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Stem cells can follow a variety of blueprints directing cell differentiation. But the final edifice is not built of stone, and differentiated cells show remarkable plasticity. [Image from the cover of Science 25 February 2000]
INTRODUCTION

Stem cell research represents an area with great scientific and therapeutic promise. Because stem cells are unique in their ability to self-renew and to differentiate into multiple cell types, they can be used to elucidate normal cell processes as well as to understand the diseased condition. In addition, stem cells from individuals with various diseases can be utilized for biotechnology advance, for example, in drug screening. There is a widespread hope that these cells will one day be used to treat human injury and disease. If, for example, stem cells can be directed to differentiate into specialized nerve cells, they could be used for the repair of a severed spinal cord or replacement of neurons destroyed by neurodegenerative conditions such as Parkinson’s disease. Directed differentiation of stem cells into muscle cells could facilitate repair of an injured heart after cardiac infarct; stem cells differentiated into insulin-producing cells could treat patients with diabetes.

Despite these grand purposes, the mention of stem cell research evokes varied images and viewpoints—both in favor of and opposed to the work. The complexity of this topic ranges from debate over usage of simple terms to heated disagreement about multifaceted moral, ethical, and political issues. The latter concerns demand stringent oversight and public dialogue regarding funding issues, informed consent, patenting, and the status of the embryo. The articles in this collection represent a small sampling of exciting advances in stem cell biology as they have been presented in Science magazine over the past few years.

The term “stem cell” does not pertain to a single, defined cell population. Instead, the description applies to a large number of very different cells: embryonic stem cells, germline stem cells, and adult somatic stem cells, including stem cells from umbilical cord blood and progenitor cells such as those in the hematopoietic and neural lineages.

As an organism grows, the developmental potency, or the range of possible cell fates, gradually decreases for most cells. However, within the organism, a supply of cells is needed for tissue regeneration and repair. Stem cells fit the bill. By definition, stem cells self-renew and have the ability to progress down multiple cell lineages. The number of cell types that can be obtained is variable, dependent on the intrinsic properties of the stem cell population and as influenced by the cell’s natural microenvironment, or niche, or by special cell culture conditions. Fertilized eggs show the greatest degree of developmental potency. They are totipotent, that is, they can form an entire organism. Embryonic stem cells also display great developmental potency. They are pluripotent and can differentiate into cells of all three germ layers, as well as the germline. A major gain in the human embryonic stem cell arena was realized in 1998 with the isolation of embryonic stem cells from human blastocysts. This work enabled research on nontransformed human cells with unlimited growth in tissue culture.

Stem cells in adult somatic tissues are generally thought to progress through strict tissue-specific cell fates. Hematopoietic stem cells, which are capable of forming the different types of blood cells in the body, have been most thoroughly investigated. We know the detailed lineage of hematopoietic cells, and hematopoietic progenitor cells have been successfully used in human therapy for decades. Adult stem cells have been reported elsewhere in the body, such as the mammary gland, prostate, lung, epidermis, intestine, and in umbilical cord blood. Claims have been made that adult somatic stem cells, such as hematopoietic stem cells, can switch to another cell fate (termed plasticity) or revert to a less differentiated state. However, in vivo the number of hematopoietic-derived cells found in nonhematopoietic tissues, for example, is quite small. In addition, cell fusion events, in which a stem cell combines with a more differentiated cell, explain some observations where implanted stem cells display differentiation markers of another cell lineage. Providing examples of reversion to a less differentiated state, studies employing somatic cell nuclear transfer (SCNT) in animals such as mice, pigs, and sheep have shown that somatic cell nuclei can be reprogrammed when placed inside an enucleated oocyte. Embryonic stem cells derived from SCNT blastocysts can give rise to cells of all three germ layers. Furthermore, if SCNT embryos are implanted in an animal uterus, development proceeds through the embryonic and fetal stages to produce viable animals; however, this method generally is inefficient and some developmental defects have been reported.

Due to the high degree of conservation of molecular and cellular mechanisms among organisms, research with animal model systems can provide biological insight for mammalian stem cells. The flatworm Planaria displays a striking regenerative capability. Other invertebrate model systems, including the fruitfly Drosophila and nematode C. elegans, and vertebrate models, such as zebrafish and mouse, are revealing conserved genes, signaling pathways, and differentiation mechanisms.

Before stem cells can be applied in human therapy, many hurdles must be jumped. In general, there are relatively few stem cells in the body. Methods for cell enrichment and isolation are being developed. It is also necessary to work out methods for directed differentiation of a particular cell type as needed for study in cell culture or for possible eventual use in a therapeutic setting. Special culture methods (utilizing growth factors, signaling molecules, microRNAs, etc.) can coax cells down a specific differentiation pathway. Other obstacles that must be eliminated include the depletion of animal products (as in media components, isolation techniques, or feeder lines), prevention of karyotypic abnormalities, methods of controlled cell growth, mitigation of transplant rejection, and demonstration of safety and efficacy.

The largest gains can be expected by studying multiple branches of stem cell science and varied model systems. It is anticipated that stem cell research will eventually lead to the use of these cells in human therapy to treat injury and disease. With the ethical sensitivity of this line of study, regulatory oversight and dialogue among researchers and the public are essential. Despite the hopes associated with stem cell research, experts caution that human therapeutic application lies well in the future, perhaps on the order of a decade or more. Until the numerous obstacles are eliminated, these cells are providing a great resource to reveal the secrets of the normal and diseased state and for biotechnology advance.

Beverly A. Purnell
Senior Editor, Science
Stem cell research is a rapidly expanding area of investigation, with the ultimate goal to prevent, diagnose, and treat human diseases, including heart disease, diabetes, cancer, stroke, and neurological disorders, such as Parkinson's and Alzheimer's disease.

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Embryonic Stem Cell Lines Derived from Human Blastocysts

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Human blastocyst-derived, pluripotent cell lines are described that have normal karyotypes, express high levels of telomerase activity, and express cell surface markers that characterize primate embryonic stem cells but do not characterize other early lineages. After undifferentiated proliferation in vitro for 4 to 5 months, these cells still maintained the developmental potential to form trophoblast and derivatives of all three embryonic germ layers, including gut epithelium (endoderm); cartilage, bone, smooth muscle, and striated muscle (mesoderm); and neural epithelium, embryonic ganglia, and stratified squamous epithelium (ectoderm). These cell lines should be useful in human developmental biology, drug discovery, and transplantation medicine.

Embryonic stem (ES) cells are derived from totipotent cells of the early mammalian embryo and are capable of unlimited, undifferentiated proliferation in vitro (1, 2). In chimeras with intact embryos, mouse ES cells contribute to a wide range of adult tissues, including germ cells, providing a powerful approach for introducing specific genetic changes into the mouse germ line (3). The term “ES cell” was introduced to distinguish these embryo-derived pluripotent cells from teratocarcinoma-derived pluripotent embryonal carcinoma (EC) cells (2). Given the historical introduction of the term “ES cell” and the properties of mouse ES cells, we proposed that the essential characteristics of primate ES cells should include (i) derivation from the pre-implantation or perimplantation embryo, (ii) prolonged undifferentiated proliferation, and (iii) stable developmental potential to form derivatives of all three embryonic germ layers even after prolonged culture (4). For ethical and practical reasons, in many primate species, including humans, the ability of ES cells to contribute to the germ line in chimeras is not a testable property. Nonhuman primate ES cell lines provide an accurate in vitro model for understanding the differentiation of human tissues (4, 5). We now describe human cell lines that fulfill our proposed criteria to define primat e ES cells.

Fresh or frozen cleavage stage human embryos, produced by in vitro fertilization (IVF) for clinical purposes, were donated by individuals after informed consent and after institutional review board approval. Embryos were cultured to the blastocyst stage, 14 inner cell masses were isolated, and five ES cell lines originating from five separate embryos were derived, essentially as described for nonhuman primate ES cells (5, 6). The resulting cells had a high ratio of nucleus to cytoplasm, prominent nucleoli, and a colony morphology similar to that of rhesus monkey ES cells (Fig. 1). Three cell lines (H1, H13, and H14) had a normal XY karyotype, and two cell lines (H7 and H9) had a normal XX karyotype. Each of the cell lines was successfully cryopreserved and thawed. Four of the cell lines were cryopreserved after 5 to 6 months of continuous undifferentiated proliferation. The other cell line, H9, retained a normal XX karyotype after 6 months of culture and has now been passaged continuously for more than 8 months (32 passages). A period of replicative crisis was not observed for any of the cell lines.

The human ES cell lines expressed high levels of telomerase activity (Fig. 2). Telomerase is a ribonucleoprotein that adds telomere repeats to chromosome ends and is involved in maintaining telomere length, which plays an important role in replicative life-span (7, 8). Telomerase expression is highly correlated with immortality in human cell lines, and reintroduction of telomerase activity into some diploid human somatic cell lines extends replicative life-span (9). Diploid human somatic cells do not express telomerase, have shortened telomeres with age, and enter replicative senescence after a finite proliferative life-span in tissue culture (10–13). In contrast, telomerase is present at high levels in germ line and embryonic tissues (14). The high level of telomerase expression expressed by the human ES cell lines therefore suggests that their replicative life-span will exceed that of somatic cells.

The human ES cell lines expressed cell surface markers that characterize undifferentiated nonhuman primate ES and human EC cells, including stage-specific embryonic antigen (SSEA)–3, SSEA-4, TRA-1-60, TRA-1-81, and alkaline phosphatase (Fig. 3) (4, 5, 15, 16). The globo-series glycolipid GL7, which carries the SSEA-4 epitope, is formed by the addition of sialic acid to the globo-series glycolipid Gb5, which carries the SSEA-3 epitope (17, 18). Thus, GL7 reacts with antibodies to both SSEA-3 and SSEA-4 (17, 18). Staining intensity for SSEA-4 on the human ES cell lines was consistently strong, but staining intensity for SSEA-3 was weak and varied both within and among colonies (Fig. 3, D and C). Because GL7 carries both the SSEA-4 and SSEA-3 epitopes and because staining for SSEA-4 was consistently strong, the relatively weak staining for...
SSEA-3 suggests a restricted access of the antibody to the SSEA-3 epitope. In common with human EC cells, the undifferentiated human ES cell lines did not stain for SSEA-1, but differentiated cells stained strongly for SSEA-1 (Fig. 3). Mouse inner cell mass cells, ES cells, and EC cells express SSEA-1 but do not express SSEA-3 or SSEA-4 (17, 19), suggesting basic species differences between early mouse and human development.

The human ES cell lines were derived by the selection and expansion of individual colonies of a uniform, undifferentiated morphology, but none of the ES cell lines was derived by the clonal expansion of a single cell. The uniform undifferentiated morphology that is shared by human ES and nonhuman primate ES cells and the consistent expression by the human ES cell lines of cell surface markers that uniquely characterize primate ES and human EC cells make it extremely unlikely that a mixed population of precursor cells was expanded. However, because the cell lines were not cloned from a single cell, we cannot rule out the possibility that there is some variation in developmental potential among the undifferentiated cells, in spite of their homogeneous appearance.

The human ES cell lines maintained the potential to form derivatives of all three embryonic germ layers. All five cell lines produced teratomas after injection into severe combined immunodeficient (SCID)–beige mice. Each injected mouse formed a teratoma, and all teratomas included gut epithelium (endoderm); cartilage, bone, smooth muscle, and striated muscle (mesoderm); and neural epithelium, embryonic ganglia, and stratified squamous epithelium (ectoderm) (Fig. 4). In vitro, the ES cells differentiated when cultured in the absence of mouse embryonic fibroblast feeder layers, both in the presence and absence of human leukemia inhibitory factor (LIF) (Fig. 1). When grown to confluence and allowed to pile up in the culture dish, the ES cell lines differentiated spontaneously even in the presence of fibroblasts. After H9 cells were allowed to differentiate for 2 weeks, both α-fetoprotein (350.9 ± 14.2 IU/ml) and human chorionic gonadotropin (hCG, 46.7 ± 5.6 mIU/ml) were detected in conditioned culture medium, indicating endoderm and trophoblast differentiation (20).

Human ES cells should offer insights into developmental events that cannot be studied directly in the intact human embryo but that have important consequences in clinical areas, including birth defects, infertility, and pregnancy loss. Particularly in the early postimplantation period, knowledge of normal human development is largely restricted to the description of a limited number of sectioned embryos and to analogies drawn from the experimental embryology of other species (21). Although the mouse is the mainstay of experimental mammalian embryology, early structures including the placenta, extraembryonic membranes, and the egg cylinder all differ substantially from the corresponding structure of the human embryo. Human ES cells will be particularly valuable for the study of the development and function of tissues that differ between mice and humans. Screens based on the in vitro differentiation of human ES cells to specific lineages could identify gene targets for new drugs, genes that could be used for tissue regeneration therapies, and teratogenic or toxic compounds.

Elucidating the mechanisms that control differentiation will facilitate the efficient, directed differentiation of ES cells to specific cell types. The standardized production of large, purified populations of euploid human cells such as cardiomyocytes and neurons will provide a potentially limitless source of cells for drug discovery and
transplantation therapies. Many diseases, such as Parkinson’s disease and juvenile-onset diabetes mellitus, result from the death or dysfunction of just one or a few cell types. The replacement of those cells could offer lifelong treatment. Strategies to prevent immune rejection of the transplanted cells need to be developed but could include banking ES cells with defined major histocompatibility complex backgrounds or genetically manipulating ES cells to reduce or actively combat immune rejection. Because of the similarities to humans and human ES cells, rhesus monkeys and rhesus ES cells provide an accurate model for developing strategies to prevent immune rejection of transplanted cells and for demonstrating the safety and efficacy of ES cell-based therapies. Substantial advances in basic developmental biology are required to direct ES cells efficiently to lineages of human clinical importance. However, progress has already been made in the in vitro differentiation of mouse ES cells to neurons, hematopoietic cells, and cardiac muscle (22–24). Progress in basic developmental biology is now extremely rapid; human ES cells will link this progress even more closely to the prevention and treatment of human disease.

References and Notes

Fig. 4. Teratomas formed by the human ES cell lines in SCID-beige mice. Human ES cells after 4 to 5 months of culture (passages 14 to 16) from about 50% confluent six-well plates were injected into the rear leg muscles of 4-week-old male SCID-beige mice (two or more mice per cell line). Seven to eight weeks after injection, the resulting teratomas were examined histologically. (A) Guttlike structures. Cell line H9. Scale bar, 400 μm. (B) Rosettes of neural epithelium. Cell line H14. Scale bar, 200 μm. (C) Bone. Cell line H14. Scale bar, 100 μm. (D) Cartilage. Cell line H9. Scale bar, 100 μm. (E) Striated muscle. Cell line H13. Scale bar, 25 μm. (F) Tubules interspersed with structures resembling fetal glomeruli. Cell line H9. Scale bar, 100 μm.

were passaged by exposure to type IV collagenase (1 mg/ml; Gibco-BRL) or by selection of individual colonies by micropipette. Clump sizes of about 50 to 100 cells were optimal. Cell lines were initially karyotyped at passages 2 to 7.


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Defining the Epithelial Stem Cell Niche in Skin

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Many adult regenerative cells divide infrequently but have high proliferative capacity. We developed a strategy to fluorescently label slow-cycling cells in a cell type–specific fashion. We used this method to purify the label-retaining cells (LRCs) that mark the skin stem cell (SC) niche. We found that these cells rarely divide within their niche but change properties abruptly when stimulated to exit. We determined their transcriptional profile, which, when compared to progeny and other SCs, defines the niche. Many of the >100 messenger RNAs preferentially expressed in the niche encode surface receptors and secreted proteins, enabling LRCs to signal and respond to their environment.

Epidermis and its appendages undergo continuous renewal and maintain reservoirs of multipotent SCs whose descendants are organized spatially and temporally. The epidermal basal layer (BL) contains putative SCs in addition to the transiently amplifying (TA) cells, which give rise to terminally differentiating suprabasal layers (1–3). The BL and the hair follicle outer root sheath (ORS) are contiguous and biochemically similar (fig. S1A). In the hair bulb, the dermal papilla (DP) maintains contact with matrix TA cells until they differentiate to form the inner root sheath (IRS) and hair shaft. Follicles periodically undergo cycles of growth (anagen), destruction (catagen), and rest (telogen). The zone between noncycling and cycling segments is a SC niche, the ORS “bulge” (4, 5).

Multipotent epithelial SCs with high proliferative potential reside in the bulge (6, 7). The bulge contains the majority of infrequently cycling, label-retaining cells (LRCs), which can respond to anagen DP signals to regenerate the follicle. After wounding or transplantation, bulge cells give rise to epidermis, follicles, and sebaceous glands. Additionally, when dissected from rat whiskers and cultured, bulge cells yield more colonies than other follicle segments (7).

It is not known what features define this specialized SC niche, what its interactions with bulge LRCs are, and whether all LRCs are SCs. To begin to address these issues, we devised a strategy based on the prediction that bulge SCs are uniquely both slow-cycling and active for a keratinocyte-specific promoter. With this strategy, we purify and characterize bulge LRCs and related keratinocyte progeny in the BL and ORS. Analyses of their transcriptional profiles reveal the skin LRC mRNAs; some of these mRNAs are found in SCs of other tissues, whereas others specify the unique environment of the skin SC niche.

To mark infrequently cycling cells of adult skin epithelium, we engineered transgenic mice to express histone H2B–green fluorescent protein (GFP) (8) controlled by a tetracycline-responsive regulatory element (TRE). A tightly regulated TRE-mCMVH2B-GFP founder animal was crossed with mice harboring a keratin 5 (K5) promoter–driven tet repressor–VP16 transgene (9), and offspring were selected for doxycycline (Tet)–controlled regulation restricted to skin epithelium (Fig. 1A). Without Tet, backskin epithelial cells exhibited ~10 to 104 units of GFP fluorescence (9, 10) (Fig. 1B). After feeding 4-week-old mice Tet for 4 weeks to 4 months (chase), only bulge cells (~1% total) retained fluorescence at ≥104 units (Fig. 1B) (fig. S1). Independent of cell surface markers, our approach marks putative bulge SCs on the basis of their slow-cycling properties. Although we used keratinocyte-specific Tet and VP16 mice, TRE-mCMV-H2B-GFP animals could be used with mice expressing other promoter/enhancer-driven, Tet-regulatable activators/repressors to isolate LRCs from other tissues.

To track LRC fate during hair cycling, we monitored GFP fluorescence intensities relative to the proliferation-associated markers Ki67, phosphorylated histone H3 (P-H3; G/M) and basonucin (BSN) (Fig. 1, B and C). Throughout the cycle, most LRCs remained in the bulge as GFP-bright and Ki67−; P-H3−; and BSN− cells (Fig. 1, B and C). During anagen, an abrupt switch in proliferation markers and H2B-GFP intensity occurred at the transition between bulge and new follicle downgrowth (large arrowheads). Overexpression verified that this downgrowth was largely GFP-positive deriving from bulge LRCs. Newly created GFP-positive populations included ORS (K5)+ matrix (Lef1+K5− ), hair (AE13+K5+), and IRS (GATA-3+K5−) (Fig. 1D). We conclude that only a few bulge LRCs initiate each new follicle, and that upon exit their progeny rapidly proliferate, change biochemistry, and regenerate all differentiated cell types.

To determine whether bulge LRCs can react to injury, we scratch-wounded 8-week-old chased mice. Within 24 to 48 hours, GFP− cells were detected outside the bulge (Fig. 1E). These were not “scattered” bulge cells, because they localized to infundibulum and displayed underlying basement membrane (anti-laminin 5 immunoreactivity; not shown). Not seen in unwounded skin, GFP-positive cells within infundibulum and epidermis expressed nuclear junB, a stress-response protein (Fig. 1E). Thus, in response to wound stimuli, LRCs change their biochemistry, exit the bulge, migrate, and proliferate to repopulate infundibulum and epidermis. The ability of LRCs to regenerate hair follicles and epidermis is a feature characteristic of bulge SCs (6, 7).

Immunofluorescence microscopy revealed that the zone harboring keratinocyte-specific, H2B-GFP–bright LRCs was more restricted than that defined by known bulge-preferred markers, including K15, K19, α6-integrin, β1- integrin, CD34, S100A4, and S100A6 (11–14) (fig. S3). To further analyze the properties of LRCs, we prepared single-cell suspensions from 8-week-old, chased transgenic skins and performed fluorescence-activated cell sorting (FACS). Of this population, 12% displayed 10 to 104 units of GFP fluorescence, with 1 to 2% exhibiting 105 to 106 units, relative to background (Fig. 2A).

Populations gated at 103 to 104 units (GFP+) and 50 to 100 units (GFP−) excluded propidium iodide and exhibited surface β1−, α6-integrins, typical of BL/ORS cells (Fig. 2B). GFP+ cells were enriched in CD34 (14), whereas GFP− cells had more CD71 (downregulated in bulge) (13). Two-color analyses indicated that ~30% of GFP−/CD34+ (also α6+) cells were GFP+ (Fig. 2B) (fig. S4B). Semiquantitative fluorescence documented that GFP+ cells corresponded in fluorescence intensity to bulge cells, whereas GFP− fluorescence placed them outside the niche (fig. S4A).

Although GFP+ and GFP− cells differed in CD34/CD71 expression, they both expressed BL/ORS keratins K5, K14, and K15 (1, 12), but not differentiation-specific K1 (1) (Fig. 2C). FACS by surface-β4 yielded a larger pool of cells that were similar to GFP− but with lower fluorescence (fig. S4B). Immunofluorescence with six markers indicated that these three FACS populations were ~90% homogeneous (fig. S4B), and semiquantitative reverse transcription polymerase chain reactions (RT-PCRs) documented their distinctive characteristics (fig. S4C). Cell cycle profiles showed that only 0.5% GFP+ LRCs were...
in G/M (Fig. 2D). Finally, although existing methods did not permit long-term culturing of adult murine bulge cells (7, 14), GFPhigh LRCs were highly enriched (>10× to 15×) for cells forming colonies, some of which were >500 cells (fig. S4D), consistent with the high proliferative capacity documented for rat whisker bulge (7).

Using microarray analyses, we obtained transcriptional profiles for the three populations, and high-stringency analyses uncovered the distinguishing features of LRCs (table S2 and Materials and Methods; ~4800 of 12,000 mRNAs were scored as present in each population. When bulge LRCs were compared with SC databases from hematopoietic (HSC), embryonic (ESC), and neuronal (NSC) tissues (15, 16), SCs were found to express 68% of mRNAs present in LRCs and ~40% of mRNAs up-regulated in LRCs relative to BL/ORS (Fig. 3A). Moreover, overlap existed between HSCs and LRCs relative to their respective progenies (table S1).

The complete database with the raw Affymetrix data files is available at www.rockefeller.edu/labheads/fuchs/database.php. Up-regulated skin LRC mRNAs included known SC markers such as stem cell factor (Kit ligand), Dab2, ephrin tyrosine kinase receptors Ephs), tenascin C (Tnc), interleukin-11 receptor, Id binding protein–2 (Idb-2), four-and-a-half lim domains (Fhl1), CD34, S100A6, and growth arrest–specific (Gas) proteins (17–21). Immunofluorescence and/or RT-PCR confirmed their bulge-preferred location (Fig. 3, B and C). Candidates to be involved in SC maintenance and/or activation, the shared LRC SC factors encompassed proteins regulating cell growth and survival; receptors able to sense and respond to growth factors, hormones, and extracellular matrix; transcription factors. This newfound relation between bulge LRCs and other SC populations (15, 16) opens important avenues for future investigation.

As shown in table S2, 154 mRNAs were up-regulated by a factor of ≥2 in all four com-

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**Fig. 1.** System for marking slow-cycling SCs in vivo and monitoring their fate. (A) Strategy. (B to D) Skin sections of mice before and after 4-week chase. (E) FACS analyses of single-cell suspensions of skins. GFP fluorescence (FL) is in arbitrary units. (F) Two-color FACS analyses for GFP and five surface markers. Arrows denote Ki67+ sebaceous gland cells in telogen. Arrowheads [(B) and (C)] denote transition zone between bulge and newly generated follicle downgrowth. Late anagen (Ki67 in red); GFP-bright cells are retained in the bulge; their progeny rapidly divide, diluting H2B-GFP. (D) Early anagen II bulb overexposed for GFP and double-labeled (small arrowheads) with Abs against each differentiation cell type. (E) Mice “after chase” were scratch-wounded and analyzed by immunofluorescence. Arrows denote likely directions of movements of GFP-positive LRCs and progeny. Abbreviations: Bu, bulge; DP, dermal papilla; Mx, matrix; hg, hair germ; Ep, epidermis; asterisk, hair shaft (autofluorescent); hf, hair follicle; Cx, cortex; ORS/IRS, outer/inner root sheaths; BM, basement membrane; In, infundibulum; W, wound. Scale bars, 50 μm.

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**Fig. 2.** Isolation and preliminary characterization of bulge LRCs and progeny. Animals were Tet-fed for 4 weeks beginning at t = 4 weeks. (A) FACS analyses of single-cell suspensions of skins. GFP fluorescence (FL) is in arbitrary units. (B) Two-color FACS analyses for GFP and five surface markers. α6/CD34 data illustrate that GFPhigh LRCs represent only ~30% of α6/CD34/KS-H2B-GFP–positive cells. White, without primary Abs; red, with Abs. Percentages of total cells scoring positive are indicated. (C) GFPhigh FACS population analyzed by immunofluorescence to illustrate homogeneity. Figure S4B provides quantification of all three fractions screened for six markers. (D) Propidium iodide (PI)–FACS cell cycle profiles by DNA content: G0/G1 (n = 1), G2/M (n = 2), and S (n = 1 or 2). Percentage of total cells in G2/M is indicated.
Comparisons of bulge LRCs to GFP<sup>+</sup> and B4-positive BL/ORS progeny; Table 1 shows functional classifications for a subset of these mRNAs. With 25 primer sets, RT-PCR verified up-regulation of 24 putative LRC mRNAs relative to at least one of the two progeny (Fig. S5). Many known bulge markers surfaced as up-regulated LRC transcripts, including CD34 (9×), S100A4 (5×) (22), S100A6 (3×) (22), Barx2 (2×) (23), and Tcf3 (3×) (24) (Table 1). Immunofluorescence confirmed their bulge-preferred location relative to BL/ORS (Fig. S3). Most LRC mRNAs were specifically expressed in bulge relative to upper ORS and BL. Some were exclusive for the bulge within the skin. Some were present not only in bulge, but also other skin cells not analyzed here. An example tektin2 (14×), a putative microtubule-binding protein, increased in bulge relative to BL/ORS and also present in arrector pili muscles (Fig. 4A).

Table 1 groups mRNAs into categories useful in considering the properties of LRCs relative to progeny cells. Several points are worthy of special mention. Consistent with their slow-cycling properties, skin LRCs expressed elevated transcripts encoding cell cycle regulatory proteins, and in particular, keratinocyte growth inhibitors implicated transforming growth factor-β (TGFβ) signaling (25). One of these, LTBP-1, is necessary for latent TGFβ activation (26) and was strongly and specifically localized to bulge (Fig. 4B). Indicative of TGFβ receptor activation, nuclear phospho-Smad2 immunoactivity was more prevalent in bulge than progeny (Fig. 4B). Activated TGFβ/phosphoSmad target genes and/or Smad interacting proteins were also included in up-regulated LRC mRNAs (Table 1) (fig. S5) (27). Conversely, transcripts down-regulated (163 total) encoded many proliferation-associated proteins, including Ki67 (3×) (Fig. 1, B and C), Cdc25C (2×), and N-myc “downstream-regulated-like” (2×). Additional up-regulated LRC mRNAs encoded members of the Wnt pathway, essential for follicle morphogenesis and hair cycle activation (28, 29). These were inhibitors, including Srfp1, Dab2, Dkk2, Ctbp2, Tcf3, and the Wnt receptors Fzd3, F2d7, and Fzd2 (Table 1) (fig. S5). Conversely, Wnt3a and Wnt3 were down-regulated >3× in LRCs. Consistent with Tcf3’s repressor function (24), a Wnt-inhibited niche would explain why at most stages in the hair cycle, the Wnt reporter gene TOPGAL is silent in the bulge (29).

Many bulge LRC–up-regulated mRNAs (43%) encoded secretory or integral membrane proteins (Fig. 5), which suggests the ability of skin LRCs to organize their niche, communicate with neighboring cells, and respond to their special environment. A case in point may be ephrin receptors (Ephs) and their membrane-bound ligands (Efns), which signal bidirectionally in cell-cell communication and tissue boundary formation (21). Although not specific, Efnb1, EphA4, and EphB4 were expressed in LRCs (Fig. 4C). Efnb1 was up-regulated further in matrix, a compartment not analyzed here, and EphA4 and EphB4 were also in a subset of lower ORS.
Table 1. Transcriptional profiling of bulge LRCs relative to their BL/OR progeny. Functional classification is shown for 66 mRNAs scored as increased in bulge LRCs relative to BL/OR progeny (full list in table S2). Average relative increase across the four comparisons is shown in parentheses; an mRNA that is present but not increased is denoted P.

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<tr>
<td>Known bulge factors</td>
<td>Cd34 (9×)<em>, S100a4 (5×)</em>, S100a6 (3×)<em>, Tcf3 (3×)</em>, β3-integrin (9×)<em>, β4-integrin (9×)</em>, o6-integrin (9×)<em>, Barx2 (2×)</em></td>
</tr>
<tr>
<td>Cell cycle</td>
<td>Gas1 (growth arrest specific 1) (4×), Ltbpb1 (latent TGFβ binding protein) (8×)<em>, Ltpb2 (10×), Ltbp3 (3×), TGFβ2 (3×)</em>, Inkbb (3×), Ak1 (3×)</td>
</tr>
<tr>
<td>TGFβ3-induced factors</td>
<td>Idrb (2×), Idrb2 (8×), Idrb3 (2×), Idrb4 (4×), Ctgf (8×), Ltbpb1 (8×), Ltpb2 (10×), Ltbp3 (3×), Igfbp5 (6×), Igfbp2 (7×)<em>, Timp2 (5×), β6-integrin (6×)</em>, Tnc (3×)*, EfnB1 (2×)</td>
</tr>
<tr>
<td>Wnt signaling</td>
<td>Sfrp1 (7×), Dab2 (9×), Dkk3 (5×), Fzd2 (5×), Fzd3 (3×), Fzd7 (4×), Ctbpb2 (2×), Fts (2×), Tcf3 (3×)*</td>
</tr>
<tr>
<td>Other signal transduction</td>
<td>Stem cell factor (Kit-l) (2×), EfnA4 (2×), EfnB1 (2×), EfnB2 (2×), Bdnf (8×)*, Tsp1 (3×), Ptprk (3×), Ppap2a (8×)</td>
</tr>
<tr>
<td>Extracellular matrix/basement</td>
<td>Gsk3β (6×), Timp2 (4×), Timp3 (4×), Cola (3×)<em>, Cola1a1 (3×)</em>, Mtn2 (2×), Bgn (2×), Agn (2×), Sdc1 (2×)*, syndecan bp (2×)</td>
</tr>
<tr>
<td>membrane proteins</td>
<td>Mad4 (2×)<em>, Max (2×)</em>, LIM domain only 1 (2×), Cited2 (2×)</td>
</tr>
<tr>
<td>Nuclear proteins/</td>
<td>Macf1 (ACF7) (3×), Tekt (14×), β6-integrin (6×), Pdlim3 (15×), Actn1 (4×), Myo1b (4×), Phn2 (3×), Krtp2-6a (7×)*, Ndn (3×)</td>
</tr>
<tr>
<td>transcription factors</td>
<td></td>
</tr>
</tbody>
</table>

* mRNAs previously reported in skin, irrespective of location.

Fig. 5. Comparison of cellular localization of bulge LRC mRNAs increased relative to BL/OR and mRNA present in skin LRCs. Left, increased in LRCs relative to BL/OR (154 total); right, present in skin LRCs (three pools of 150 mRNAs each of the 4839 present were analyzed). Black, expressed sequence tags; red, intracellular/cytosolic; blue, nuclear; gray, integral to membrane; white, secreted.

cells in full anagen (Fig. 4C) (39).

Although most markers labeled bulge LRCs irrespective of whether follicles were in anagen or telogen, Bdnf and TGFβ2 changed LRC expression with the hair cycle (31, 32). Transient stimuli mediated by DP may be particularly important in influencing signaling pathways within the niche. Another example is β6-integrin, present only in early anagen and not telogen (Fig. 4D). βvβ6 makes an attractive candidate for SC activation/migration, as it uses tenascin-C as ligand and is activated during skin wounding and tumorigenesis. Similarly, although not yet formally tested, DP-induced changes in Wnt signaling could explain why TOPGAL is transiently activated in the bulge at early anagen (29). If signaling pathways (e.g., Wnts) are generally important in SC self-renewal, as they are in hematopoietic SCs (33, 34), then differences in their status could have an impact on rates at which SCs divide and are mobilized from their niche.

In summary, we have uncovered a constellation of distinguishing features of bulge LRCs relative to related keratinocyte progeny, which, together with their localization, likely accounts for their special properties. Our findings suggest that the bulge SC niche is a growth and differentiation–restricted environment. The LRC-related changes that have thus far surfaced are already suggestive of a broad interaction between environmental stimuli and the SC niche.

References and Notes

28. T. Han, et al., data not shown.
33. We thank L. Degenerstein, J. Fan, and L. Polak for help with mice; A. Glick for the K5Tet reporter mice; S. Maelz and T. Shengelia for flow cytometry; and all who assisted with mice. A. Glick for the K5Tet reporter mice; S. Maelz and T. Shengelia for flow cytometry; and all who assisted with reagents (see supporting online material). Affymetrix hybridizations were conducted by the IHMII Stanford Microarray facility. Supported by postdoctoral fellowships from the Life Sciences Foundation (T.T.), Human Frontier Science Program (G.G., C.B.), EMBO (V.G.), Austrian Science Foundation (M.R.), BAIF and NATO (C.B.), and NIH (W.G.). E.F. is an investigator of the Howard Hughes Medical Institute.

Supporting Online Material

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Materials and Methods

Figs. S1 to S5 are available as supporting material on Science Online.

Tables S1 and S2 are available as supporting material on Science Online.

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Endothelial Cells Stimulate Self-Renewal and Expand Neurogenesis of Neural Stem Cells

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Neural stem cells are reported to lie in a vascular niche, but there is no direct evidence for a functional relationship between the stem cells and blood vessel component cells. We show that endothelial cells but not vascular smooth muscle cells release soluble factors that stimulate the self-renewal of neural stem cells, inhibit their differentiation, and enhance their neuron production. Both embryonic and adult neural stem cells respond, allowing extensive production of both projection neuron and interneuron types in vitro. Endothelial coculture stimulates neuroepithelial cell contact, activating Notch and Hes1 to promote self-renewal. These findings identify endothelial cells as a critical component of the neural stem cell niche.

Stem cell expansion and differentiation are regulated in vivo by environmental factors encountered in the stem cell niche (1).

In the adult, neural stem cells lie close to blood vessels: in the hippocampus (2), the subventricular zone (SVZ) (3), and the songbird higher vocal center (4). In the developing central nervous system (CNS), ventricular zone cells produce vascular endothelial growth factor, which attracts vessel growth toward them (5). Thus, vascular cells are close to CNS germinal zones throughout life (fig. S1), and it has been suggested that they form a niche for neural stem cells (2).

To examine a possible functional interaction, we cocultured neural and vascular cells (Fig. 1A). Neural stem cells from mouse cerebral cortex from embryonic day 10 to 11 (E10-11) were plated at clonal density on the base of culture wells. The upper transwell compartment was seeded with purified vascular-associated or other feeder cells: primary bovine pulmonary artery endothelial (BPAE) cells, a mouse brain endothelial (MbEND) cell line, vascular smooth muscle (VSM) cells, NIH3T3 fibroblasts, or as a control, high-density age-matched cortical cells (CTX). CD31+ (platelet endothelial cell adhesion molecule c-1, PECAM-1) endothelial cells were never found in the lower compartment when BPAE or MbEND cells were plated in the transwell upper compartment (Fig. 1B), confirming that the feeder cells could not migrate through the 0.4-μm-diameter membrane pores.

As expected (6), embryonic stem cell clones cocultured with CTX began producing neurons within a day. Most neuron production was over by 7 days, and growth after this time was largely in glial lineages. Clones cocultured with BPAE or MbEND cells behaved differently (Fig. 1D and fig. S2), growing into sheets of largely flat...
Endothelial cells stimulate proliferation and neurogenesis of neural stem cells from a variety of embryonic CNS regions and from different stages. E15.5 cortical and adult SVZ stem cells grown in endothelial coculture generated sheets of LeX+ Nestin+ cells. After differentiation, E15.5 endothelial-expanded cortical cells and adult SVZ cells produced more neurons compared to control cells (Fig. 2, F and H).

In vivo, most projection neurons are born in the early embryonic period, whereas glia and interneurons arise later; adult stem cells are primed to generate interneurons (8, 9). To examine the neuron subtypes generated from E10-11 cortical stem cells expanded in endothelial coculture, differentiated clones were stained for glutamic acid decarboxylase (GAD67), a GABAergic marker typically expressed in interneurons, or Tbr1, an early pyramidal neuron marker that preferentially labels projection neurons. E15.5 cortical cells grown as neurospheres in fibroblast growth factor 2 (FGF2) for 7 days were plated in adherent conditions and cocultured for 3 days with endothelial cells or with age-matched cortical cells, then differentiated by withdrawal of feeder cells for 4 days. Stem cells exposed to endothelial factors produced 22% neurons, compared to 2% neurons in control CTX cocultures (Fig. 2G).

Many more stem cell clones growing in BPAE cocultures contained a high percentage of neurons, up to 64%, compared to clones grown in CTX coculture (Fig. 2, D and E), and neuron production was prolonged (supporting online text and fig. S3). Increased neurogenesis from endothelial cocultured neural stem cells did not occur at the expense of gliogenesis: The percentage of glial fibrillary acidic protein (GFAP+) astrocytes generated was similar, and although oligodendrocyte differentiation (indicated by staining with the early oligodendrocyte marker O4) was reduced in BPAE cocultures compared to CTX cocultures, the difference could not account for the enhancement of neuron generation (Fig. 2, C and E). NIH3T3 cells enhanced oligodendrocyte generation. Coculture with VSM or NIH3T3 cells reduced neurogenesis compared to CTX (Fig. 2E), showing that the endothelial effect is cell-type specific.

Endothelial cells stimulate proliferation and neurogenesis of neural stem cells from a variety of embryonic CNS regions and from different stages. E15.5 cortical and adult SVZ stem cells grown in endothelial coculture generated sheets of LeX+, Nestin+ cells. After differentiation, E15.5 endothelial-expanded cortical cells and adult SVZ cells produced more neurons compared to control cells (Fig. 2, F and H).

Neurosphere-expanded stem cells responded to endothelial factors. E15.5 cortical cells grown as neurospheres in fibroblast growth factor 2 (FGF2) for 7 days were plated in adherent conditions and cocultured for 3 days with endothelial cells or with age-matched cortical cells, then differentiated by withdrawal of feeder cells for 4 days. Stem cells exposed to endothelial factors produced 22% neurons, compared to 2% neurons in control CTX cocultures (Fig. 2G).

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That projection neurons typical of the early embryo arise in E10-11 cocultures after many cell divisions suggests that endothelial factors promote stem cell self-renewal and inhibit the normal progression in which older stem cells preferentially produce glia or interneurons. We found few Tbr1+ neurons produced from E15.5 stem cells and none from adult SVZ cells, indicating that endothelial factors are permissive, not instructive, for this fate: They cannot reverse the restriction.

Supporting the hypothesis that endothelial factors promote stem cell self-renewal, time-lapse video recording of dividing clones revealed that stem cells grown with endothelial cells underwent symmetric, proliferative divisions generating Nestin progeny, in contrast to the asymmetric division patterns seen in control conditions (6, 11) (Fig. 4A). Cytological stem cells cocultured with endothelial cells for 4 days generated more secondary stem cell clones, neurospheres, and neuron-generating progenitor cells than did those cocultured with CTX clones (Fig. S4).

The most obvious effect of endothelial factors is that they promote neural stem cell growth as epithelial sheets with extensive junctional contacts (Fig. 1C), which could promote self-renewal by influencing β-catenin signaling pathways (12, 13), mode of cell division (14), and Notch activation (15). Indeed, stem cells cocultured with endothelial cells and then exposed to β-secretase inhibitor II, which inhibits Notch1 activation (16), showed a similar extent of cell–cell contact, division, and differentiation to those in CTX cocultures (Fig. 4B and fig. S5). In neural stem cells cultured with endothelial factors, the Notch effector Hes1 was up-regulated, but Hes5 was not (Fig. 4C), consistent with involvement of Hes1 in neural stem cell self-renewal (17, 18).

Our results identify endothelial cells as critical components of the neural stem cell niche, as they secrete soluble factors that maintain CNS stem cell self-renewal and neurogenic potential. Thus, although FGF2 promotes neural stem cell proliferation, it cannot alone maintain their self-renewal; endothelial factors acting with FGF2 accomplish this.

In the presence of endothelial cells, a neural stem cell undergoes symmetric, proliferative divisions to produce undifferentiated stem cell sheets that maintain their multipotency and, upon endothelial cell removal, generate neurons as well as astrocytes and oligodendrocytes. No CD31+ cells were detected in clones, showing that, at least under these circumstances, neural stem cells do not generate endothelial progeny.

Growth with endothelial cell–derived factors may be an important tool for promoting neural stem cell self-renewal and neurogenesis, allowing efficient production of neural stem cells and a variety of CNS neurons for use in replacement therapies.

References and Notes
15. S. Hitoshi et al., Genes Dev. 16, 846 (2002).
19. We thank H. Singer for VSM cells, Y.-P. Hseuh for Tbr1 antibody, and C. Fasano, Y. Wang, C. Butler, and K. Kirchofer for help in manuscript preparation. Supported by the National Institute of Neurological Disorders and Stroke and the New York State spinal cord research program.

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Fig. 4. Endothelial factors stimulate self-renewal of neural stem cells. (A) Comparison between typical lineage trees reconstructed from time-lapse video recordings of single E10 cortical stem cells grown with endothelial cells and those grown under control conditions. In endothelial coculture, the cortical stem cell divided symmetrically and did not make neurons during the recording period (all progeny were Nestin+ as shown in the fluorescence and phase images of the final clone). In contrast, a cortical stem cell grown under control conditions generated an asymmetric lineage tree, generating neurons [β-tubulin-III+ (red), designated as N in the lineage tree] as well as Nestin+ progenitor cells (green). Neurogenic progeny are numbered to show the match of cells in the final clone to the lineage tree. Arrows indicate neurons in the field that did not originate from this clone. (B) After treatment of 4-day-old cocultures with γ-secretase inhibitor II for 6 hours, β-catenin staining is significantly decreased and β-tubulin-III staining significantly increased in BPAE cocultured clones, whereas there was no effect on CTX cocultured clones (ANOVA; *, P < 0.01 by post-hoc tests). DMSO, dimethyl sulfoxide. (C) Hes1 is up-regulated after endothelial coculture, but Hes5 expression was similar to that in control coculture. Reverse transcription–polymerase chain reaction gel band densities were normalized to expression levels of glyceraldehyde phosphate dehydrogenase (GAPDH).
MicroRNAs (miRNAs) Modulate Hematopoietic Lineage Differentiation

Chang-Zheng Chen,1 Ling Li,1 Harvey F. Lodish,1,2* David P. Bartel1,2*

MicroRNAs (miRNAs) are ~22-nucleotide (nt) noncoding RNAs that can play important roles in development by targeting the messages of protein-coding genes for cleavage and repression of posttranscriptional regulation (1–3). Examples include the lin-4 and let-7 miRNAs, which control the timing of Caenorhabditis elegans larval development (4–6); Bantam miRNA, which regulates Drosophila tissue growth by stimulating cell proliferation and preventing apoptosis (7); and mir-14, which affects Drosophila fat metabolism and prevents apoptosis (8). Humans have between 200 and 255 genes that encode miRNAs, an abundance corresponding to almost 1% of the protein-coding genes (9). Based on the evolutionary conservation of many miRNAs among the different animal lineages, it is reasonable to suspect that some mammalian miRNAs might also have important functions during development (10–14). Moreover, genes for miR-142, miR-15, and miR-16 are at sites of translocation breakpoints or deletions linked to human leukemias (15–18). However, no mammalian miRNAs have established functions (19).

As a first step toward testing the idea that miRNAs might play roles in mammalian development, and more specifically that some might regulate mammalian hematopoiesis, we cloned ~100 unique miRNAs from mouse bone marrow, using the protocol of Lau et al. (20). Most had already been identified as vertebrate miRNAs, but their expression in bone marrow had not been examined. miR-181 (9, 12, 21), miR-223 (9), and miR-142s (18) were carried forward for further analyses, because they, unlike miR-16 and most of the other miRNAs cloned, were differentially or preferentially expressed in hematopoietic tissues (Fig. 1).

miR-181 was very strongly expressed in the thymus, the primary lymphoid organ, which mainly contains T lymphocytes. It was also strongly expressed in the brain and lung and was detectable in bone marrow and the spleen. miR-223 was nearly exclusively expressed in bone marrow, the primary hematopoietic organ, which consists of hematopoietic stem cells and myeloid, erythroid, and lymphoid cells at various differentiation stages. miR-142s, whose gene is at the site of a translocation associated with an aggressive B cell leukemia (16, 18), was highly expressed in all the hematopoietic tissues tested, with little or no expression in nonhematopoietic tissues. Expression at embryonic day 13 in fetal liver, an embryonic hematopoietic organ, suggests that miR-142 might also function in early hematopoietic development.

Because the bone marrow, spleen, and thymus each have specialized functions in adult hematopoiesis and comprise largely different cell types, the differential expression of the miRNAs in these complex tissues suggested that individual hematopoietic cell types might differentially express the miRNAs. When cells within bone marrow were sorted based on lineage markers, they were found to differentially express the hematopoietic miRNAs (Fig. 2). In contrast, expression of miR-16, an miRNA seen in broad range of tissues, was more constant.

Mature miR-181 expression in bone marrow cells was detectable in undifferentiated progenitor cells (Lin−) and up-regulated in differentiated B lymphocytes, which are marked by the B220 surface antigen. In other differentiated lineages, miR-181 expression did not increase over that seen in Lin− cells. Sorted lineage cell populations are ~85% pure; thus, some miRNA signals in the other lineages might be caused by residual B220+ cells. miR-142s expression was highest in the erythroid (Ter-119+ and Fy+ lymphoid [CD3ε+] lineages and most of the other miRNAs cloned, were differentially or preferentially expressed in hematopoietic tissues (Fig. 1).

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where it is cleaved by Dicer to generate the mature miRNA (22). We reasoned that the miR-223(67) transcript was not cleaved by Dicer, because it did not derive from a properly processed primary transcript, and that sequences flanking the hairpin precursor were needed for nuclear processing of the primary transcript. To include the sequences needed for proper processing, constructs with increasing genomic sequence flanking the 67-nt predicted hairpin were generated. All constructs with at least 40 nt on each side of the 67-nt hairpin were efficiently processed into the mature miRNA (Fig. 3B), with a heterogeneity pattern of 21- to 24-nt RNAs similar to that seen for the mature miR-223 in bone marrow cells (Figs. 1 and 2). In light of the recent report that Drosha is responsible for the nuclear processing of miRNA primary transcripts (23), our results can be explained by the idea that elements needed for Drosha recognition reside within the sequences that flank the miR-223 predicted hairpin.

Having determined that sequences flanking the hairpin were needed for detectable miRNA expression, we speculated that a general strategy for miRNA expression would be to use ~270-nt primary transcripts that included the ~22-nt mature miRNA and 125 nt of genomic sequence flanking each side of the miRNA. This strategy has proven successful for all 13 of the miRNAs that we have attempted to express in hematopoietic lineages from mouse bone marrow cells (BM).
express, including the three hematopoietic miRNAs (Fig. 3, C to E). Mature miRNAs ectopically expressed in 293T cells or bone marrow cells had length distributions indistinguishable from those of the endogenous miRNAs, as shown for miR-142s (Fig. 3E). miR-30 is unusual in that it can be expressed from transcripts in which its 71-nt hairpin is flanked by heterologous sequence (24). Nonetheless, when expressed from our vector, miR-30 was much more efficiently processed when presented in the context of its native flanking sequence (Fig. 3F).

To uncover the effects of ectopic expression of miRNAs on hematopoietic lineage differentiation, Lin hematopoietic progenitor cells from mouse bone marrow were infected with viral vectors that expressed either miR-181, miR-223, miR-142s, miR-30 (a control miRNA), or no miRNA (25). miR-30 was selected as a control because its expression was detectable in lung and kidney but not hematopoietic tissues. The cells were then seeded onto S17 bone marrow stromal cells and supplemented with a cocktail of cytokines and growth factors (25, 26). Cells descending from infected progenitor cells were distinguished on the basis of the green fluorescent protein (GFP) marker, and differentiation of Lin cells to lymphoid cells was characterized by the expression of lineage-specific surface antigens (25).

Ectopic expression of the hematopoietic miRNAs substantially altered lineage differentiation (Fig. 4). Expression of miR-181 resulted in a doubling of cells in the T-lymphoid lineage with no significant change in the T-lymphoid lineage, as measured by the fractions of cells that express the Thy-1.2 or CD19 cell surface antigens, which are markers for the T- and B-lymphoid lineages, respectively (Fig. 4, A and B). Ectopic expression of miR-142s or miR-223 had opposite effects—a 30 to 40% increase in the T-lymphoid lineage with little or no reduction in the B-lymphoid lineage. At the two extremes, the ratio of T- to B-lineage cells ranged from about 1:1 to about 4:1 (Fig. 4) when miR-181 and miR-142s were expressed, respectively.

Modest effects were also seen when analyzing cells for myeloid lineage markers (fig. S3). In contrast, ectopic expression of miR-30 had little or no effect on the output of lymphoid and myeloid cells, indicating that merely expressing an arbitrary miRNA does not influence lymphoid differentiation.

Because miR-181 ectopic expression had the greatest effect in vitro, we examined its effect in vivo. Mouse Lin bone marrow cells were infected with either the retrovirus that expressed miR-181 or the control vector that expressed no miRNA and were then transplanted into lethally irradiated mice, where they reconstituted all blood lineages. After 4.5 weeks, the lineage composition of peripheral blood cells descending from infected stem/progenitor cells (GFP+ cells) was examined (25). As seen in vitro, miR-181 expression in vivo led to a substantial increase in B-lymphoid (CD19+) cells, with the median fraction of these cells in peripheral blood increasing to 80% from the control value of 32% (Fig. 5A). This increase was accompanied by a substantial (~88%) decrease in T-lymphoid (Thy-1.2+) cells, particularly the CD8+ T cells, for which the median percentage decreased from 16% to 1.2% (Fig. 5, A and B). There were relatively small or insignificant decreases in CD4+ T cells and myeloid lineage cells (Fig. 5, B and C).

Hematopoietic lineage differentiation, the process of continuous development of hematopoietic stem cells into at least eight different blood lineages, is known to be controlled or modulated by complex molecular events that simultaneously regulate the commitment, proliferation, apoptosis, and maturation of hematopoietic stem/progenitor cells. The demonstration that certain miRNAs are differentially expressed in hematopoietic lineages in vivo and are able to alter lineage differentiation provides solid evidence that miRNAs represent a class of molecules that regulate mouse hematopoiesis and, more broadly, mammalian development.

The ability of ectopically expressed miR-181 to increase the fraction of B-lineage cells in vitro and in vivo (Figs. 4 and 5) coincides with its preferential expression in B-lymphoid cells in mouse bone marrow (Fig. 2), suggesting that miR-181 is a positive regulator for B-cell differentiation. One explanation for the effect of miR-181 expression on the differentiation of both B cells (CD19+) and cytotoxic T cells (CD8+), which are not developmentally linked during hematopoietic lineage commitment, is that miR-181 acts independently in the two lineages, perhaps through the repression of different target...
genes. Indeed, miR-181 is highly expressed in the thymus, supporting the idea that it also modulates T cell development in this organ (Fig. 1). The observation that the differentiation of myeloid and other lymphoid cell types was not totally blocked when the B-lymphoid lineage increased suggests that miR-181, at least when considered singly rather than in combination with other miRNAs, appears to function more as a lineage modulator than as a switch.

In the known invertebrate examples, miRNAs repress the productive translation of their mRNA targets (1). To facilitate further exploration of the roles of hematopoietic miRNAs in modulating lineage differentiation, computational and molecular experiments are under way to determine their regulatory targets. If we assume a mode of regulation analogous to that observed in invertebrates, miRNA modulation of hematopoietic lineage differentiation supports the notion that the roles of translational regulation in hematopoiesis and more broadly, vertebrate development might have been underappreciated. Studies on the gene expression profiles of uncommitted hematopoietic stem cells and intermediate progenitor cells reveal that stem cells exhibit a “promiscuous beginning,” a so-called priming state in which many lineage-specific genes required for subsequent unique lineages are coexpressed (27). Thus, selective gene silencing might be a key event during subsequent hematopoietic lineage differentiation events. Clearly, progressive silencing of lineage-specific genes could be mediated by changes in the activation of master transcription factors or by chromatin remodeling. Our work adds to this list a set of hematopoietic-specific miRNAs that presumably act by pairing to the miRNAs of their target genes to direct gene silencing processes critical for hematopoiesis.

Fig. 5. Effect of miR-181 ectopic expression on hematopoietic lineage differentiation in vivo. (A) Percentage of T-lymphoid (Thy-1.2+) and B-lymphoid (CD19+) lineage cells in GFP+ nucleated peripheral blood cells, in mice reconstituted with bone marrow cells transduced with control (black) or miR-181 (red) retroviral vectors. Box plots describe the distribution of individual lineage composition from all positively reconstituted recipients (those with more than 1.0% GFP+ cells in peripheral blood). The ends of the boxes define the 25th and 75th percentiles, a line indicates the median, and bars define the 5th and 95th percentiles. Individual outliers are also shown. P values were determined with the Mann-Whitney rank sum test. (B) T cell subtypes marked by CD4 and CD8 surface antigens. (C) Neutrophils and monocytes marked by Mac-1 and Gr-1 double-positive cells (Mac-1+ Gr-1+) and Mac-1 positive and Gr-1 negative-to-low cells (Mac-1+ Gr-1−), respectively. Mac-1 and Gr-1 double-negative cells (Mac-1− Gr-1−) are nonmyeloid cells. (D) Representative FACS analyses for the same experiment. Gating was on GFP+ cells. For each quadrant, the fraction of cells relative to the total number of GFP+ cells is indicated as a percentage.

References and Notes
19. The recent report that miR-23 regulates Hes1 during retinoic acid-induced differentiation (28) has been called into question, because it mistakenly reported the analysis of Homolog of E11 rather than of Hes1.
25. Materials and methods are available as supporting material on Science Online.
30. We thank N. Lau and B. Reinhardt for reagents and advice on the cloning of endogenous Dicer products. E. Weinstein for bioinformatic analysis. V. Carey for help on statistical analysis, and members of the Lodish and Bartel laboratories and P. Zanone for comments on the manuscript. Supported in part by grants from NSF (H.F.L.) and NIH (D.P.B.) and by a Donaldson, Lufkin, and Jenrette postdoctoral fellowship from the Cancer Research Institute (C.-Z. C.).

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Because ethical restrictions limit in vivo studies of the human hematolymphoid system, substitute small animal xenotransplantation models have been employed. Existing models, however, sustain only limited development and maintenance of human lymphoid cells and rarely produce immune responses. Here we show that intrahepatic injection of CD34+ human cord blood cells into conditioned newborn Rag2−/− γc−/− mice leads to de novo development of B, T, and dendritic cells; formation of structured primary and secondary lymphoid organs; and production of functional immune responses. This provides a valuable model to study development and function of the human adaptive immune system in vivo.

Biomedical research in humans is restricted largely to in vitro assays that lack the components and complexity of a living organism. To overcome this limitation, substitute in vivo models have been developed in which human hematopoietic cells and tissues are transplanted into mice that are compromised in their capacity to reject xenogenic grafts. Engraftment was first reported after transfer of mature human peripheral blood leukocytes in severe combined immunodeficient mice (hu PBL–SCID mice) (1) and transplantation of blood-forming fetal liver cells, fetal bone, fetal thymus, and fetal lymph nodes in SCID mice (SCID-hu mice) (2, 3). Subsequently, some level of human hematopoietic development was achieved by transplantation of blood-forming cells in NOD/SCID, NOD/SCIDβ2m–/–, or NOD/ SCIDγc−/– mice (4–7). However, transfer of human cells in immunodeficient mice has, so far, not appeared to result in the de novo formation of a functional human adaptive immune system (1, 7–16).

The liver contributes to perinatal hematopoiesis, and the hematolymphoid system expands most significantly during the first weeks of life. Thus, we reasoned that human hematopoietic stem and progenitor cells transplanted into the liver of immunodeficient newborn mice might find better conditions to engraft, expand, and reconstitute a human immune system. We transplanted newborn Rag2−/− γc−/− mice, a mutant strain that lacks B, T, and NK cells (17, 18), intrahepatically (i.h.) with CD34+ cord blood cells (19). Mice were subsequently analyzed between weeks 4 and 26 of age, and human CD45+ hematopoietic cells were detected in all animals (Fig. 1A). An increase in splenic and thymic cellularity was detectable, and all mice beyond 8 weeks developed mesenteric lymph nodes, several within size and cellularity of wildtype controls (Fig. 1B).

Most CD19+ cells in bone marrow (BM) of engrafted mice were negative for surface immunoglobulin M (IgM) and CD20 expression, whereas spleen, lymph node, and blood CD19+ cells expressed these antigens (fig. S1A). This was consistent with generation of B cells in BM and subsequent migration to spleen and lymph nodes. Human IgM was detectable in serum of young transplanted animals and increased in most over time; IgG was detected in older animals, demonstrating class switching of Ig isotypes (fig. S1B). Ig-producing cells were located in BM and spleen and correlated closely with numbers of CD19+CD27+CD138+ plasma cells (fig. S1, C and D). Thus, in contrast to transplanted NOD/SCID mice where human B cells fail to produce Ig (13, 14), full B cell maturation occurred in reconstituted Rag2−/− γc−/− mice.

All thymus contained double-positive, as well as CD4 and CD8 single-positive, T cells in 1:1 to 4:1 ratios, with thymus of young mice containing fewer mature thymocytes than thymus of older mice (Fig. 1C). Of T cell receptor (TCR)β+ T cell receptor (TCRαβ) was upregulated normally during transition from double-negative to single-positive stages (Fig. 1C), and typical thymic cortex and medulla structures were apparent (fig. S2). Some thymus in animals beyond 25 weeks still contained >70% double-positive cells, indicating continuous T cell generation over time. Interestingly, CD25 and Foxp3 expression on some CD4+ thymocytes suggested that human regulatory T cells might also be generated via thymic development (fig. S3, A and B) (20, 21).

Mature T cells with a broad Vβ repertoire were detectable in thymus, spleen, mesenteric lymph nodes, and bone marrow (Fig. 1D; fig. S3C). More than 40% of T cells displayed a naïve phenotype as assessed by CD45RA/CCR7 expression (22) (Fig. 1D). To test whether human T cells in mouse secondary lymphoid organs underwent similar numbers of post-thymic cell divisions as T cells in human newborn blood, TCR-rearrangement excision circles (TRECs) were measured. TREC levels were highest in mouse thymi, whereas somewhat reduced levels were detected in mouse spleen and lymph nodes, which correlated closely with TREC levels in nontransplanted human cord blood (fig. S3D). Thus, human T cells in mouse peripheral tissues appeared to have undergone similar numbers of divisions as T cells in cord blood. Together these data imply that in the thymi of reconstituted Rag2−/− γc−/− mice human T cells with a broad repertoire develop over at least 6 months and home to secondary lymphoid organs without being massively activated.

The proliferative capacity of T cells in response to mouse or human major histocompatibility complex (MHC) antigens was next tested using mixed lymphocyte reactions (MLRs). Human T cells isolated from mouse lymph nodes and spleen proliferated vigorously when stimulated with human allogeneic dendritic cells (DCs) but weakly, or not at all, when stimulated with autologous human DCs (fig. S4A). This indicates that developing T cells had undergone some level of selection on human MHC, possibly within the mouse thymus. Reactivity to mouse DCs was generally low, possibly reflecting suboptimal xenogenic cell interactions (fig. S4B). However, the small difference in response to host BALB/c (Rag2−/− γc−/−) versus mismatched C57BL/6 DCs would also be consistent with the possibility that some thymic selection had occurred on mouse MHC.

Human DCs can be divided into CD11c+ DCs and CD11c− CD123+ plasmacytid, type I interferon producing pre-DCs (23, 24). Both subsets were present in bone marrow, spleen, liver, and, at low levels, thymus and lymph nodes of reconstituted mice (Fig. 2, A and B). Upon maturation ex vivo, CD11c+ DCs displayed typical DC morphology and became potent stimulators of allogeneic T cells (Fig. 2, C and D; fig. S5B). CD11c− cells, stimulated ex vivo with influenza virus, produced high amounts of α-interferon (IFNα) (Fig. 2E).

Therefore, reconstituted animals supported development of both functional DCs and plasmacytid pre-DCs.

Spleens from animals older than 16 weeks possessed white pulp-like structures, consisting of central arterioles surrounded by human

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Fig. 1. Human hematopoietic cell engraftment, organ enlargement, and T cell development in transplanted animals. (A) Bar graphs represent percent of human CD45$^+$ cells in bone marrow and spleen of consecutively analyzed mice ($n = 30$) at indicated weeks after transplantation. Bars are broken up into human CD45$^+$CD3$^-$ T cells, CD45$^+$CD19$^+$ B cells, and CD45$^+$CD19$^-$CD3$^+$ cells. Letters X, Y, Z indicate numbers of CD34$^+$ cells transplanted per animal: X, $3.8 \times 10^4$; Y, $7 \times 10^4$; Z, $9 \times 10^4$. *Indicates animal of which organs are shown in (B). (B) Representative photographs of spleen, thymus, and mesenteric lymph nodes of 19-week-old BALB/c, transplanted Rag2$^{-/-}$$ \gamma_c^{-/-}$, and Rag2$^{-/-}$$ \gamma_\delta^{-/-}$ control mice. (C) Contour plots show representative human thymocyte staining profiles of a young and older animal. Histogram shows TCR$\alpha$ expression on gated double-negative (DN), double-positive (DP), and single-positive CD4 (CD4 SP) and CD8 (CD8 SP) thymocytes from the same animal. (D) Representative mesenteric lymph node profile for CD4 and CD8, and CCR7 versus CD45RA expression on gated CD3$^+$ cells. For comparison, same staining on cord blood cells is shown.
T and B cells (Fig. 3, A and B). Although B and T cell–deficient mice lack follicular dendritic cells (FDCs), immunoreconstitution with mouse BM induces their formation, likely from resident (host-derived) nonhematopoietic cells (25). This has been shown to be dependent on the presence of lymphotixin α-expressing B cells (25). As expected, no human FDCs were detectable in reconstituted animals, although mouse FDCs (FDC-M1+) were induced (Fig. 3B). Furthermore, in some cases, typical germinal center–like structures could be observed (Fig. 3C). These findings provide strong evidence that functional interactions between immune cells were occurring, both within the engrafted human cell populations and across the species barrier.

To more directly test the immune response after reconstitution, mice were vaccinated with tetanus toxoid (TT) or were infected with Epstein-Barr virus (EBV). In three animals vaccinated with TT at 8 weeks, no specific human IgG antibodies could be detected. It is possible that failure to respond at this stage may have been due to the immaturity of the immune system. In contrast, when TT vaccinations were started at 12 to 17 weeks of age, three of five mice produced measurable anti-TT IgG antibodies (table S1) and memory B cells could be detected in lymph nodes (table S2). Although antibody levels were lower than those achieved in human adults, anti-TT IgG to total IgG ratios compared closely (table S1).

Engrafted animals were next infected with increasing doses of EBV and were analyzed 5 to 10 weeks later (Fig. 4; table S3). In all animals, EBV was detectable by polymerase chain reaction (PCR) in BM, spleen, and liver. CD123+BDCA-4+ bone marrow cells produce high amounts of IFNγ upon viral stimulation. CD123+BDCA-4+CD45+ (1) and CD123+BDCA-4+CD45− (3) cells from bone marrow of a transplanted animal, and BDCA-4+CD19−CD14+ cells from peripheral blood of a healthy adult (2) were sorted and stimulated overnight with influenza virus (3.5 × 10⁶ cells each). IFNγ in supernatants was evaluated by enzyme-linked immunosorbent assay (ELISA). Representative experiment of three.

**Fig. 2.** Functional human dendritic cell subsets develop in transplanted animals. (A) Identification of human CD11c+CD123+ plasmacytoid pre-DC and CD11c+ DC in bone marrow, spleen, and liver of an 11-week-old transplanted animal. (B) Histograms show plasmacytoid pre-DC and DC associated marker expression (solid lines) and isotype controls (dashed lines) on CD11c−CD123+ and CD11c+ gated cells in bone marrow. (C) Sorted bone marrow CD11c+ cells, activated with lipopolysaccharide (LPS) and granulocyte-macrophage colony-stimulating factor (GM-CSF) show typical dendritic cell morphology. (D) CD11c+ but not CD19+ bone marrow cells induce strong proliferation of allogeneic peripheral blood T cells. Histogram shows overlay of CD3+ gated cells stimulated with CD11c+ cells (solid line, closed histogram) or CD19+ cells (dashed line). T cell proliferation was evaluated at day six of culture. One of two experiments depicted. CFSE, carboxyfluorescein diacetate succinimidyl ester. (E) CD123+BDCA-4+ bone marrow cells produce high amounts of IFNγ upon viral stimulation. CD123+BDCA-4+CD45+ (1) and CD123+BDCA-4+CD45− (3) cells from bone marrow of a transplanted animal, and BDCA-4+CD19−CD14+ cells from peripheral blood of a healthy adult (2) were sorted and stimulated overnight with influenza virus (3.5 × 10⁶ cells each). IFNγ in supernatants was evaluated by enzyme-linked immunosorbent assay (ELISA). Representative experiment of three.

**Fig. 3.** Spleens of Rag2−/−γc−/− mice transplanted with human CD34+ cord blood cells develop structured white pulp. (A) Focal accumulation of T (CD3+) and B (CD20+) cells around arterioles. (B) Close-up of (A) showing white pulp containing predominantly B cells (CD20+) and T cells (CD3+), as well as naive B cells (CD23+ and mouse FDCs (FDC-M1+). Human FDCs (CD21+) were not detected. (C) Close-up showing a germinal center–like structure with accumulation of highly proliferating CD20+ bcl6−bcl2− B cells and CD3+ T cells, in this case in a vaccinated animal. Representative analysis, 26 weeks ([A] and [B]) and 25 weeks (C) after reconstitution.
lymph node B cells (26). Two animals, infected with the highest EBV doses, developed LMP1+ B cell proliferation in spleen, liver, and kidney (Fig. 4A, table S3), whereas five animals infected with lower EBV doses did not develop obvious B cell proliferation. In the latter, spleen T cells increased substantially (Fig. 4; table S3). Three of these five mice and both mice with B cell proliferation developed LMP1+ B cell proliferation in spleen, liver, and kidney (Fig. 4A; table S3), whereas five animals infected with lower EBV doses did not. Moreover, CD8+ T cells from EBV-infected animals proliferated strongly when stimulated with autologous EBV-transformed B cells in vitro (Fig. 4C). These data suggest that upon in vivo challenge with moderate infectious doses, T cell responses were initiated that could control EBV, at least for the time periods observed. It will be important to next determine the type of EBV infection, as well as the duration, epitope, and MHC specificity of the T cell response.

Taken together, reconstitution of newborn Rag2−/− mice with cord blood CD34+ cells was found to lead to ortotopic de novo generation and maturation of human DCs, B cells, and T cells with a broad repertoire. Although it remains to be clarified how human T cell selection on mouse thymic background occurs, the T cells generated discriminate self from allogeneic MHC. Together, human cells were able to collaborate in forming lymphoid organ structures and induce the differentiation of mouse FDCs, thus providing evidence for an unexpectedly robust cellular interaction across xenogenic barriers. These findings provide a technically straightforward in vivo model with which it will be possible to characterize development, homeostasis, and functional cooperation of the human adaptive immune system. Furthermore, the model should provide a valuable tool to study pathogens that specifically target the human immune system and test potential therapeutic interventions.

![Fig. 4. EBV infection of CD34+ cord blood reconstituted Rag2−/−γc−/− mice. (A) Spleen histology showing CD20+ LMP1+ B cell proliferation in an animal infected with high doses of EBV five weeks earlier. (B) Representative fluorescence-activated cell sorter (FACS) analysis of spleen and lymph node T and B cells in a noninfected sibling control and in EBV-infected animals that did and did not develop B cell proliferation. (C) Lymph node T cells of EBV-infected animals proliferate when stimulated ex vivo with autologous EBV-transformed B cells. Lymph node T cells from noninfected siblings do not proliferate when stimulated under the same conditions. Proliferation was measured by CFSE dilution at day five of cultures.](https://www.sciencemag.org/cs/content/full/304/5667/1040/DC1)

References and Notes

7. M. Ito et al., Blood 100, 3175 (2002).
19. Materials and methods are available as supporting material on Science Online.
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Supporting Online Material

www.sciencemag.org/cgi/content/full/304/5667/1040/DC1
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Derivation of Oocytes from Mouse Embryonic Stem Cells

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Continuation of mammalian species requires the formation and development of the sexually dimorphic germ cells. Cultured embryonic stem cells are generally considered pluripotent rather than totipotent because of the failure to detect germline cells under differentiating conditions. Here we show that mouse embryonic stem cells in culture can develop into oogonia that enter meiosis, recruit adjacent cells to form follicle-like structures, and later develop into blastocysts. Oogenesis in culture should contribute to various areas, including nuclear transfer and manipulation of the germ line, and advance studies on fertility treatment and germ and somatic cell interaction and differentiation.

In the early mammalian embryo, the germ line and soma are indistinguishable from each other. In the mouse, germ cell competence is induced at embryonic day 6.5 in proximal epiblast cells by signals emanating from the extraembryonic ectoderm (1, 2). Even during the specification period, precursor cells give rise to primordial germ cells and certain somatic cells, such as extraembryonic mesoderm and allantois. The potential of embryonic stem (ES) cells to generate all lineages of the embryo in vivo has been widely reported in the literature, in striking contrast to the lack of data describing the derivation of germ cells from ES cells in vitro. We attributed the inability to demonstrate the derivation of germ cells from ES cells in culture to the lack of a suitable reporter system for the noninvasive visualization of germ cell formation.

**Induction of germ cells in culture.** Elucidation of the various known regulatory elements within the germ-line-specific gene

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Fig. 1. Analysis of early stages of germ cell formation. (A) Schematic representation of the Oct4 reporter gene (gcOct4-GFP) showing four conserved sequences (CR1 to 4) in the 5' regulatory region with the deleted area boxed. The conserved sequences overlap with two regulatory elements: a distal enhancer (DE, germ cell–specific) and a proximal enhancer (PE, epiblast-specific) that have been described previously (5). (B) Phase contrast image of E14 ES cells transformed with gcOct4-GFP and growing on an embryonic fibroblast feeder layer (42). The clone presented here resulted in 3.5 dpc and 6.5 dpc mice embryos that lacked GFP expression but showed specific expression in germ cells of 12.5 dpc fetuses. (C) Merged fluorescent and phase contrast image of gcOct4-GFP ES cells 7 days (d7) after ~1 × 10⁴ to 2.5 × 10⁴ cells/cm² were plated without feeder cells or growth factors in ES cell medium. Bar scale for (B) and (C), 75 μm. (D) Expression analysis of four distinct cell populations of d7 cultures sorted by fluorescence-activated cell sorting (FACS) using markers GFP and c-kit. RT-PCR results for Oct4, the Oct4-GFP reporter, c-kit, Vasa, DMC1, SCP3, and β-actin are shown. (E) Scheme of differentiating early germ cells in vitro. Only positive markers are presented. (F) FACS analysis of a d9 culture using the gcOct4-GFP reporter. The R3 population was further sorted in (G) on the basis of c-kit staining, which shows that, at d9, only a minor fraction of gcOct4-GFP⁺ cells are also positive for c-kit. R5 represents the population of c-kit negative cells, whereas R2 denotes cells in which c-kit is weakly or moderately expressed.
Oct4 (3, 4) was instrumental in the development of a system that can visualize the initial steps of germ cell formation in vitro. Comparative analysis of the mouse, human, and bovine Oct4 genes highlighted three conserved regions, CR2 to CR4, that lie within the known germ cell (DE) and epiblast (PE) enhancers (Fig. 1A) (5). To restrict expression of an Oct4-based reporter to germ cells during mouse development, we deleted CR2 and CR3 from a genomic Oct4 fragment driving an inserted green fluorescent protein (GFP) rather than Oct4 (gcOct4-GFP, Fig. 1A) (6). ES cells were transfected with gcOct4-GFP, and three positive clones were expanded and tested for specific expression in transgenic animals. Two transgenic lines showed specific gcOct4-GFP expression in germ cells and no signal in blastocysts or epiblast-stage embryos (7).

The same gcOct4-GFP ES cells (Fig. 1B) that had been used to generate transgenic mice were subsequently used to establish the conditions required to induce germ cell differentiation in vitro. ES cells were plated on tissue culture dishes and maintained in ES cell medium without any feeder cells or growth factors besides the factors present in the heat-inactivated serum. Expression of gcOct4-GFP was detected in some cells after 4 days (d4), in ~25% of all cells at d7 (Fig. 1C), and in 40% of all cells at d8 (not shown). Day 7 cultures were sorted into four cell populations on the basis of the levels of expression of the gcOct4-GFP reporter and endogenous c-kit, both being markers of early germ cells (Fig. 1D). After sorting, we further characterized cells by determining the mRNA expression of Oct4, gcOct4-GFP, c-kit, Vasa (a marker of postmigratory germ cells) (8), and two meiosis-specific markers, namely the synaptonemal complex protein 3 (SCP3) and the mouse homolog of the yeast meiosis-specific homologous recombination gene (DMC1) (9). The SCP3 protein is part of the axial-lateral element of the synaptonemal complex to which the chromatin loops are attached and is an excellent marker for detection of the meiotic transition in mammals because its expression is required for the onset of the first meiotic division (10). The DMC1 protein is supposed to function during chromosome synopsis and homologous recombination events (11, 12).

Three of the sorted cell populations (GFP+/c-kit−, GFP−/c-kit+, and GFP−/c-kit−) contained cells at different stages of germ cell development (Fig. 1, D and E). The GFP+/c-kit− fraction expressed Oct4, gcOct4-GFP, and c-kit but little Vasa mRNA (Fig. 1D, lane 2). Because Vasa is a marker for postmigratory germ cells until postmeiotic stages (8), these results suggest that these cells correspond to premigratory or migratory primordial germ cells. Cells sorted as GFP+/c-kit− (Fig. 1D, lane 3) were found to express Oct4, gcOct4-GFP, and Vasa, but not c-kit, and thus may represent cells of an early postmigratory germ cell stage. GFP−/c-kit− cells did not express Oct4, gcOct4-GFP, or c-kit mRNA but expressed Vasa mRNA (Fig. 1D, lane 4). These cells likely represent postmigratory germ cells that are about to enter meiotic prophase I, because these cells did not express the meiotic markers DMC1 and SCP3 (9). GFP−/c-kit− cells seem therefore to exhibit the same expression pattern as that in vivo, in which both Oct4 and c-kit are down-regulated in female germ cells before the zygotene-pachytene stage of meiotic prophase I, around 15.5 days postcoitum (dpc) (13). The fourth group (GFP−/c-kit+) was composed of cells that were not part of the germ line (Fig. 1D, lane 1) (6, 14). Further analyses are required to define the identity of these c-kit+ cells that 2 days later were almost absent (d9) (Fig. 1, F and G).

**Early germ cell differentiation.** In our cultures, colonies of variable size had formed by d12 (Fig. 2, A to C, represent a large colony). Large colonies in general exhibited reduced GFP expression but contained a high percentage of Vasa+ cells (Fig. 2B). Within these colonies, three distinct types of cells were identified: (i) cells only expressing GFP, (ii) cells expressing both GFP and Vasa, and (iii) cells only expressing Vasa. Strings of GFP+ and Vasa− cells were also found in the cultures, reminiscent of migratory germ cells (Fig. 2D) (15). GFP+/Vasa− cells were always found in the vicinity of both GFP+/Vasa+ and GFP−/Vasa− cells and may correspond to early postmigratory germ cells in vivo (Fig. 2B). GFP-expressing cells had nuclei with a more diffuse DNA staining, whereas cells solely expressing Vasa were more condensed, round, and physically separated from each other (Fig. 2A), which is typical of postmigratory germ cells. Outside the colonies, all cells were negative for germ cell markers and therefore most likely represent somatic cell types.

![Image 2](https://example.com/image2.jpeg)

**Fig. 2.** Large d12 colony with different early germ cell stages. (A) Fluorescent image showing DNA localization (Hoechst staining, blue). (B) Merged fluorescent image of gcOct4-GFP (green) and immunoreactive Vasa protein (red). (C) Phase contrast image of gcOct4-GFP+ cells (arrow). (D) Merged fluorescent GFP and phase contrast image of the supernatant from a d12.5 culture. (E) Merged fluorescent (green, GFP; red, Vasa) and phase contrast image of one aggregate with a single GFP+ cell. All pictures represent whole mount stainings. Bar scales, 50 μm in (A) to (C) and (E) and 25 μm in (F).
Individual cells or groups of cells detached simultaneously from the large colonies, and in both cases these predominantly Vasa⁺ germ cells tended to form small aggregates in the supernatant, with very few GFP⁺ cells (Fig. 2, E and F). It is likely that these cells detach because of the loss of cell-cell contacts, as noted in the lower center area of the colony (Fig. 2, A to C). A reduction in cell-cell adhesion among these Vasa⁺ germ cells in culture is quite similar to that of postmitatory germ cells in vivo (8). Interestingly, the aggregates were frequently attached to GFP⁻/Vasa⁻ cells, which often resulted in a more compact structure than that within the original colony (Fig. 2F). These tightly knit structures are quite similar to the histotypic structures obtained after disaggregation by mild trypsin treatment of male or female genital ridges (16, 17). Furthermore, cultures of suspended ovarian cells of newborn mice and rat can also yield primordial follicles (18).

**Formation of follicular structures.** Aggregates were collected by centrifugation and cultured in new plates. Well-organized structures formed, and some of these were morphologically similar to early ovarian follicles (Fig. 3, B and C) (6). During the next 2 weeks, advanced follicle-like structures formed both in the master plate and aggregate cultures (Fig. 3, E, F, and H). The majority of these structures degenerated upon further cultivation, and ~20% of oocytes larger than 40 μm.

**Characterization of oocytes.** As early as d26 of culture, oocyte-like cells were released from the vicinity of their companion somatic cells (Fig. 3I) and found floating in the supernatant. The oocyte-like cells were enclosed in a coat resembling the zona pel-
lucida, which was fragile and easily lost when manipulated with a micropipette (compare Fig. 4, B and C). Most of these cells were 50 to 70 μm in size, which is in the size range of natural oocytes (23). However, some looked swollen, reaching a size of 130 μm, and had a thinned zona (Fig. 4C). They were GFP+ in accordance with Oct4 being reexpressed after birth in diplotene-arrested oocytes (Fig. 4D) (13). The large cells also exhibited cytoplasmic staining for zona pellucida proteins 2 and 3 (ZP2 and ZP3, respectively) (24) at a location predominantly adjacent to or in the cell membrane, which is distinct from that of the GFP signal (Fig. 4E, ZP2 shown in red; ZP3 not shown). To support the immunocytochemical analysis, we examined the cultures for mRNA expression of several oocyte-specific markers, including ZP1, ZP2, ZP3, a factor in the germ line (Fig. 5a), and GDF-9 (20, 21, 24–27). Fig. 5a, a transcription factor required for the expression of ZP1, ZP2, and ZP3, was absent in the ES control cells (not shown) and expressed at similar levels between d16 and d30 (Fig. 4A). As expected, expression of both ZP2 and ZP3 was also observed; however, ZP1 expression was not detectable (Fig. 4A, lanes 2 to 4). This may indicate that factors required for specific expression of ZP1 are not properly expressed in our cultures. Because ZP1 serves as a linker for ZP2 and ZP3 (28), its absence may account for the fragile zona observed on the ES-derived oocytes. Loss of ZP1 has been reported to perturb but not impair folliculogenesis (28).

Expression of the murine oocyte-specific GDF-9 in the d16 and d22 adherent cultures and the increase in GDF-9 mRNA levels in the replated aggregate cultures (d16F; with a high oocyte/somatic cell ratio) provide additional evidence for folliculogenesis (Fig. 3D). Loss of GDF-9 expression (15.2-fold decrease) between d16 and d22 in the adherent cultures is also consistent with loss of oocyte GDF-9 expression as follicular growth occurs (20).

Evidence of meiosis. DMC1 expression and lack of SCP3 expression as determined by RT-PCR at d16 indicated that the ES-derived germ cells were about to enter meiosis (6). To further substantiate entry into meiosis, we mildly disaggregated d16 cultures with trypsin, replated them, and collected single cells of varying sizes after the majority of the cell population had reattached (Fig. 5A). Expression and distribution of SCP3/COR1 (Fig. 5, B, D, F, and I) or SCP1/SYN1 (not shown) within these cells were analyzed with the use of the respective antibodies (29, 30). Germ cells indistinguishable in size from somatic cells showed SCP3/COR1 staining (Fig. 5, A, white arrows, and B) that was very similar to that of female germ cells isolated from day 15.5 embryos (Fig. 5C), suggesting that these cells are in a stage before leptotene. Germ cells up to 25 μm in size showed SCP3/COR1 staining that colocalized with the nucleus (Fig. 5, A, black-white arrow, and D to F). In contrast, SCP3/COR1 was undetectable in accompanying smaller cells (Fig. 5, D to F) or in germ cell controls in which the primary antibody had been omitted (not shown). In large germ cells (Fig. 5A, black arrow), SCP3/COR1 (Fig. 5, G to I) and SCP1/SYN1 (not shown) were localized predominantly in the cytoplasm, which is indicative of a more advanced meiotic stage (28). Addition of gonadotropins, i.e., pregnant mare serum gonadotropin and human chorionic gonadotropin...
Blastocyst-like structures derived from ES cells. At about d43, structures are found in the cultures that, with respect to morphology (Fig. 6, A to M) and expression of molecular markers (Fig. 6, M and N), are similar to mouse preimplantation embryos. A defined zona around the embryo was only detected in a few cases (for example, Fig. 6B), which is likely a consequence of its fragile structure. A zona was identified after it had detached from the embryo (compare Fig. 6, C and D, showing the same embryo on 2 subsequent days). This embryo had defined features of a 16-cell morula (Fig. 6, E to G). Nuclear staining by Hoechst, the cytoplasm-to-nuclear ratio, a compacted morphology, and Oct4 protein expression and distribution, which at this stage starts to be localized predominantly to the nucleus [Fig. 5C in (31)], are characteristic for a mouse morula. Several structures were found in our cultures that resembled blastocysts (Fig. 6, H to L, show a representative collection of five different blastocyst-like structures).

It is likely that the structures resembling preimplantation embryos represent parthenotes, as suggested by the similarity of our follicle outgrowths (Fig. 6A) to parthenogenetically activated oocytes of the LT/Sv mouse strain (32). Future experiments will address whether cleavage was induced by the culture conditions. It is well known that agents such as ethanol or cations or thermic shock of the oocytes during the observations may induce parthenogenesis with or without extrusion of a second polar body. Addressing these and related questions may provide a better understanding of the physiological problems of parthenogenesis in vivo. In addition, our system may contribute to a better understanding of the regulation of spontaneous ovarian teratocarcinogenesis and the molecular mechanism by which genes such as c-mos block the second meiotic metaphase arrest (22, 33, 34).

Summary and conclusions. It is not surprising that the derivation of oocytes and blastocyst-like structures could be accomplished with both female and male ES cells. In the absence of appropriate SRY expression in the gonads, male primordial germ cells enter the female pathway and often undergo the first step of oogenesis, entering meiotic arrest at prophase I [for review see (35)]. Male-to-female sex reversal even happens in the mammalian embryo when single genes such as Sry, Sox9, or Fgf9 are not properly regulated or deleted (36). Because we detected Sry expression by RT-PCR only in the later stages of our cell cultures (not shown), the gene may not be efficiently regulated in the areas where germ cells differentiated into female germ cells.

The reports from 1978 cited above in the context of dissociation and reaggregation of gonadal cells are interesting also in this con-
text (16, 17). Disintegration and reaggregation of male mouse and rat testes resulted in long, twisting tubular structures as well as spherical aggregates (16, 17). One type of aggregate was indistinguishable in appearance from isolated medium-sized ovarian follicles in suspension and thus termed “folliculoids.” The yield of folliculoids in these cultures was markedly increased when treated with antibodies specific for H-Y, an antigen that is responsible for the differentiation of the undetermined gonadal anlage into a testis [for review see (17–19)]. Because even cells of natural gonads form such “folliculoids,” the formation of similar structures from male ES cells seems not too farfetched to us.

Two main arguments have been put forth to explain why ES cells are not totipotent in vitro. Mouse ES cells have not been observed to give rise to germ cells in vitro, nor have they been shown to differentiate along the trophodermal lineage. Expression of trophodermal markers, like Troma-1 protein (Fig. 6M) or Pl-1, Hand-1, and Tpb mRNAs (Fig. 6L) (38–40), together with the morphology of the structures observed, strongly suggests that a trophoblast is formed from ES cells in vitro. Notably, our blastocyst-like structures looked better than most of those obtained by nuclear transfer of somatic nuclei. In addition, they had the correct Oct4 expression, which is in contrast to most clones, and yet the latter are generally considered to represent blastocysts (41). We conclude that mouse ES cells are capable of differentiating into oocytes and form structures very similar, if not identical, to blastocysts, thereby demonstrating that these cells are actually totipotent even in vitro. Future experiments will reveal whether the oocytes we have generated in culture from ES cells can be fertilized, whether they have undergone a gender-specific resetting of the epigenetic marks (imprinting), and whether they can be used as starting material to derive ES cell lines after nuclear transfer.

References and Notes
6. Materials and methods are available as support material on Science Online.
34. N. Hashimoto et al., Nature 370, 368 (1994).
43. We thank J. Dean for generously providing the monoclonal ZP2 and ZP3 antibodies, P. Moens and B. Spyropoulos for SCOP3/CO1 and SCOP1/SYN1 antibodies, S. Eckardt and J. McLaughlin for providing a female ES cell line, T. Noce for providing the polyclonal Vasa antibody, E. Gonzales for helping with the immunocytochemistry, and A. Malapetsas for editing the manuscript. The Troma-1 hybridoma antibody, developed by P. Brulet and R. Kemler, was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the National Institute of Child Health and Human Development (of NIH) and maintained by the University of Iowa, Department of Biological Sciences. Supported, in part, by the Marion Dilley and David George Jones Funds, the Commonwealth and General Assembly of Pennsylvania, grant NIH 1 RO1 HD 42011-01 (K.H., M.B., J.K., R.R., and H.S.), grant HD06274 (L.C., J.W., and J.S.), grant T32 HD 07305 (J.W.), and grant 4484 (G.F.) from the Association pour la Recherche sur le Cancer.

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Figs. 51 and 52

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Consent from Donors for Embryo and Stem Cell Research


Research on human embryos and embryonic stem cells holds great promise for understanding human reproduction and development and for regenerative medicine. The need for informed consent from research participants is well established (1). Under U.S. federal regulations, persons who provide biological materials for research are research subjects who need to provide consent (1–3). In research involving human embryos, informed consent is particularly important because of the diverse opinions and strong emotions that surround these issues (4, 5). Some potential donors consider all such research to be unacceptable; others only support some forms of research (6–8). A donor might consider infertility research acceptable but object to research that could lead to stem cell lines, patenting, or commercial products (9, 10). Governmental bodies in several countries have considered the issue of consent for embryo and stem cell research (5, 11–13).

In the United States, federal regulations permit a waiver of informed consent for the research use of anonymous biological material that cannot be linked to donors even through a code (2, 14). However, people commonly place great emotional and moral significance on their reproductive materials (15, 16). Using gametes or embryos for certain kinds of research without consent, even after identifiers have been removed, could be regarded as a wrong or offensive (17, 18).

The consent of the woman or couple in the assisted reproductive technology (ART) program is clearly required for research with frozen embryos remaining after completion of infertility treatment (19, 20–22). Frozen embryos may be created with sperm or oocytes from donors who do not participate any further in assisted reproduction or child rearing. Guidelines in the United Kingdom and Canada require consent from gamete donors, as well as infertility patients, for research with frozen embryos (12, 23). However, current U.S. guidelines do not consider whether these gamete donors also have an autonomy-based interest in the use of such embryos for research (20, 21). The argument against requiring such consent is that in donating to ART patients, gamete donors have ceded their right to direct further usage of their gametes, particularly when they received financial compensation. However, gamete donors who are willing to help women and couples bear children may object to the use of their genetic

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materials for research. In one study, 25% of women who donated oocytes for infertility treatment did not want the embryos created to be used for research (18). Little is known about the wishes of sperm donors concerning research. Moreover, if stem cell lines are created, it is the donor’s genetic material that will be propagated indefinitely.

We suggest that gamete donors’ wishes should be determined and respected; informed consent from both oocyte and sperm donors should be obtained for an embryo to be used in research (19). Our position does not depend on ascribing personhood to embryos or on classifying gametes or embryos per se as research subjects. There are logistical and practical differences in obtaining consent to embryo research from oocyte and sperm donors. ART clinics can readily discuss donation for research with oocyte donors during visits for oocyte stimulation and retrieval. However, most ART clinics obtain donor sperm from sperm banks and typically have no direct contact with the donors. Commercial agencies and nonacademic ART centers may be reluctant to include research in their consent discussions with sperm donors. The current consent form used by one of the largest sperm banks in the United States makes no provisions for use of sperm other than for “the primary purpose of causing pregnancy” (24). Furthermore, sperm is often donated anonymously, with strict confidentiality provisions, and frozen sperm must be quarantined for 6 months (25). Thus, recontacting sperm donors may be difficult or impossible and may violate donor privacy. Sperm banks and researchers need to collaborate to change the consent process for future sperm donation to include consideration of donation for research.

Basic and clinical scientists, ART clinicians, and leaders of research institutions should together stimulate broad public discussion to create guidelines for informed consent that protect donors while allowing important research to proceed.

References and Notes

26. B. Lo, L. Wolf, and V. Chou are supported by the Greenwall Foundation.
**Introduction**

Embryonic stem cells have an infinite capacity for self-renewal and are pluripotent (i.e. can differentiate into any cell type represented by the three germ layers—ectoderm, endoderm, and mesoderm). A great deal of hope is associated with the potential application of hES cells in functional genomics, cell therapy and regenerative medicine. Successful derivation of hES cells was first reported in 1998. In that study, hES cells were isolated from the inner cell mass of blastocysts and plated onto mitotically inactivated murine embryonic fibroblast (MEF) feeder cells. Initial hES cell derivation and culturing techniques originated predominantly from methodology developed for murine (mES) cells. While MEF feeders have proved to be a robust surface for long-term culture of hES cells, they also present a number of limitations. Concerns about contamination of hES cells from animal-derived pathogens makes this method of culturing unsuitable for clinical applications. From a practical standpoint, it is inconvenient and tedious to grow and maintain two cell types. Furthermore, it is difficult to transfect and genetically manipulate the compacted hES cell colonies on feeders. For all these reasons, efforts have been initiated to develop feeder-free conditions for culturing hES cells.

To date, many signaling pathway regulators have been identified that are thought to play an important role in mediating self-renewal and differentiation of ES cells. These include bFGF, Wnt, Activin A/Nodal, and BMP. Manipulation of specific signaling regulators has facilitated development of feeder-free conditions required for propagating undifferentiated hES cells. Recent studies have clearly delineated that hES cells can be grown in the absence of feeders as long as they are plated on an appropriate extracellular matrix (ECM) and cultured in media either conditioned on feeder cells or supplemented with appropriate soluble factors.

**Discussion**

BD Matrigel™ Matrix is a reconstituted basement membrane isolated from the Engelbreth-Holm-Swarm (EHS) mouse sarcoma. This matrix is predominantly composed of laminin, collagen IV, entactin, and heparin sulfate proteoglycan. In 2001, the first feeder-free hES culture was predominantly composed of laminin, collagen IV as substrates for culturing various hES lines (WiCell Research Institute, Inc.) with MEF-conditioned media (MEF-CM). BD Matrigel Matrix and laminin were equivalent in their ability to support undifferentiated hES cell growth for extended periods of time. Cells grown under these conditions were found to have a normal karyotype, demonstrated a stable proliferation rate, and high telomerase activity. Cells expressed characteristic undifferentiated hES cell markers and formed teratomas in severe combined immunodeficient (SCID) mice and differentiated into cells from all three germ layers. Since then, BD Matrigel Matrix coupled with different feeder-conditioned media has been widely accepted as an alternative method for culturing hES cells. More recently, others demonstrated that hES cells could be propagated in an undifferentiated state on BD Matrigel Matrix using conditioned media from different types of feeders. The major limitations of using conditioned media are that it involves a tedious process of generating media from feeder cells and can vary in performance depending on the condition and type of feeders used. Therefore, the logical next step in feeder-free media development was focused on supplementing basal media with key factors necessary to maintain self-renewal of hES cells.

bFGF is known to play a central role in maintenance of hES cell self-renewal. Recent studies illustrated that hES cells cultured on BD Matrigel™ Matrix with unconditioned media (UM) supplemented with knockout serum replacement and high concentrations of bFGF was sufficient for maintaining self-renewal. BMP activation has been shown to promote differentiation of hES cells. Noggin and other BMP antagonists have been found in hES media conditioned by MEFs and are thought to be important for reducing differentiation of hES. A combination of exogenous Noggin and bFGF appears to have a synergistic effect in suppressing hES differentiation and can be used to supplement unconditioned media and maintain hES cells in an undifferentiated state for long periods of time on BD Matrigel Matrix. Another signaling pathway deemed important for maintaining self-renewal of hES cells is the Wnt pathway. A specific inhibitor of glycogen synthase kinase-3, 6-bromoindirubin-3’-oxime (BIO), which activates the canonical Wnt pathway, maintained hES cells in an undifferentiated state for long periods of time. Cells grown under these conditions were found to have a normal karyotype, demonstrated a stable proliferation rate, and high telomerase activity. Cells expressed characteristic undifferentiated hES cell markers and formed teratomas in severe combined immunodeficient (SCID) mice and differentiated into cells from all three germ layers. Since then, BD Matrigel Matrix coupled with different feeder-conditioned media has been widely accepted as an alternative method for culturing hES cells. More recently, others demonstrated that hES cells could be propagated in an undifferentiated state on BD Matrigel Matrix using conditioned media from different types of feeders. The major limitations of using conditioned media are that it involves a tedious process of generating media from feeder cells and can vary in performance depending on the condition and type of feeders used. Therefore, the logical next step in feeder-free media development was focused on supplementing basal media with key factors necessary to maintain self-renewal of hES cells.

**Table 1: Summary of BD Matrigel Matrix as ECM substrate for feeder-free human ES cell culture.**

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Serum or SR</th>
<th>Media</th>
<th>Characterization*</th>
<th>Culture Duration**</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CM or Key components</td>
<td>Marker</td>
<td>Pluripotency</td>
<td>Karyotype</td>
</tr>
<tr>
<td>H1, H7, H9 and H14</td>
<td>✓</td>
<td>MEF-CM</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>H1, H7, H9</td>
<td>✓</td>
<td>MEF1-CM</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>H1 and HES-NCL1</td>
<td>✓</td>
<td>HES-df-CM</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>SA 002, SA 038, SA 121, SA 1677</td>
<td>✓</td>
<td>k-VitrOEDS media</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>SA 002, SA 038, SA 121, SA 1677</td>
<td>✓</td>
<td>k-VitrOEDS media</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>H1, H9, H14</td>
<td>✓</td>
<td>40 ng/ml of bFGF+500 ng/ml of Noggin</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>H7, H9, H14</td>
<td>✓</td>
<td>NIH/3T3-Nog-CM, 40 ng/ml of bFGF+500  ng/ml of Noggin</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>H1, H7, H9, H14</td>
<td>✓</td>
<td>&gt; 100 ng/ml of bFGF</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
</tbody>
</table>

Abbreviations: SR: serum replacement; Mkr: Marker; Pluri: Pluripotency; Kary: Karyotype; TER: Teratoma formation in SCID mice; EB: Embryoid body formation; DF: Differentiation to specific lineage; Ref.: Reference; MEF: Mouse embryonic fibroblast; CM: Conditioned media; HES1-CM: Conditioned media from mitotically inactivated HES1-NTERT cells (human ES derived fibroblast, stably transfected with TERT); HES-df-CM: Conditioned media from hES cell derived fibroblast; k-VitroES media: MEF conditioned VitroES media. *Characterization may be reported on selected cell lines listed; **: The longest culturing time for one of the cell lines listed; ***: Table entry is modified from Table 1 in Ref. 17.
Recently, BD Matrigel™ Matrix has been shown to support long-term culture of hES cells with three independent chemically defined media.10-16 One of the defined media (TeSR1) contains all human supplements and was completely defined (except for human serum albumin). TeSR1 media coupled with BD Matrigel Matrix has been a successful combination for culturing different WiCell hES lines for two-six months. In the same study, various ECMs (collagen IV, fibronectin, laminin, and vitronectin) were combined to form a human surface capable of supporting long-term proliferation of hES cells using this media. Another defined media17, when used with BD Matrigel Matrix, sustained the highest number of undifferentiated hES cells compared to all other single ECM substrates tested (collagen, fibronectin, and laminin). In this study, a combination of fibronectin and collagen was required to match the performance of BD Matrigel Matrix. A summary of BD Matrigel Matrix applications for culturing hES cells is provided in Table 1.

There are a number of advantages in using BD Matrigel™ Matrix as a feeder-free substrate for culturing hES cells. For instance, DNA, RNA, and protein isolation is easier on the BD Matrigel Matrix surface due to lack of potential contamination from the feeder cells. In addition, genetic manipulation of hES cells requires efficient transfection capabilities. Transfection efficiencies of hES cells grown on a BD Matrigel Matrix surface are improved compared to those grown on feeders.

When cultured on BD Matrigel Matrix, hES cells form monolayer-like colonies, making individual cells more accessible for penetration with DNA or siRNA.18-21 Although other ECM proteins have also been successfully used for feeder-free cultures, several studies have suggested that a combination of ECM proteins is required to perform as well as BD Matrigel Matrix.18-19 Moreover, purified proteins are generally more expensive and not as well tested for long-term performance as BD Matrigel Matrix.

Despite the wide acceptance of BD Matrigel™ Matrix as a viable substrate for long-term, feeder-free culturing of hES cells, it has a key limitation. Since BD Matrigel Matrix is derived from the EHS tumor, lot-to-lot variability in protein concentration and composition is inherent. In order to limit the amount of time researchers spend today pre-screening lots of BD Matrigel Matrix and coating plates on a weekly basis, BD Biosciences has developed a stable, ready-to-use BD BioCoat™ Matrigel™ 6-well Plate for ES culture. This culturing system was optimized using MEF-CM due to a lack of commercial media that is qualified and defined. Much of hES cell culturing success depends on methods used for ES cell derivation, dissociation, and cryopreservation. Moreover, protocols that have been validated over long periods of time are essential. We have optimized a culturing protocol using BD BioCoat Matrigel 6-well Plates which includes pre-qualified reagents such as BD™ human bFGF as a media supplement. Using this protocol, H9 and H1 cell lines from WiCell have been cultured on the optimized BD BioCoat Matrigel Matrix surface for over 40 population doublings with MEF-CM supplemented with 8 ng/ml bFGF (Figure 1). hES cells typically spread out more on the BD BioCoat Matrigel Matrix surface and form larger colonies (Figure 1b) compared to those cultured on MEF feeders (Figure 1a). Cellular morphology was comparable to colonies cultured on freshly coated BD Matrigel Matrix Plates and consistent with previous reports.2-5 Cells grown on the newly optimized BD BioCoat Matrigel Matrix surface stained positive for expression of well-characterized, undifferentiated markers (Oct-3/4 and SSEA-4) (Figure 1c, d). H9 cells cultured continuously on this surface for 10 passages retained their ability to form embryoid bodies (Figure 1e) suggesting that the ability of these cells to differentiate has not been altered. Independent studies suggest that the BD Matrigel Matrix surface will likely be compatible with a number of hES culturing media (Table 1) and that this surface may also be used to culture other types of ES cells.22

Conclusion
In summary, BD Matrigel™ Matrix has served as an optimal surface for propagating hES cells, maintaining their self-renewal and pluripotency characteristics for extended periods of time in culture. To the best of our knowledge, it has worked with every type of media tested thus far, feeder-conditioned or defined. BD Matrigel Matrix has provided an equivalent, and more often superior, performance compared to other single EC.Ms tested. Two independent studies have demonstrated that a combination of ECMs was required to match the performance of BD Matrigel Matrix as a substrate for maintaining integrity of hES cells in culture. BD Matrigel Matrix is a more affordable option compared to purified ECMs. The BD BioCoat Matrigel 6-well Plate (Cat. No. 354671) provides lot-to-lot consistency needed for standardized cultures. Many basic research questions can be addressed by using recently developed media in conjunction with the BD Matrigel Matrix-coated culture surface.

References
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