and Pitx2) signaling, but not LIF receptor/gp130 signaling. Even though the FGF receptors are relatively abundant in these cells, the distribution of these receptor subtypes was highly heterogeneous (70), as is likely to be the case for most other signaling components commonly associated with hES cells.

### B. Serial Analysis of Gene Expression

In the first attempt to quantify the functionally active genome of ES cells, we employed serial analysis of gene expression (SAGE; see Fig. 8), which is a sequence-based technique that relies on short sequence tags to identify transcripts present in a cell (373). Although we initially used SAGE to define the transcriptomes of P19 EC and R1 ES cell lines (9, 10), only two other mouse SAGE libraries were available at that time for comparative purposes, precluding a clear analysis of the molecular basis for the embryonic stem cell phenotype. Recently, two SAGE libraries were constructed from hES cells (296). Like the microarray data presented earlier, the human data suffered from considerable heterogeneity among cell lines. In one of the cell lines, for example, transcripts encoding Rex-1 were highly abundant, but absent in the second. Although the authors suggested that Rex-1 might be dispensible for the derivation of human ES cells, it is more likely that the hES cell line lacking Rex-1 was more closely associated with primitive ectoderm (339), which does not normally express Rex-1 at least in mouse. Com-

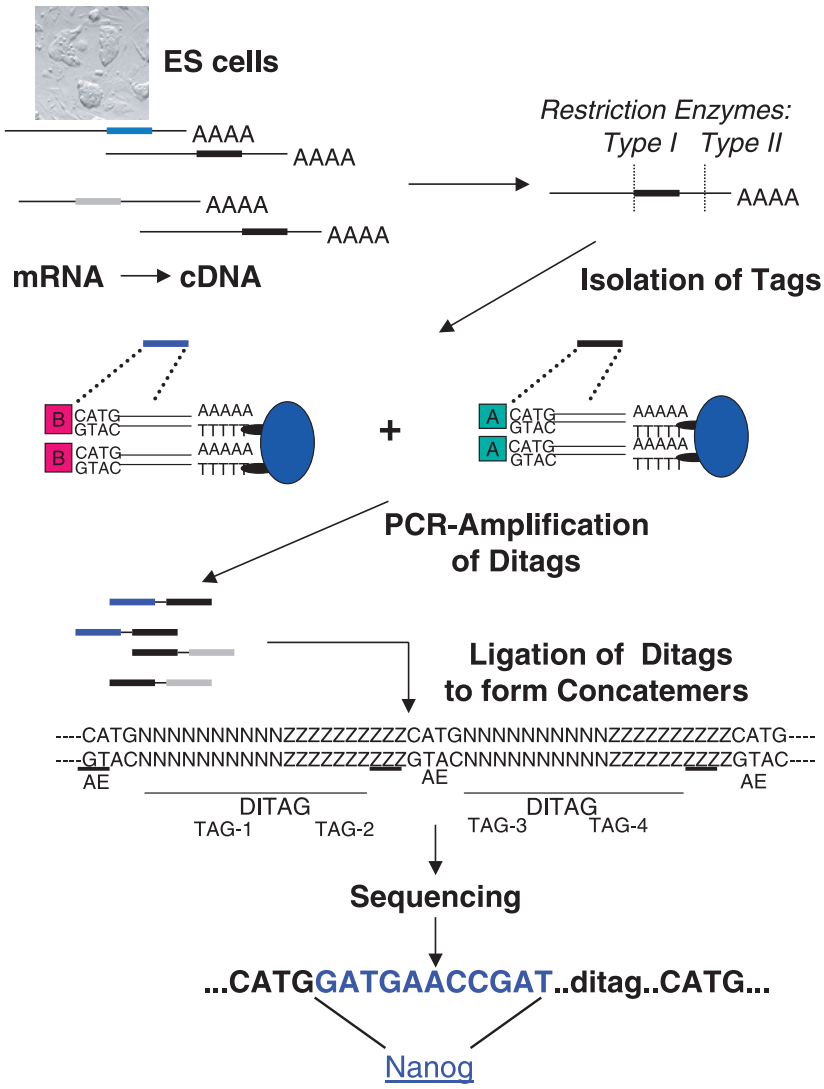


FIG. 8. Principal steps of serial analysis of gene expression (SAGE). Two assumptions are critical for SAGE analyses: *1*) short DNA sequences (10–14 bp) are sufficient to identify individual gene products and *2*) concatenation (linking together) of short DNA sequences or tags increases the efficiency of identifying expressed mRNAs in a sequence-based assay. To generate the sequences, purified mRNA from ES cells (or any other cell line) is used to generate double-stranded cDNA. With the use of streptavidin-coated magnetic beads, double-stranded cDNA is purified, followed by digestion with a type I restriction enzyme or anchoring enzyme that recognizes specific sites located in the double-stranded DNA recognition sequence (CATG for *Nla*III). The fragments, located closest to the biotinylated primer, are purified by binding to magnetic beads, divided in half and ligated to two linker/primer sets. SAGE tags are generated by digestion of the cDNA molecules with a type II restriction enzyme or tagging enzyme, which cleaves DNA several bases away from the recognition sites. The SAGE tags are joined to form ditags and amplified by PCR with a set of primers that recognize linkers A and B. The ditags are separated from the linkers and ligated together to form concatemers of purified ditags. These are then subcloned into a plasmid vector, amplified, and sequenced. The individual tags can then be extracted by identifying the CATG anchoring enzyme sequences. Each individual tag sequence is then run against GenBank databases to identify the corresponding gene product, and comparisons among SAGE libraries (http://www.ncbi.nlm.nih.gov/sage/) facilitate the identification of factors implicated in ES cell identity. [Adapted from Boheler and Wobus (43).]

parisons with the mouse R1 ES cell SAGE library indicated considerable differences between the transcriptomes of mouse and human ES cells. Members of the LIF signaling pathway (STAT3, LIFR, and gp130) were much more highly expressed in mouse than in human ES cells, whereas Oct-3/4 and Sox2 were more highly abundant in human than mouse ES cells.

Because SAGE data are quantitative in nature, we were able to use the R1 mouse SAGE dataset to estimate the total number of transcripts present in ES cells. For statistical reasons, it proved difficult to estimate accurately the total number of unique transcripts, but a simple correction indicated that >54,000 unique transcripts must be present, and model simulations indicated that 130,000 unique transcripts were compatible with the R1 ES cell sampling profile (343). Because ~10% of the tags in this SAGE library did not map with any previously described EST dataset, we estimated that the number of unique transcripts (splice variants or novel gene transcripts) that have not yet been identified in ES cells remain quite high (~6,000–13,000), underscoring a potential limitation in our ability to define the molecular basis of ES cell identity.

Since our initial SAGE analysis of mouse R1 ES cells, over 40 mouse SAGE libraries, including two additional ES cell lines (D3 and ESF 116) and one from an EG cell line (EG-1), have been deposited in the public domain, which have permitted us to identify transcripts with expression patterns similar to that of Oct-3/4 (unpublished data). We have been able to exploit the comparative power of SAGE (http://www.ncbi.nlm.nih.gov/SAGE), which increases as a function of the number of publicly available libraries, to confirm or refute the authenticity of other stemness-associated transcripts. As an example, we have taken a subset of known and putative stemness factors identified from microarray analyses and compared

the abundance (tags per million) of each transcript among 40 SAGE libraries. Based on these analyses, we would conclude that Mdr1 and the LIF receptor are not stemness-restricted factors but that factors like UTF-1, Dppa-5, Sox2, and Tdgf (in addition to Oct-3/4 and Nanog) are authentic embryonic stemness-related transcripts, whereas other transcripts, like those to Thy1 (see Table 2), would be excluded from our stemness list because of its elevated expression levels in testes and cerebellum.

Based on all available transcriptome (microarrays and SAGE) evidence, it is likely that ES cells contain a relatively small set of novel molecular markers/transcripts implicated in stemness. It is also likely that molecular determinants of pluripotentiality versus differentiation will involve a constellation of factors working in concert to regulate a stem cell's choice, but functional studies similar to those described for Nanog (233) and Wnt signaling (314) will be required before any specific signature factor can be unequivocally associated with stemness or a defined progeny.

### C. Proteomic Analyses

The molecular basis of ES cells and their ability to differentiate into cell lineages is a complex process that involves altered protein abundance resulting from changes in gene expression (transcription, polyadenylation, splicing, etc.) as well as protein regulatory events associated with translation (initiation, elongation, termination) and PTMs. Proteomic approaches have therefore been deemed essential to the visualization and interpretation of the cellular phenotype of undifferentiated ES cells. As a first step in this analysis, Elliott et al. (106) have established a proteomic database of mouse R1 ES cells analyzed by two-dimensional gel electrophoresis coupled with mass spectrophotometric techniques. Of the 700 spots analyzed, 241 distinct protein species were identified that corresponded to 218 unique proteins, approximately one-half of which were specifically associated with DNA maintenance, transcription, translation, and protein processing. Almost 21% of the proteins exhibited some form of PTM (e.g., phosphorylation, palmitoylation), and several of the proteins (e.g., peptidyl prolyl *cis-trans* isomerase A and FK506-binding protein 4) had not been previously associated with PTMs in other tissues. Although it is difficult to conclude how widespread these events are until comparisons have been made among ES cell lines of mouse and human origin, these data confirm that highly abundant proteins in mouse ES cell lines in vitro undergo substantial PTMs and that transcriptome analyses alone are insufficient to account for the molecular and cellular basis of embryonic stemness.

## VII. USE OF EMBRYONIC STEM CELLS IN PHARMACOLOGY AND EMBRYOTOXICOLOGY

The therapeutic potential of stem cells has been widely discussed, but stem cells also represent a dynamic system suitable to the identification of new molecular targets and the development of novel drugs, which can be tested in vitro for safety or to predict or anticipate potential toxicity in humans (94). Human ES cell lines may, therefore, prove clinically relevant to the development of safer and more effective drugs for human diseases. Three aspects are relevant to this issue. *1*) At present, insufficient methods exist in some areas of in vitro toxicology to predict target organ toxicity. *2*) In embryotoxicology, interspecies variation complicates data analysis, and human cell systems may enhance the identification of hazardous chemicals. *3*) Human ES-derived cells cultured in vitro may reduce the need for animal testing in pharmacotoxicology.

In the short-term, the application of hES cells in pharmacology and embryotoxicology could have a direct impact on medical research, but to date, such an approach has primarily been used with mouse ES cells.

The first pharmacological investigations with mES cells were performed on ES cell-derived cardiomyocytes to test the chronotropic activity of cardiovascular drugs (398). Cardiac-specific agonists and antagonists were also applied to characterize the physiological properties of cardiomyocytes dependent on the developmental stage (216). The functional properties of cardiomyocytes enabled the establishment of a semi-automated imaging system for screening of cardiac-specific drugs (395). The MEA approach (see Ref. 155) fostered insights into the physiological properties of ES-derived cardiomyocytes, such as action potential propagation and the development of arrhythmias. Similarly, patch-clamp studies have been employed to characterize the pharmacological properties of ES-derived neuronal cells (350) and dopaminergic neurons (191, 206).

ES-derived systems are of special importance for the investigation of embryotoxic properties of teratogenic agents. One of the most effective teratogenic agents known so far is RA, a drug that has already been used to induce differentiation of EC cells into neuronal cells (178). RA when applied to ES cells at various stages of EB formation significantly affect the differentiation of ES cells in a time- and concentration-dependent manner. High concentrations of RA applied during early EB development induce the differentiation of neurons, while lower concentrations applied at later EB stages promote the differentiation of skeletal and cardiac muscle cells (397). ES cells have also been employed to analyze the antiangiogenic capacity of drugs in an EB model (381). Moreover, by using the ES cell system, Sauer et al. (316)

presented experimental evidence for the primary molecular mechanisms responsible for the teratogenic effects of thalidomide, which inhibited angiogenesis in ES-derived EBs by the generation of hydroxyl radicals.

The ES cell test, EST, was established based on the observations that EB formation at least, partially, parallels developmental processes of early embryogenesis (204, review in Ref. 306) and RA affects lineage-dependent development within EBs (397). The EST includes a set of cytotoxicity and differentiation tests. Specifically, embryotoxic agents are applied during differentiation, and the cytotoxic and differentiation inhibiting activity of the compounds are analyzed (340). On the basis of these data, a prediction model has been proposed, which allows the discrimination of chemical agents into three classes as "nonembryotoxic," "low embryotoxic," and "high embryotoxic" compounds (137). Importantly, the in vitro data have a high correlation with in vivo embryotoxicity (319).

Because the EST is rather labor intensive and requires skilled personnel, alternative strategies including those suitable for a high-throughput screening of chemicals for embryotoxicity test systems have been proposed. For example, FACS analysis of ES cell derivatives labeled by fluorescence markers (EGFP) controlled by tissue-specific promoters can be used to test for toxic effects of chemicals (50, 341). A further modification includes the use of a combined system of metabolic competent cells and ES cells for the analysis of proteratogens, such as cyclophosphamide (49). The ES cell system can be applied to analyze the effects of physical factors, such as electromagnetic fields (EMF), on cellular functions of ES-derived populations. Recent studies from our lab indicate that wild-type ES cells after EMF exposure did not alter transcript levels for stress response and immediate early genes, whereas loss of p53 in ES cells affected transcript levels of regulatory genes (88, 89).The application of genomics and proteomic technologies to stem cell-based systems will also offer new molecular approaches for pharmacotoxicity and embryotoxicity screening on a large scale (see Refs. 10, 284 and sect. VI).

Besides embryotoxicity tests, cytotoxicity and mutagenicity in vitro test systems have been adapted using ES, EC, and EG cells (for review, see Ref. 305). In this context, it is interesting to note that ES and somatic cells differ in their mutation frequency. Mutations were less frequent in ES cells than in somatic cells; however, extended culture of mES cells led to an accumulation of cells with mutations (uniparental deficiency) rather than loss of heterozygosity (72).

## VIII. REQUIREMENTS OF STEM CELL-BASED THERAPIES

Today's most urgent problem in transplantation medicine is the lack of suitable donor organs and tissues, and treatments to replace, repair, or enhance the biological function of damaged tissue through cell transplantation/replacement therapy have until recently been limited to a few systems (41; review in Refs. 132, 384). Potential sources of cells for repair are self (autologous), same species (allogeneic), different species (xenographic), primary or immortalized cell lines, and adult stem cell-derived donor cells. The ability to cultivate, multiply, and manipulate these cell types has either limited or encouraged their use in specific treatment protocols (132). Presently, only allogeneic or matched donor-derived stem cells have been used in human cell-grafting therapies. While the differentiation potential of some adult stem cells (hematopoietic and mesenchymal) are well-characterized in vivo (HSC) or in vitro (MSC), the transdifferentiation potential of most adult stem cells remains controversial (235, 378), partly as a consequence of culture conditions (175) and contaminations or cell fusion events (3, 358). Regardless of these limitations, it is to be anticipated that human (embryonic and adult) stem cell research may help millions of people who are affected by a wide range of intractable human ailments (Parkinson's disease, spinal cord injuries, heart failure, and diabetes; see Table 5).

The in vitro developmental potential and the success of ES cells in animal models demonstrate the principle of using hES-derived cells as a regenerative source for transplantation therapies of human diseases. Before transfer of ES-derived cells to humans can proceed, a number of experimental obstacles must be overcome. These include efficient derivation of human ES cells in the absence of mouse feeder cells, and an understanding of genetic and epigenetic changes that occur with in vitro cultivation. It will be necessary to purify defined cell lineages, perhaps following genetic manipulation, that are suitable for cell-based therapies. If manipulated, then it will be important to guard against karyotypic changes during passaging and

TABLE 5. *Persons in the United States affected by diseases that may be helped by human pluripotent stem cell research*

| Condition | Number of Persons Affected |
|---|---|
| Cardiovascular diseases | 58 Million |
| Autoimmune diseases | 30 Million |
| Diabetes | 16 Million |
| Osteoporosis | 10 Million |
| Cancer | 8.2 Million |
| Alzheimer's disease | 4 Million |
| Parkinson's disease | 1.5 Million |
| Burns (severe) | 0.3 Million |
| Spinal cord injuries | 0.25 Million |
| Birth defects | 150,000 (per year) |
| Total | 128.4 Million |

Data from the Patients' Coalition for Urgent Research, Washington, DC (according to Perry, Ref. 267).

preparation of genetically modified ES-derived cells. Once introduced into the tissue, the cells must function in a normal physiological way. Finally, assurances against the formation of ES cell-derived tumors and donor/recipient immunocompatibility are additional requirements of stem cell-based therapies. As pointed out, significant progress has been made in the isolation of defined cell lineages in mouse, and important advances have already been seen with hES cells. Before therapeutically applicable, any ES-based treatment must, however, show limited potentials for toxicity, immunological rejection, or tumor formation, and at present, human ES cell research has not reached this threshold.

### A. Genetic and Epigenetic Concerns

About 70 human ES cell lines (excluding those held in the private sector and established more recently) have been described that are available for research, but at present, only ~22 of them can be propagated in culture (see http://escr.nih.gov/). Although some of the hES cell lines can be cultivated indefinitely and demonstrate a normal chromosomal complement after 2 or more years of passaging, this does not necessarily mean that these cells are genetically stable during long-term culture (and correspondence by 62, see Ref. 100). In somatic cells from humans and other animals, approximately one mutation occurs every cell division. A cell that has divided 200 times in culture would therefore be expected to contain ~200 mutations (195). The majority of these mutations may occur without consequence, but in those instances where protooncogenes or regulatory sequences are affected, the consequences may render the cells unsuitable for therapeutics.

Epigenetic modifications, such as DNA methylation, acetylation, histone modification, and other changes in chromatin structure that do not alter the genomic sequence, would also be expected to play an important role in the developmental potential of ES cells. We have already described how batches of serum or serum withdrawal, which causes epigenetic modifications (30), can affect the differentiation potential of mES cells and how altered functional levels of Oct-3/4 would be expected to modify development (see sect. II). In fact, epigenetic changes that decrease Oct-3/4 levels cause a decrease in cell number in mouse clone blastocysts that would be expected to adversely affect development (44). The fact that the vast majority of cloned embryos die during embryonic development, despite their normal chromosome complement, also suggests that epigenetic reprogramming in reconstructed oocytes is incomplete (297). The consequences of uncontrolled epigenetic modifications are only now being analyzed in hES cells.

Based on these data, it is likely that the current supply of human ES cell lines may be insufficient to adequately test their potential for cellular therapeutics. Additional or freshly isolated ES cell lines may be a constant requirement, but with the current legal constraints, this may not be possible in all countries. The generation of ES cell-derived germ cells (136; see Refs. 164, 365) may represent one possible alternative source for these cells, but before this can occur, it will be necessary to determine whether gametes can be obtained from hES cells that are capable of forming blastocyst-like structures. This of course brings up one additional concern: gametes generated from ES cells will have undergone prolonged cultivation times with accumulating genetic and epigenetic defects, which may render these cells of limited value, except in the context of nuclear transfer (see sect. IX*B*).

### B. Tumorigenesis

It is well established that undifferentiated, early embryonic cells commonly generate teratomas or teratocarcinomas when transplanted to extrauterine sites (346; see sect. I). This is not surprising, because ES cells display many features characteristic of cancer cells (57) including unlimited proliferative capacity (351), clonal propagation, and a lack of both contact inhibition and anchorage dependence. Tumor growth in immunodeficient animals appears to depend primarily on the presence of an undifferentiated stem cell population. Benign teratoma formation would therefore be expected at the site of injection and potentially at other locations whenever undifferentiated ES cells are present. Short-term, tumor formation does not appear to be a significant problem; however, few long-term animal experiments have been performed to demonstrate that transplantation of ES cell-derived donor cells do not give rise to tumors. Importantly, it is not simply the transplantation of mouse (396) and human (362) ES cells that results in the growth of teratomas, but also the transplantation of ES-derived differentiated cell populations (38, 191). The passive elimination of undifferentiated cells via lineage selection protocols as described below may therefore prove insufficient to eliminate the cancer risk. It may be necessary to develop additional strategies for the active elimination of tumorigenic cells by directing the expression of suicide or apoptosis-controlling genes in graft tissue.

### C. Purification and Lineage Selection

Because of the potential tumorigenicity of human ES cells (362), protocols have been established to purify committed cells of the desired phenotype and exclude nondifferentiated cells from cell grafts. In this context, early tissue-restricted stem and progenitor cells, characterized by a limited potential for self-renewal (i.e., cells

may not be tumorigenic), a high proliferative capacity, and the ability to generate a number of differentiated cell progeny, are of special interest. Two major experimental schemes have been devised to isolate such progenitor or tissue-specific stem cells from differentiating ES cells: *1*) selection of specified progeny through the use of cell surface markers coupled with flow cytometric fluorescence-activated (FACS) or magnetic-activated cell sorting (MACS) selection and *2*) genetic manipulation to introduce selectable markers and/or therapeutic genes.

As examples, Li et al. (210) employed a drug-resistance gene under the control of a lineage-specific promoter. In this "lineage selection" experiment using mES cells, a neomycin cassette was targeted to the neuron-specific SOX2 gene. After selection with neomycin (G418), only those cells expressing the neomycin gene under the control of the SOX2 promoter survived, resulting in the development of an apparently pure population of neuroepithelial cells, which subsequently differentiated into neuronal networks. Similar strategies have been employed for the isolation of skeletal muscle cells using MyoD as a target gene (95). For the selection of cardiac cells (from a low yield of ~3–5% cardiomyocytes in ES-derived populations), targeting of the cardiac $\alpha$-MHC gene promoter has yielded populations consisting of 99.6% cardiac myocytes (192). Recently, a lineage selection strategy combined with specific culture conditions was successfully employed to generate a neural progenitor population of high purity (15).

FACS sorting of cells expressing enhanced green fluorescent protein (EGFP) offers an alternative (and substitute) to drug selection and has been used to isolate cardiac myocytes from D3 ES cells expressing the EGFP under the control of the cardiac $\alpha$-actin promoter (193). A similar strategy has proven successful for the isolation of ventricular cells following targeting of the MLC-2v promoter by ECFP (enhanced cyan fluorescent protein) and EGFP (229, 237). The direct sorting of differentiated cells using fluorescent antibodies and magnetic microbead-tagged antibodies by MACS is especially feasible for cell types, which express defined surface antigens, as is the case for cells of the hematopoietic lineages (159).

Because no single drug-resistance or fluorescence-based enrichment procedure generates a 100% pure population of cells, it may prove useful to combine the two using antibiotic resistance and EGFP expression (by FACS or MACS) together with cultivation in specific growth factors as done by Marchetti et al. (220). Attempts are underway to test similar selection systems with hES cells.

### D. Tissue-Specific Integration and Function

One of the critical questions concerning the potential therapeutic use of ES-derived cells is whether cells produced by a particular in vitro differentiation protocol can integrate into the recipient tissue and fulfil the specific functions of lost or injured cells. This seems to be possible for at least some mES-derived progeny, since a degree of specific function has been reported following transplantation (Table 6, see sect. IX). In pilot experiments designed to analyze the potential of human ES-derived neural progenitor cells to integrate into the developing brain, the transplanted cells integrated into the developing nervous system of mice (292, 415). Similarly, colonies

TABLE 6. *Transplantations of mouse and human ES-derived cells into animal models*

| Cell Type | Transplantation Into | Reference Nos. |
|---|---|---|
| Cardiomyocytes (m) | Myocardium of dystrophic mice | 192 |
| RA-induced GABAergic neurons (m) | Rat striatum: integration | 95 |
| Neural progenitors (m) | Embryonic rat ventricles | 55 |
| | Rat striatum: integration | 12 |
| | Mouse cerebrum: integration | 6 |
| FACS-sorted postmitotic neurons (m) | Telencephalic vesicle of embryonic rat: integration | 386 |
| Glial precursors (m) | Myelin-deficient rat (Pelizaeus-Merzbacher disease): integration and function | 54 |
| RA-induced neurons (m) | Injured rat spinal cord: function | 226 |
| Motor neurons (m) | Chicken spinal cord: integration and muscle innervation | 389 |
| Midbrain dopaminergic neurons (m) | Parkinson rat model: function | 25, 191 |
| Neural progenitors (h) | Mouse brain ventricles | 292 |
| | Neonatal mouse brain: integration | 415 |
| Insulin-producing cells (m) | Streptozotocin-treated diabetic mice: normalization of blood glucose levels | 38, 162, 338 |
| Hepatocytes (m) | Mice with $CCl_4$ intoxicated liver damage: regeneration | 405 |
| Hematopoietic precursors (m) | Irradiated mice: myeloid and lymphoid engraftment | 196 |
| Undifferentiated mES cells | Spleen of immunosuppressed nude mice | 80 |
| | Infarcted myocardium of rats | 231 |
| Undifferentiated hES cells | Immunocompromised mice | 252 |
| | Somites of chick embryos (E1.5-2d) | 139 |

m, mouse; h, human.

of hES cells have been grafted directly adjacent to the host neural tube of chick embryos. These cells subsequently differentiate into primary structures with morphologies and molecular characteristics typical of neural rosettes and differentiated neurons (139). Although it is too early to conclude normal, full, or protracted functioning of transplants derived from hES cells, these earliest findings are clearly encouraging, but extensive experimentation in large-animal models will be required before application in humans.

### E. Immunogenicity and Graft Rejection

One major problem potentially associated with the use of hES-derived cells for tissue regeneration is the immunological (in)compatibility between donors and recipients. Clearly, uncontrolled immune reactions would lead to rejection of mismatched grafts. Although the levels of MHC-I expression on hES cells are low, they increase moderately after differentiation either in vitro or in vivo, and markedly following interferon treatment (101). The absence of MHC molecules may also lead to natural killer cell rejection of the transplanted cells. Several approaches to reduce or eliminate ES-derived graft rejection have therefore been proposed.

*1*) One could reduce the host reactivity to allogeneic ES-derived transplants by classic immunosuppression, as is routinely employed for organ transplantation (132). Unfortunately, most of the immunosuppressive drugs currently used are associated with complications, including opportunistic infections, drug-related toxicities, skin malignancies, and posttransplantation lymphoproliferative disorders. A more specific suppression of immune rejection may be achieved by the cotransplantation of both therapeutic tissue and hematopoietic stem cells generated from the same parental ES cell line (see Ref. 252) or by preimmunization of recipients with preimplantation-stage stem cells, as has been recently reported to induce long-term allogeneic graft acceptance (110).

*2*) A tempting alternative to suppressing the immune rejection would be to avoid it completely by eliminating the genes responsible. The first report of successful homologous recombination in hES cells is an important step towards the generation of genetically modified ES cells for transplantations (419). One possibility is that the elimination of major histocompatibility complex (MHC) class I expression in hES cells may generate a "universal cell" that would be suitable for all patients (41, 101). Homologous recombination has been used to "knock out" MHC class I and class II molecules in mES cells; however, the consequences of such extensive gene targeting are difficult to assess (144). Additionally, loss of the MHC class I and class II molecules do not necessarily protect against rejection, because of indirect allo-recognition-mediated rejection and/or natural killer cell-mediated cell destruction.

*3*) Another option relies on the generation and storage of HLA-isotyped and/or genetically manipulated hES cell lines in a cell bank. Only humans with similar HLA molecules could be donors for other hosts. Practically this would require determination of allogeneic compatibility. For ES cells derived from one human individual, all HLA molecules would be clonal. As such, banks of ES cells with known HLA backgrounds could be established. According to some calculations based on organ transplantation data, a minimum of 200 or more ES cell lines generated from independent HLA subtypes would be required. The requirement of isolating multiple pure populations of ES cells with defined HLA molecules represents an enormous amount of work, may be unattainable, and under current law, i.e., in the United States, could only be performed in the private sector.

*4*) The fourth principal possibility relies on the generation of autologous donor cells through a process known as "therapeutic cloning" (201; see sect. IX*B*), which, in principle, follows the strategy used to create the sheep Dolly (392). In the therapeutic cloning approach, somatic cell nuclei of the patient would be fused to enucleated human eggs, which in vitro would be cultivated into blastocysts. From these cells, hES cell lines would be established and differentiated into the desired cell types for transplantation (201). Recently, two South Korean groups demonstrated the proof of principle for this strategy (165) (see sect. IX*B*). Such cells should be immunologically compatible, because they contain (except in the mitochondrial genome) the same genetic information as the patient. However, it is evident that the unlimited use of human oocytes for the generation of autologous donor cells would generate numerous ethical and legal problems (252; see also sects. IX and XI).

## IX. EMBRYONIC STEM CELL-BASED THERAPIES

Currently, no ES cell-based therapies are on going in humans. Only allogeneic or matched donor-derived adult stem cells have been employed in human cell-grafting therapies, the best examples of which are bone marrow transplantations for the treatment of leukemia after myeloablative therapies. The availability of human ES cells, however, represents an extraordinary opportunity for cell transplantation that may be applicable to a wide range of human ailments. Three properties make ES cells relative to adult stem cells very attractive for replacement therapies (158). *1*) Human ES cells can be grown indefinitely in culture. *2*) ES cells can be genetically manipulated, and loss of function genes (e.g., CTFR) can theoretically be repaired by the introduction of transgenes into ES cells

either by random transgenesis or through gene targeting. Importantly, homologous recombination could be used to correct specific genetic mutations that would not lead to random mutations in tumor-promoting genes. *3*) Numerous differentiation protocols have already been established that permit the generation of almost any cell type, either through the use of established culture conditions or when coupled with genetic manipulations. In theory, hES cells could be applied to a wide range of human ailments, but the proof of principle has largely come from the use of mouse ES cells.

### A. Animal Models for Cell Therapy

Mouse and human ES-derived progeny have been analyzed in various animal models of human diseases (see Table 6), and some examples are discussed with respect to cardiovascular and neurorepair and for the treatment of diabetes.

#### *1. ES cells for cardiac repair*

As described earlier, cardiac-restricted promoters have been used to select cardiomyocytes from differentiating ES cells (115, 192, 229, 237). Loren Field's group (192) published the first therapeutic demonstration of mouse ES cell derivatives. In this study, purified (99.6%) cardiomyocytes were injected into the ventricular myocardium of adult dystrophic (*mdx*) mice and were found to be present in the grafts for at least 7 wk after implantation, without tumor formation (192). Min et al. (231) subsequently reported improved left ventricular function in postinfarcted rats after transplantation of "beating cells" derived from ES cells. The engrafted cardiomyocytes expressed sarcomeric $\alpha$-actin, $\alpha$-myosin heavy chain, and troponin I and were rod-shaped with typical striations, suggesting differentiation into mature cardiomyocytes. ES cell-derived cardiomyocytes thus expressed myofilament proteins and were able to form "normal coupling units" with endogenous cardiomyocytes. It was unclear from these studies if the transplanted cardiomyocytes coupled normally with endogenous cells, or whether any beneficial effects of cell integration were long-term.

These results validate the potential use of ES-derived cardiomyocytes for cardiac therapy; however, experiments to generate 100% pure and stable cardiac grafts have not proven successful (see sect. IV*B*). Recent studies from our laboratories have also indicated that culture conditions can significantly affect the quality of the cardiac tissues generated from ES cells. We have found that changes in plating conditions can lead to ES-derived cardiac cells that are immature, arrhythmic, show signs of cell stress, and are predisposed to cell death via a p53-mediated cascade (unpublished data). These findings underscore the role of regulated cell-cell and cell-ECM interactions and the need to establish histotypic culture conditions for the generation of suitable cardiac grafts from in vitro-differentiated ES cells. The generation of cardiomyocytes growing in three-dimensional aggregates may offer an alternative.

#### *2. ES cells used for the in vitro formation of vascular structures*

Recently, human ES cells have been employed to isolate endothelial cells. During differentiation, transcripts characteristic for endothelial cells were detected, including GATA-2, PECAM1, Flk1, and VE-cadherin. PECAM1 antibodies were used to isolate endothelial cells from the 13-day EBs after enzymatic dissociation. The isolated PECAM1$^+$ cells were seeded onto highly porous PLLA/PLGA biodegradable polymer scaffolds (280), and the sponges were implanted subcutaneously into SCID mice (208). After development in SCID mice as well as after in vitro differentiation in Matrigel, microvessels developed from these hES cells. Similarly, rhesus monkey ES cells differentiated into endothelial cells and when introduced into a Matrigel plug and implanted subcutaneously into mice formed intact vessels and recruited new endothelial cell growth in vivo (184).

#### *3. ES cells for neurorepair*

The successful generation of apparently "normal" neural cell types from in vitro differentiated ES cells has naturally led to intense interest in their potential use to repair or limit the damage associated with infarct or neurodegenerative diseases. Brustle et al. (55) first demonstrated that ES cell-derived neural cells could survive, respond to environmental signals, and exhibit aspects of region-specific differentiation when introduced into developing mouse brain. They showed in a later study, and following in vitro generation of precursors for oligodendrocytes and astrocytes, that these transplanted cells interact with host neurons and myelinate the axons in brain and spinal cords in a rat model of human dysmyelinating Pelizaeus-Merzbacher disease (54). The resulting remyelination of axons led to a recovery of the pathological phenotype in the animals.

In transplantation experiments in which dissociated neural progenitors have been introduced to appropriate sites, mouse ES cells have also been found to differentiate into dopaminergic neurons, and to promote partial recovery in a rat model of Parkinson's disease (191). The efficient generation of midbrain dopaminergic neurons from human ES cells opens the possibility to test their therapeutic effects in animal models (see Ref. 266). mES-derived GABAergic neurons were found to survive after transplantation into a rat model of Huntington's disease

(95), while oligodendrocytes myelinated host axons after transplantation that could partially restore function in rodents with spinal cord injury (211, 225). These experiments provide a clear indication that mES cells can serve as a valuable source of specific neuronal and glial cells for transplantation (see Fig. 9 and Table 6). It remains to be seen whether transplanted hES-derived neural cells can persist and function over long periods. This issue has been highlighted by a clinical study, in which the transplantation of neural cells derived from fetal brain to Parkinson's patients showed no significant benefit; moreover, 2 years after surgery, some treated patients developed persistent dyskinesia (128). Nonetheless, this study provided important information about the ability of dopaminergic neurons to survive in humans.

### *4. ES cells for the treatment of diabetes*

As described in section IV*C*, mES cells differentiate into functional isletlike cells that are able to rescue experimentally induced diabetes in mouse models (38, 162, 207); however, similar strategies must be established with hES cells. Efficient selection methods with pancreatic lineage-specific promoters will be necessary to overcome current limitations, such as tumor formation of grafts and low (therapeutically irrelevant) insulin levels. In parallel with a common "lineage-selection" strategy, selection of differentiated pancreatic cells expressing glycolipids or other cell surface markers of pancreatic β-cells [i.e., A2B5 (105), 3G5 (276), IC2 (53)] might be feasible; however, it has to be shown that the composition of cell surface markers of ES-derived pancreatic cells is similar to those expressed in islets. The engineering of pancreatic islets in vitro clearly requires a further maturation of ES-derived cell clusters. Insulin-producing β-cells depend on specific signals from nonpancreatic cells: cell-to-cell interactions and characteristic "biosociology" are necessary for tissue-specific function of β-cells (272). This could be achieved by histotypic culture systems (38) that are additionally supported by vascularization. Finally, the maturation process could be enhanced by delivery of specific pancreatic transcription factors or developmental control genes in a "gain-of-function" approach.

Although the data presented so far illustrate the capacity of both mouse and human ES cells to differentiate into therapeutically useful cell types, it is still unclear whether hES-derived progeny would function normally in the body especially with respect to long-term functionality.

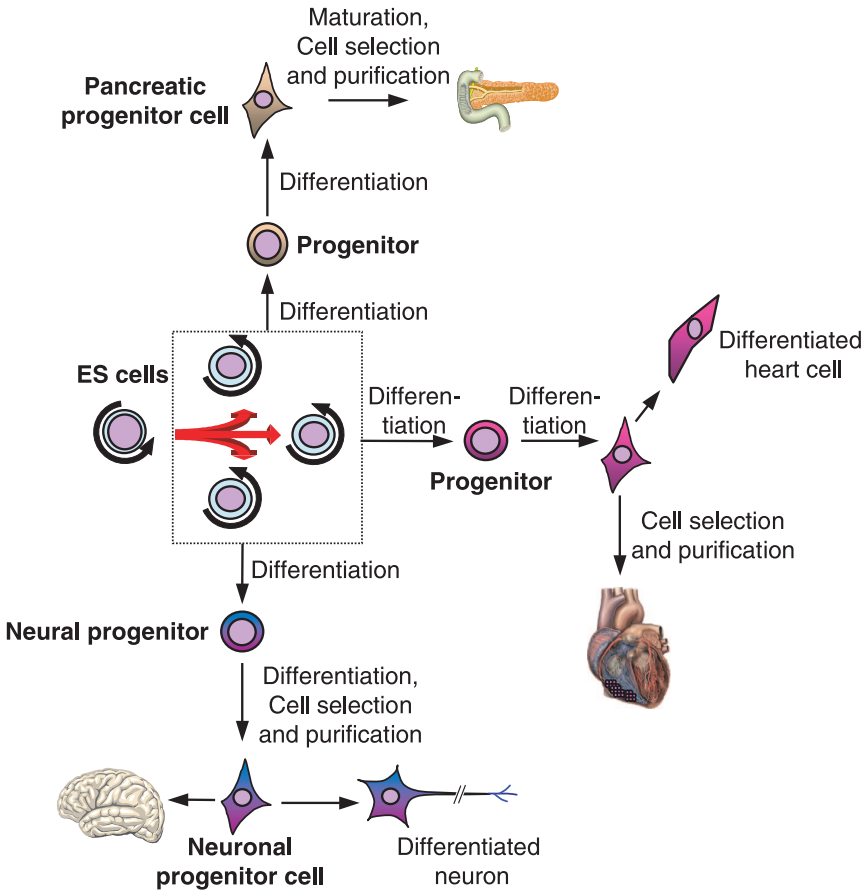


FIG. 9. Proposed strategies of cell therapy using human ES cells for the treatment of heart and central nervous system diseases and diabetes. Pluripotent human ES cells must first be propagated in vitro. ES cells may then be selectively differentiated into cardiac, neural, or pancreatic progenitor cells, which have the capacity for terminal differentiation in vitro. Defined progenitor cells are selected and purified followed by further differentiation/maturation and transplantation into the injured or damaged tissue to integrate and develop into functional cardiomyocytes, neurons, and pancreatic endocrine cells, respectively. For the treatment of cardiac infarcts or diabetes, mature cells may be necessary, whereas for the treatment of neurodegenerative diseases, neuronal progenitor cells could be applicable. [Adapted from Boheler and Wobus (43).]

### B. Therapeutic Cloning

Cloning is defined as the production of a set of individuals with the same genotype. This occurs naturally by asexual reproduction in hydra, sea anemone, planarians, and annelids (407), but cloning is also possible with mammalian somatic cells, as illustrated by the birth of Dolly the sheep in 1997 (392). Two forms of cloning are generally described from adult somatic cells: reproductive and therapeutic. Therapeutic cloning utilizes nuclear transfer techniques (238) to produce pluripotent ES cells with the genome of the nucleus of origin. Specifically the nucleus of an adult donor cell is introduced into an enucleated donor oocyte to generate a cloned embryo. The somatic cell nucleus, at a low frequency and depending on the donor cell type, may regain its pluripotentiality to initiate the earliest stages of embryonic development. If these cells are transferred to the uterus of a female recipient, then the developing embryo would have the potential to grow into an infant, in a process known as "reproductive cloning," i.e., Dolly. If the developing mass of cells is however left in culture, ES cells can be isolated from the inner cell mass of a developing blastocyst. The ES cells derived in this manner are genetically identical to the donor cells, except for the mitochondrial genome, and can be induced to differentiate into replacement cells for transplantation. This process is commonly referred to as "therapeutic cloning." Importantly, the differentiated cells generated in this manner are autologous, thus eliminating the problem of immuno-incompatibility and the requirement for immunosuppression (see Fig. 10). Moreover, ES cells provide a renewable source for replacement cells.

As pointed out earlier, ES cells are amenable to genetic manipulation. When combined with therapeutic cloning, ES cell derivatives offer the potential for both gene- and cell-based therapies. A demonstration of this potential was published in 2002 (298). Nuclear transfer (nt) ES cells were produced from tail tip cells of immu-

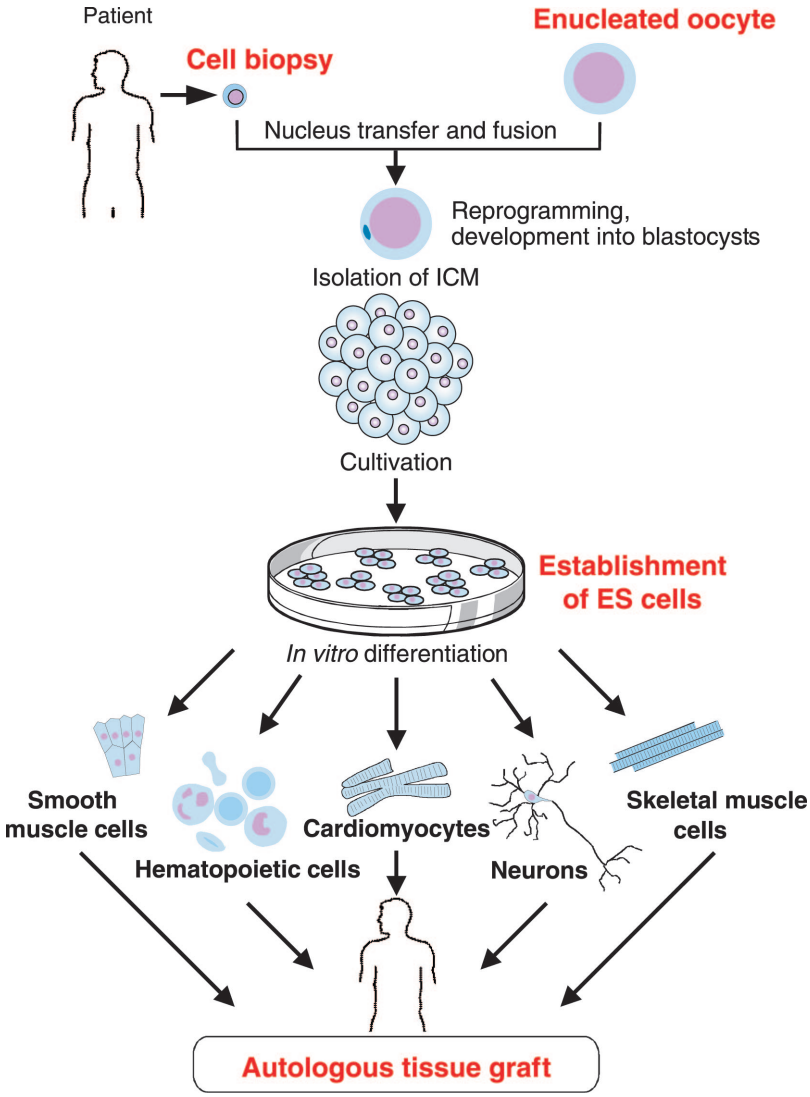


FIG. 10. Strategy of human "therapeutic cloning" to generate autologous tissue grafts. Somatic donor cell nuclei are fused to enucleated oocytes. In the context of the oocyte cytoplasm, the genome of adult cells is reprogrammed to an embryonic status. From this embryo, blastocysts are developed that are used to establish human ES cells. These ES cells are subsequently differentiated in vitro into the desired cell type to generate an autologous tissue graft for transplantation. [Adapted from Lanza et al. (201).]

nodeficient mice, homozygous for a knockout mutation in the "recombination activating gene" 2 (RAG2 mice). These mice lack mature B and T cells. By homologous recombination, this genetic mutation was "cured," and the targeted ntES cells were differentiated in vitro into EBs and into hematopoietic precursor cells by expressing HoxB4. When reintroduced into irradiated Rag2-deficient animals, these precursor cells partially repopulated the deficient immune system, and functional B and T cells were detected in these mice. Unexpectedly, the initial attempts at engraftment with these cells failed, because of an increase in natural killer cells. Immunosuppression was therefore required to rescue this phenotype. This experiment, however, serves as a proof of principle therapy where nuclear transfer was combined with gene therapy to treat a genetic disorder. More recently, Barberi et al. (25) showed that transplantation of ntES cell-derived dopaminergic neurons could correct the phenotype of a mouse model of Parkinson's disease.

Based on these findings, therapeutic cloning in combination with established hES cell protocols could offer a means to obtain autologous cells for the treatment of a variety of diseases. Proof of principle of this strategy has been reported. The application of the somatic cell nuclear transfer (SCNT) technology using human oocytes and cumulus (nucleus donor) cells resulted in the derivation of a pluripotent ES cell line from a cloned human blastocyst (165). After continuous proliferation for more than 70 passages, SCNT-hES cells maintained a normal karyotype, were genetically identical to the somatic nuclear donor cells, and showed differentiation capabilities in vivo (teratoma formation) and in vitro (165).

However, Mombaerts (234) has suggested that it will be prohibitively expensive to pursue this approach until the efficiency of nuclear transfer is improved or an alternative source of human oocytes can be found. The recent report of ES cell-derived oocytes suggests that the latter may be feasible (164), and if so, some of the ethical problems associated with therapeutic cloning may be overcome. Another possibility would be the reprogramming of adult somatic nuclei by fusion with hES cells (96). Before these principles can be applied clinically, it will be necessary to minimize epigenetic reprogramming of ES cells in culture, determine the genetic consequences of using aged nuclei, evaluate the effects of oocyte-derived mitochondrial proteins in somatic cells obtained by nuclear transfer, and reconcile the potential immunological rejection of these cells (158, 407). It is therefore important to continue research in this field to determine the potential of these therapies for humans.

## X. PROSPECTS FOR STEM CELL THERAPIES

ES cells are not the only source for possible therapeutics. Adult stem cells (ASC) can be coaxed into differentiated cells not normally associated with their "committed" state (131). Examples include hematopoietic stem cells from bone marrow that developed into neural, myogenic, and hepatic cell types, neural or skeletal muscle stem cells that developed into the hematopoietic lineage (33, 83, 131, 133, 148, 170, 269), stromal stem cells differentiating into cardiac myocytes (215), and mesenchymal stem cells into adipocytic, chondrocytic, or osteocytic lineages (273). The question therefore arises whether adult stem cells are the cell type of choice for cell therapies. While the differentiation potential of some adult stem cells (hematopoietic and mesenchymal) are well-characterized in vivo (HSC) or in vitro (MSC), the transdifferentiation potential of most adult stem cells remains controversial (235, 378, 379), partly as a consequence of culture conditions (175), contaminations, and cell fusion events (3, 358). Conversely, a major advantage in the use of ASC for cell replacement therapy is that they will not provoke immune-system rejection, should not become malignant, and may differentiate into a finite number of cell types.

Based on our present knowledge, ASCs, compared with ES cells, do not have the same developmental capacity. Injection of ASCs (hematopoietic or neuronal) into a mouse blastocyst can contribute to a variety of tissues, but the contribution differs in each embryo. Injection into animal models also leads to varying tissue contributions, the degree of which may depend on previous cultivation steps, since freshly isolated HSCs do not seem to transdifferentiate with high efficiency (378). Obviously, somatic stem cells of the adult organism may yet have a high plasticity, and their developmental potential may not be restricted to one lineage, but could be determined by the tissue environment in the body (383). The identification of such reprogramming factors will be one of the challenges of the future. These studies will show whether it may be possible to reprogram, not only adult somatic nuclei by fusion to enucleated eggs (64), but also to (retro- and/or trans-)differentiate adult somatic stem cells in response to "reprogramming" factors (see Ref. 379).

Finally, four therapeutic concepts using stem cells are currently being envisaged.

*1*) The direct administration of stem cells includes strategies for the administration of (adult) stem or progenitor cells directly to the patient, either locally or systemically, in such a way that the cells colonize the correct site of the body and differentiate into the desired cell type ("homing") under the influence of tissue-specific factors ("niche"). This strategy cannot be applied with ES cells, without prior isolation of ES-derived adultlike stem or progenitor cells (see Fig. 9), because of tumor formation (see sect. VIII*C*), but it has been successfully employed in rodent models with a variety of stem cells isolated as primary isolates, following cultivation in vitro or following genetic modification (219, 384).

*2*) Transplantation of differentiated stem cell progeny is a strategy that involves stem cell cultivation in vitro, differentiation and selection prior to transplantation into a target organ. As stated earlier, this may result in a number of genetic or epigenetic modifications, but it has an advantage, in that purified cell progeny can be isolated. The normalization of blood glucose levels by insulin-secreting cells represents one example. In the case of diabetes, it would be necessary that a cellular graft respond to high glucose levels in the bloodstream by controlled insulin release. At the present time, hES cells do not show this ability (13, 283, 324). The first attempts using genetically modified mouse ES cells in (streptozotocin-treated) diabetic mice are encouraging (38, 207), but at present, we are far from applicable cell therapy strategies for the treatment of diabetes.

*3*) Recent progress in tissue engineering using stem cells offers the possibility of organizing the cells into three-dimensional structures that can be used to repair damaged tissues. Tissue engineering often takes advantage of biodegradable scaffolds or novel peptide-based biomaterial scaffolds to form three-dimensional structures, which can be seeded with cells (stem cells and their progeny), grown in culture and subsequently grafted into the organ as needed. Examples include bone, cartilage, tendon, and muscle. The principles behind tissue engineering have been extensively reviewed elsewhere (119, 161, 200, 371).

*4*) The stimulation of endogenous stem cells is based on the possibility that self-repair could be induced or augmented by stimulating the patient's own stem cells by administrating growth factors. Bone marrow cells, for example, can be mobilized by stem cell factor and granulocyte-colony stimulating factor. In the case of myocardial infarction, these mobilized cells seem to be home to an infarcted region to promote myocardial repair (257). It is currently unclear whether the activation process or the release of factors from activated stem cells is more important to this therapeutic approach. A recent study showed that transplantation of adult bone marrow-derived cells reduces hyperglycemia in diabetic mice by initiating endogenous pancreatic tissue regeneration. Engraftment of bone marrow-derived cells to ductal and islet structures was accompanied by rapid proliferation of recipient pancreatic cells and neogenesis of insulin-producing cells of recipient origin. This strategy may represent a previously unrecognized means by which bone marrow-derived cells can contribute to tissue restoration (156). Many potential endogenous stem cell sources (liver, brain, skin, heart, bone marrow, intestine) are now recognized to be present in humans. Stimulation of endogenous sources of stem cells is currently only achievable from bone marrow. With the rapid advance of stem cell research, it is likely, however, that further advances will be made so that endogenous supplies can be mobilized to more readily repair and replace damaged tissues following injury.

## XI. OUTLOOK

Studies of human ES cells have demonstrated an enormous potential for generating tissues of therapeutic value, but we have also highlighted problems associated with inefficient differentiation, tumorigenicity, and immunogenicity in addition to the complexity of the ethical issues surrounding the isolation of cells from in vitro fertilized human embryos.

Five fundamental ethical principles are applicable to hES cell research: *1*) the principle of respect for human dignity, *2*) the principle of individual autonomy (informed consent, respect for privacy, and confidentiality of personal data), *3*) the principle of justice and of beneficence (improvement and protection of health), *4*) the principle of freedom of research (balanced against other fundamental principles), and *5*) the principle of proportionality (no alternative more acceptable methods are available) (370).

Ethical judgements about the use of human ES cells in research and therapies rely on the status of the embryo. If one feels that an embryo is a human being or should be treated as one because it has the potential to become a person, then it would be considered unethical to do anything to an embryo that could not be done to a person. At the opposite end of the spectrum, one could express the view that the embryo is nothing more than a group of cells that can be treated in a manner similar to tissues used in transplantation. An intermediate position would be to ascribe a special status to the embryo, and depending on its stage of development, the embryo could be considered less than human life and deserving of respect. Such a special status would necessarily impose some limits or restrictions on its use.

On the basis of these fundamental issues and in conjunction with specific sociocultural and religious traditions, different opinions reflect the various positions of countries involved in stem cell research and stem cell biotechnology. Most countries have passed bioethical regulations or laws about principal requirements of human embryo and hES cell research (see Ref. 335). These regulations differ mainly because countries have different views regarding the status of the human embryo, which determine whether early embryonic stages are subject to manipulation. Because scientific success in stem cell research is developing so rapidly, such rules are under continuous change [for special regulations of hES cell research, see http://www.aaas.org/spp/sfrl/projects/stem/main.htm; www.nih.gov/news/stemcell/ (USA); www.nibsc.ac.uk/divisions/cbi/stemcell.html (UK); http://www.shef.ac.uk/eurethnet/news/index_news.htm (European coun-

tries including UK); http://www.aph.gov.au/house/committee/laca/humancloning/contents.htm; Australia].

Parallel to the extensive research activities using hES cells over the past 4–5 years, numerous reports of the presence of multipotential stem cell activity in adult tissues have raised hopes that these may offer an alternative and more acceptable source of regenerative tissue for transplantation purposes. However, as discussed in section IX, recent studies also highlight a number of uncertainties concerning the true extent and nature of the differentiation/transdifferentiation capacity of adult stem cells.

One of the major challenges for the emerging field of stem cell research will be the development of in vitro culture conditions that tease out and maximize the required regenerative potential from cultured stem cells. This is likely to require an understanding of the extrinsic signals, which recruit and direct stem cells in vivo, and of the intrinsic (endogenous) circuits, which both define and limit the ability of a stem cell to respond to a given set of conditions. A detailed understanding of these processes will require continued studies of the mechanisms of embryonic and adult stem cell biology and the identification of those factors and signaling components that are necessary to generate and to manipulate stem cell progeny for therapeutic applications. Although we cannot currently use ES cell-based therapeutic strategies in humans, the recent technical achievements of cell and molecular biology will positively influence stem cell research and, in the future, should result in the generation of functional tissue grafts for clinical applications.

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