Stem Cells in Review

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The advances in our knowledge and understanding of stem cells have fundamentally changed the way we see human biology and physiology, not to mention disease. It has become clear in recent years that not only are our bodies more plastic than previously thought, but the number and type of stem cells that play a role in the generation and regeneration of human tissue far exceed original expectations.

In order to characterize stem cells, they must first be identified. Markers for many different stem cell populations have been elucidated, but challenges still remain. The papers presented in this booklet by Notta et al. (p. 4) and Takeda et al. (p. 10) highlight these hurdles. For instance, in Takeda’s words, “The location and identity of ISCs [intestinal stem cells] have been a subject of much research and debate…” The identification of the precise location of these stem cells, which support the rapid replacement of cells in the intestine, is integral to understanding both normal gut homeostasis, as well as what occurs during disease, particularly cancer. Notta and colleagues take on a similar challenge with hematopoietic stem cells (HSCs) as they demonstrate that a specific adhesion protein, CD49f, can be used to separate true HSCs from their multipotent progeny.

Embryonic stem cells (ESCs), as well as multipotent and unipotent stem and stem-like cells, all hold promise for potential disease treatments. Induced pluripotent stem cells (iPSCs) that have been generated in the lab by genetic reprogramming of a somatic cell have the advantage that they are near identical to ESCs, but can be artificially created and therefore circumvent many of the ethical concerns with the extraction and use of ESCs. In a recent paper published in *Science Translational Medicine*, Sharkis et al. (p. 17) outline potential applications for iPSCs in disease therapy and regenerative medicine, while also remaining realistic about residual impediments to their broad clinical application, particularly safety concerns.

Advances in cell culture techniques have allowed for the more skillful manipulation of stem cells in vitro to generate almost any differentiated cell type required. The balance is delicate, but once achieved, stem cells can be bent to the will of the researcher to create any number of lineages for further study or even transplantation. On page 22, Tursen et al. describe one such example, in which they use just a single transcription factor to generate a number of differentiated neuronal cell types from mitotic germ cells in *C. elegans*.

The field of regenerative medicine is moving forward at an increasingly rapid pace. As in vitro cultured cells for human treatment become a reality, and the techniques for recreating even complex organs becomes possible, the opportunity to apply stem cells to the treatment of many different diseases and conditions seems more attainable than ever.

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*Science*
Inspiration for Life Science

Cell culture procedures and cell-based assays are among the most powerful investigative tools available to life science researchers. Consequently, cell culture has become one of the most widely used techniques in life science research with new and increasingly intricate protocols appearing regularly. Furthermore, practical applications of cell culture such as iPS technology have laid the groundwork for new and promising approaches to regenerative engineering medicine. While the rapidly increasing commercialization of innovative cell-based technologies permits their widespread dissemination, their successful use in the field requires strong support in the marketplace by reliable product providers.

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Mature blood cell lineages are generated from a network of hierarchically distinct progenitors that arise from self-renewing hematopoietic stem cells (HSCs). The extensive regenerative potential of HSCs makes them attractive targets for cellular and genetic therapies. The molecular regulation of specific HSC properties such as long-term self-renewal is beginning to be elucidated for murine HSCs (1). However, the biology of human HSCs remains poorly understood because of their rarity and the lack of methods to segregate HSCs from multipotent progenitors (MPPs). The demarcation of human HSCs and MPPs will enable the investigation of the molecular determinants of HSCs, with a goal of developing stem cell–based therapeutics.

The bulk of HSCs are CD34+, as evidenced by human transplantation and xenograft re-population assays; however, most CD34+ cells are lineage-restricted progenitors and HSCs remain rare. HSCs can be enriched further on the basis of CD45RA (2), Thy1 (3–5), and CD38 (6, 7) expression. Loss of Thy1 expression in the CD34+CD38−CD45RA− compartment of lineage-depleted cord blood (CB) was recently proposed to be sufficient to separate HSCs from MPPs (5). However, more than a third of Thy1− primary recipients gave rise to engraftment in secondary animals, raising uncertainty about whether Thy1 can absolutely segregate HSCs from MPPs. To resolve the relationship between these two subsets, the number of cells in each subset that are capable of short-term and long-term engraftment must be quantified at clonal resolution. We recently optimized the HSC xenograft assay by using intrafemoral injection into female NOD-scid-IL2Rgc−/− (NSG) mice (8–10). Flow-sorted CB HSCs (CD34+CD38−CD45RA Thy1−; Thy1+) (Fig. 1, P5) and MPPs (CD34+CD38 CD45RA− Thy1−; Thy1−) (Fig. 1, P2) fractions were functionally characterized with our HSC assay. A priori, HSCs were operationally defined by lymphomyeloid engraftment that persisted for at least 20 weeks after transplant. This duration represents a stringent test of long-term repopulation and encompasses the total engraftment time of primary and secondary transplants historically used to assess the self-renewal capacity of human Isolation of Single Human Hematopoietic Stem Cells Capable of Long-Term Multilineage Engraftment

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Lifelong blood cell production is dependent on rare hematopoietic stem cells (HSCs) to perpetually replenish mature cells via a series of lineage-restricted intermediates. Investigating the molecular state of HSCs is contingent on the ability to purify HSCs away from transiently engrafting cells. We demonstrated that human HSCs remain infrequent, using current purification strategies based on Thy1 (CD90) expression. By tracking the expression of several adhesion molecules in HSC-enriched subsets, we revealed CD49f as a specific HSC marker. Single CD49f+ cells were highly efficient in generating long-term multilineage grafts, and the loss of CD49f expression identified transiently engrafting multipotent progenitors (MPPs). The demarcation of human HSCs and MPPs will enable the investigation of the molecular determinants of HSCs, with a goal of developing stem cell–based therapeutics.

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HSCs in xenograft models. At nonlimiting cell doses, recipients of Thy1\(^+\) and Thy1\(^-\) cells had similar levels of human chimerism and lineage distribution (injected femur: \(P = 0.17\); Fig. 2, A and B; fig. S1; and table S1). To assess whether Thy1\(^-\) cells would persist beyond the allotted 20-week primary transplant period, we performed secondary transplants for an additional 12 to 14 weeks. This revealed that Thy1\(^-\) cells could be serially transplanted, albeit with lower efficiency than Thy1\(^+\) cells (table S2), which is consistent with previous work (5). These data suggest that cells with extensive self-renewal potential exist in both Thy1\(^+\) and Thy1\(^-\) subsets, although the basis for the disparity in secondary transfer efficiency between these subsets remained unknown.

We next performed limiting dilution analysis (LDA) to measure the frequency of HSCs within Thy1\(^+\) and Thy1\(^-\) fractions. One in 20 Thy1\(^+\) cells (5%) clonally initiated long-term hematopoiesis in NSG mice as compared to 1 in 100 (1%) Thy1\(^-\) cells (\(P = 0.0003\), Fig. 2C). Double sorting and high-stringency sort modes used in our experimental design...
ruled out the possibility that HSC activity from Thy1− cells was due to residual contamination from Thy1lo− cells (figs. S2 and S3). The inability of prior studies to detect engraftment from Thy1− cells was probably due to the less sensitive xenograft models employed (3). Thus, although Thy1+ enriches for HSCs, long-term repopulating activity persists in the Thy1− fraction previously believed to represent MPPs (5).

To examine the hierarchical relationship between the Thy1 subsets, we cultured sorted Thy1+ and Thy1− cells with stroma cells known to express HSC-supportive ligands (II). Both Thy1+ and Thy1− cells (>70%) remained CD34−CD38− on stromal cultures (fig. S4, column 3). Unexpectedly, Thy1− cells consistently generated Thy1+ cells on stroma (Fig. 2D, right panel) and also in vivo within the bone marrow microenvironment of NSG mice that received transplants (Fig. 2E). Thy1+ cells arising from Thy1− cells after culture, as well as Thy1+ cells that retained their Thy1 expression, had robust repopulating activity in NSG mice 20 weeks after transplantation. Engraftment and lineage potentials were identical for Thy1+ cells derived from either Thy1 subtraction (Fig. 2F and fig. S5). Cells that remained Thy1− after being cultured on OP9 stroma did not sustain a long-term graft (Fig. 2F, right panel); however, they transiently repopulated (fig. S6). These results demonstrate that the Thy1− compartment is heterogeneous and contains a small fraction with repopulating activity and a larger fraction with MPP-like activity.

To further purify HSCs in both Thy1+ and Thy1− subsets, we searched for a different cell surface marker. We hypothesized that integrins would mark human HSCs, because they mediate niche interactions and have been used to isolate murine HSCs and other somatic stem cells (12, 13). We compared the surface expression of several adhesion molecules between HSC-enriched (Thy1+) and -depleted (Thy1−) fractions. Among our candidates, only ITGA6 (integrin α6, termed CD49f) was differentially expressed (Fig. 3A and fig. S7), with 50 to 70% of Thy1+ cells expressing CD49f versus 10 to 20% of Thy1− cells.

To determine whether human HSCs could be delineated using CD49f expression, we partitioned Thy1+ cells into CD49fhi (here called Thy1+CD49fhi; Fig. 1, P9) and CD49flo− (here called Thy1+CD49flo−; Fig. 1, P8) subfractions and evaluated their capacity for long-term multilineage chimeraism in NSG recipients. Mean chimerism in the injected femur was 6.7-fold higher for Thy1+CD49fhi than for Thy1+CD49flo− cells (22.7% versus 3.4%, P < 0.0001, Fig. 3B, left panel), and only Thy1+CD49flo− cells could be serially transplanted (table S3). LDA revealed that 9.5% (1 in 10.5) of Thy1+CD49fhi cells had long-term repopulating activity as compared with 0.9% (1 in 111.3) Thy1+CD49flo− cells (P = 9.9 × 10−9, Fig. 3C, and tables S1 and S4). Because we had found that the Thy1− fraction was heterogeneous, we tested whether CD49f expression also marked Thy1− HSCs. Indeed, only Thy1+CD49flo− cells reconstituted NSG mice 20 weeks after transplant (Fig. 3B, right panel). LDA indicated that approximately 4.5% (1 in 22.1) of cells in this fraction had long-term multilineage engraftment potential as compared to 0.13% (1 in 735.2) of Thy1+CD49fhi cells (Fig. 3C and table S4). No difference in lineage potential was observed between Thy1+CD49flo− and Thy1−CD49flo− cells (fig. S8), although recipients of Thy1+CD49flo− cells trended toward higher levels of chimerism at similar cell doses (table S1). We estimate that although most human HSCs are Thy1+, consistent with prior work, 1 in 5.5 CB HSCs lack Thy1 expression. These data indicate that human HSCs are marked by CD49f, and they establish the existence of Thy1− HSCs.

The absence of long-term grafts in Thy1− CD49flo− recipients raised the possibility that the loss of CD49f demarcated human MPPs in the Thy1− subset (5). To test this idea, we temporally monitored the peripheral blood and marrow of NSG recipients transplanted with all four Thy1 and CD49f subsets for 30 weeks. Although levels of chimerism gradually increased in the peripheral blood of mice transplanted with CD49f− HSC subsets, engraftment of Thy1+CD49f− cells peaked between 2 and 4 weeks and then declined (Fig. 3D). The bone marrow of Thy1+CD49f− recipients displayed significantly higher levels of chimerism at 2 weeks than did CD49f+ HSCs in both the injected.
femur and noninjected bones, indicating that Thy1−CD49f− cells have a higher engraftment and differentiation potential than HSCs immediately after transplant (Fig. 3E). These results also rule out the idea that Thy1−CD49f− cells have impaired capacity to home and proliferate in the marrow. B cells, monocytes, granulocytes, and eryth-rocyes were detected in the bone marrow of Thy1−CD49f− mice (Fig. 3E and fig. S9). HSC-enriched fractions displayed a delay in engraftment until 4 weeks (Fig. 3F). The engraftment kinetics of Thy1−CD49f− cells were intermediate to HSC and Thy1−CD49f− subsets (Fig. 3, D to F). These data demonstrate that Thy1−CD49f− cells can give rise to all major hematopoietic lineages but fail to engraft long-term, indicating that these are bona fide MPPs. To remain consistent with previous work (5), we defined transiently engrafting Thy1−CD49f− cells as MPPs. However, considering that Thy1−CD49f− cells...
differ from CD49f HSCs solely in the ability to engraft durably, an alternate interpretation is that Thy1 CD49f cells are short-term HSCs.

To provide an independent line of evidence for distinguishing our functionally defined HSC and MPP populations, we carried out global gene expression analysis of sorted CD49f+ and Thy1−CD49f− subsets. Unsupervised hierarchical clustering revealed that the CD49f+ HSCs clustered together irrespective of Thy1 status (fig. S10A). No significant differences in gene expression were detected between these subsets, although Thy1−CD49f+ HSCs displayed an intermediate pattern to Thy1+CD49f+ HSCs and MPPs (fig. S10B), consistent with the lower frequency of HSCs within this population. In contrast, the Thy1−CD49f− MPPs clustered independently from both CD49f+ HSC subsets (fig. S10A). Seventy differentially expressed genes segregated Thy1−CD49f− MPP versus HSC subsets (fig. S10B and table S5), consistent with the lower frequency of HSCs within this population. In contrast, the Thy1−CD49f− MPPs clustered independently from both CD49f+ HSC subsets (fig. S10A).

Although sorting based on CD49f enables the highest reported purity of human HSCs, this test still falls short of the most definitive assessment of HSC potential: single-cell transplantation. A single long-term mouse HSC provides lifelong blood production (16). Because only 9.5% of Thy1+CD49f+ cells were HSCs by LDA, additional strategies were needed to efficiently assess single human HSCs. High efflux of the mitochondrial dye rhodamine-123 (Rho) could enrich for HSCs within the Lin−CD34+CD38− fraction (17). To test whether Rho efflux marked HSCs in conjunction with Thy1, we transplanted limiting numbers of Thy1+Rho− cells sorted based on high (Thy1+Rho−; Fig. 1, P6) and low (Thy1+Rho−; Fig. 1, P7) Rho efflux. Recipients of Thy1+Rho− cells exhibited 40-fold higher chimerism in the injected femur (fig. S11) and displayed twofold enrichment for HSCs as compared to Thy1+ alone (table S1).

We next questioned whether the addition of Rho to Thy1+CD49f+ would permit...
robust engraftment of single human HSCs. We flow-sorted single Thy1<sup>+</sup>Rho<sup>lo</sup>CD49f<sup>+</sup> cells and transplanted them into NSG recipients (Fig. 4A). In our first experiment, 5 of 18 recipients (28%, Fig. 4B) transplanted with single cells displayed multilineage chimerism 20 weeks after transplant (Fig. 4C and fig. S12). Serial transfer was successful in two of four secondary recipients despite the fact that only 20% of total marrow was used for transplantation (fig. S13), indicating that individual Thy1<sup>+</sup>Rho<sup>lo</sup>CD49f<sup>+</sup> cells extensively self-renew. In a second experiment, we observed a lower frequency (14%) of single-cell transfer, perhaps reflecting the genetic heterogeneity of CB donors (Fig. 4B). Human cell engraftment at marrow sites distant from the injected femur indicated that single Thy1<sup>+</sup>Rho<sup>lo</sup>CD49f<sup>+</sup> cells could give rise to systemic grafts (Fig. 4D to E), fulfilling a key criterion of HSCs. Based on historical data showing xenotransplantation inefficiency (18), we are probably underestimating HSC frequency. Engraftment of single Lin<sup>−</sup>CD34<sup>−</sup>CD38<sup>−</sup>CD45RA<sup>−</sup>Thy1<sup>+</sup>Rho<sup>lo</sup>CD49f<sup>+</sup> cells provides evidence that human HSCs express CD49f.

In this study, we purified human HSCs at single-cell resolution, separated HSCs from MPPs, and identified CD34<sup>+</sup>Thy1<sup>+</sup> HSCs. The ability to investigate two highly enriched and related multipotent cell populations that differ in their capacity for self-renewal opens the way for studies to elucidate developmental programs specific to human HSCs. Because the cell number required for chromatin immunoprecipitation coupled with high-throughput sequencing (ChIP-seq) and DNA methylation profiling is constantly decreasing (19–21), it will be critical to subject HSCs and MPPs to these technologies. Such analyses will aid in identifying gene regulatory networks that govern human HSC function and in turn facilitate manipulating and expanding human HSCs ex vivo to overcome barriers to successful transplantation.

References and Notes

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Supporting Online Material
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Figs. S1 to S14.
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References
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Interconversion Between Intestinal Stem Cell Populations in Distinct Niches

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Intestinal epithelial stem cell identity and location have been the subject of substantial research. Cells in the +4 niche are slow-cycling and label-retaining, whereas a different stem cell niche located at the crypt base is occupied by crypt base columnar (CBC) cells. CBCs are distinct from +4 cells, and the relationship between them is unknown, though both give rise to all intestinal epithelial lineages. We demonstrate that Hopx, an atypical homeobox protein, is a specific marker of +4 cells. Hopx-expressing cells give rise to CBCs and all mature intestinal epithelial lineages. Conversely, CBCs can give rise to +4 Hopx-positive cells. These findings demonstrate a bidirectional lineage relationship between active and quiescent stem cells in their niches.

The multicellular epithelium of the intestine is replaced every few days, and this renewal process is maintained by multipotent intestinal stem cells (ISCs) (1, 2). The location and identity of ISCs have been a subject of much research and debate, with implications for understanding gastrointestinal cancer, repair after intestinal injury, and normal physiology. Numerous reports have suggested that ISCs are located at the +4 position relative to the crypt base (3, 4), while a separate body of work has identified a distinct stem cell niche at the crypt base where crypt base columnar (CBC) cells are interspersed between Paneth cells (5–7). The +4 cells correspond to the location of slow-cycling, label-retaining cells (LRCs) (3, 8) and colocalize with Bmi1-expressing cells (4), as well as those expressing an mTert transgene (9, 10). CBC stem cells, by contrast, are marked by Lgr5 (5). Although +4 cells and CBCs are clearly distinct, lineage-tracing studies have shown that both can give rise to all the various cell types comprising the small intestine epithelium: goblet cells, neuroendocrine cells, Paneth cells, and epithelial absorptive cells. However, the relationship between these two distinct stem cell populations remains incompletely understood. A recent report suggests that +4 cells can compensate for the loss of CBCs to maintain homeostasis after experimental ablation of Lgr5-expressing cells (11). However, a bidirectional lineage relationship between active and quiescent populations of stem cells in multiple tissues has also been postulated (2), though experimental evidence to support this proposal has been lacking. Here, we show that quiescent +4 ISCs express the atypical homeobox gene Hopx and give rise to Lgr5-expressing CBCs. Conversely, rapidly cycling CBCs expressing Lgr5 give rise to +4 cells expressing Hopx. These findings reconcile controversies regarding the location and identity of ISCs and demonstrate interconversion between organ-specific stem cell niches.

Hopx encodes an atypical homeodomain-containing protein that has previously been studied in the heart and neural stem cells (12–14). Analysis of the intestines of Hopx lacZ knock-in (Hopx lacZ/+) mice revealed robust expression of β-galactosidase (β-Gal) in intestinal crypts along the entire length of the intestine (Fig. 1A and fig. S1A). Expression

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was strongest in the +4 region and included label-retaining cells identified after irradiation and pulse labeling with 5-bromodeoxyuridine (BrdU) (9, 15, 16) (Fig. 1B and fig. S1B). Eighty-six percent (68/79) of non-Paneth BrdU-retaining cells expressed β-Gal, and nearly all were at or near the +4 position (Fig. 1C). Similar results were found in unirradiated animals; 92% (35/38) of non-Paneth BrdU-retaining cells expressed β-Gal (fig. S1C). To track the fate of Hopx cells, we generated a tamoxifen-inducible Cre (ERCe) knock-in targeted to the 3′ untranslated region of the Hopx locus following an internal ribosomal entry sequence (IRES) (HopxERCe+) (fig. S2) and crossed them with R26RstoplacZ mice (R26LacZ/+) (fig. S3) (17, 18). Two months after tamoxifen induction, localized staining indicative of β-Gal activity in cells derived from Hopx-expressing precursors was evident in intestinal crypts with a proximal-to-distal gradient (Fig. 1D). Under the conditions used, tamoxifen induction was only partially...
efficient; nevertheless, entire crypt-villus structures were labeled, suggesting clonal origins. *Hopx* descendants were present at least 13 months after induction (Fig. 1, E and F), the latest time point we examined, even though the entire intestinal epithelium replenishes every ~5 days, suggesting that *Hopx* cells self-renew and/or give rise to multipotent ISCs. All differentiated intestinal epithelial cell types can derive from *Hopx* cells, including Paneth, goblet, neuroendocrine, and absorptive cells (fig. S4).

Eighteen hours after a single pulse of tamoxifen to *Hopx*ERCre/+;R26LacZ/+ mice, distinct single cells at the +4 position expressed green fluorescent protein (GFP) (Fig. 1G). Serial analysis of *Hopx* descendants after initial pulse labeling of *Hopx* clones indicated that *Hopx* cells at the +4 position gave rise to progeny that populated the crypt base and the villus epithelium (Fig. 1H). The entire crypt base, including regions occupied by CBCs expressing *Lgr5*, expressed β-Gal.

The location of *Hopx*-expressing cells in the intestine was distinct from that of *Lgr5*-positive cells (compare Fig. 1, G and I). The position of β-Gal–positive cells in 334 crypts was recorded 18 hours after tamoxifen induction, confirming a propensity for the +4 position (Fig. 1J). In contrast, a similar analysis performed with *Lgr5*EGFP-ERCre/+;R26LacZ/+ mice (*Lgr5*EGFP-ERCre/+;R26LacZ/+)(5) placed *Lgr5*-positive cells predominantly between Paneth cells in the +1/+2 position (Fig. 1, I and J). Serial analysis over 13 months indicated that increasing numbers of crypt-villus structures were entirely labeled over time (Fig. 1K, purple). We observed a gradual decrease in the number of crypts that had two or more LacZ-positive cells without ubiquitous labeling (Fig. 1K, green), and an increase in crypts with a single labeled periodic acid–Schiff–positive Paneth cell (Fig. 1K, red), consistent with the relatively long...
Figure 3. FACS and gene expression of Hopx descendants. (A and B) FACS-sorted Hopx descendants after a pulse of tamoxifen (HopxERCre/+, R26mT-mG/+) . Representative plots from one of three experiments are shown. Analysis of Hopx descendants at the indicated time periods after a single pulse of tamoxifen to HopxERCre/+, R26mT-mG/+ mice demonstrates a gradual increase in the number of cells expressing GFP (orange), representing Hopx derivatives, and a concomitant loss of the tdTomato signal (blue, ubiquitously expressed until inactivated by HopxERCre/+ expression). Percentages of cells expressing GFP are shown in the gated population in (B). (C) Gene expression of GFP-positive cells was determined by quantitative reverse transcription–polymerase chain reaction (normalized to glyceraldehyde-3-phosphate dehydrogenase). Results are expressed relative to the level of gene expression observed 18 hours after tamoxifen induction, n = 3 experiments. (D) Rate of growth per organoid, n = 5 organoids. Hopx derivatives 18 hours (blue) or 4 days (brown) after tamoxifen induction of HopxERCre/+, R26mT-mG/+ mice, and Lgr5hi (green) cells from HopxLaZ+/+, Lgr5EGFP-ERCre/+ mice were used (error bars: ±1 SD).
survival of Paneth cells compared with other epithelial cell types. Parallel experiments with Lgr5EGFP-ERCre/+;R26lacZ/4 mice showed more rapid acquisition of ubiquitous crypt-villus labeling (Fig. 1L), consistent with the interpretation that Lgr5-positive cells are more rapidly cycling stem cells (n = 3 mice for each genotype; >190 crypts were scored for each time point). Taken together, these data indicate that Hopx-positive cells at the +4 position are multipotent, slow-cycling, label-retaining ISCs, which are distinct from Lgr5-positive cells at the crypt base.

CBCs have been shown to form crypt-villus structures and generate all intestinal epithelial cell types in organoid culture (19). Cultures of crypt epithelial cells from HopxlacZ/+ mice, which constitutively express β-Gal from the Hopx locus (18), produced two types of organoids: those that express β-Gal and those that do not (Fig. 2A). Over time in culture, the percentage of organoids expressing β-Gal increased from ~70% at day 2 to >95% by day 14 (Fig. 2B). β-Galactosidase produced from the Hopx locus was relatively stable and perdured, as has been reported in other cases [for example, see (20)]. Thus, all cells expressing Hopx and their early descendants express β-Gal. The presence of unlabeled organoids in these experiments indicated the existence of ISCs that do not express, and did not recently express, Hopx. Examination of organoids from HopxlacZ/+;Lgr5EGFP-ERCre/+ mice indicated that β-Gal-negative organoids expressed GFP from the Lgr5 locus (fig. S5, A and B).

In cultures in which β-Gal expression was identified, labeled cells were initially located immediately above Paneth cells (identified by their dense granules). Labeled cells expanded to generate crypt-villus "buds" in organoid culture (Fig. 2C). Similar experiments in which HopxERCre/+;R26lacZ/+ cultures were exposed to a single pulse of tamoxifen confirmed the clonal origin of developing crypt-villus structures and epithelial derivatives (Fig. 2D). Cultures derived from HopxlacZ/+;Lgr5EGFP-ERCre/+ mice also produced organoids in which β-Gal (persisting in Hopx-derived cells) overlapped with expression of GFP derived from the Lgr5 locus (fig. S5, C and D).

Hopx cells at the +4 position and Hopx descendants located between Paneth cells at the crypt base were isolated by laser capture microdissection (LCM) from HopxERCre/+;R26lacZ/4 mice 18 and 48 hours after tamoxifen induction, respectively, and expression levels of stem cell markers were compared. Lgr5 was robustly expressed in Hopx descendant CBCs, whereas Hopx and Bmi1 were more strongly expressed by +4 cells (figs. S6 and S7). In separate experiments, Hopx-positive cells and their descendants were isolated by fluorescence-activated cell sorting (FACS) from HopxERCre/+;R26mT-mG/4 mice 18 hours, 2 days, and 4 days after tamoxifen treatment (Fig. 3, A and B), and the expression level of stem cell and differentiation markers was analyzed. Expression of Hopx, Bmi1, Msi1, and Tert, markers of +4 cells (4, 9, 10, 21), decreased over time, whereas that of Lgr5, Olfm4, and Ascl2, expressed by CBCs (5, 22, 23), increased (Fig. 3C). Genes expressed by differentiated epithelial derivatives including Alpi, Lyz1, and Muc2, also increased (Fig. 3C). Notably, single Hopx-expressing cells isolated 18 hours after tamoxifen induction in these experiments remained quiescent in culture. For example, in one experiment only 1 of 7500 cells isolated by FACS expanded substantially during 4 days of culture in the presence of Wnt3A (100 ng/ml). By contrast, cells isolated 4 days after tamoxifen treatment proliferated at a rate equivalent to that of Lgr5hi cells [those with the most robust GFP expression (19)] derived from Lgr5EGFP-ERCre/+ mice (Fig. 3D). Taken together, these findings are consistent with the interpretation that Hopx labels a quiescent population of ISCs that can give rise to more rapidly proliferating Lgr5-expressing ISCs. This conversion may take place more prominently in vivo than in vitro, perhaps due to signals present in the niche.

Lgr5-positive cells can also give rise to Hopx-expressing cells. Single Lgr5hi cells derived from HopxlacZ/+;Lgr5EGFP-ERCre/+ mice produced organoids with an efficiency of 13.0 ± 3.0% and 3.08 ± 0.57% with and without Wnt3A (100 ng/ml), respectively (n = 2000 cells in each group from three different mice) (Fig. 4A), similar to results reported previously by others using Lgr5EGFP-ERCre/+ mice (19, 24). After 21 days in culture, robust β-Gal expression was evident in a
Figure 4. Lgr5-positive cells can give rise to Hopx-positive cells. (A and B) Organoid cultures from single Lgr5<sup>hi</sup> cells (Hopx<sup>LacZ</sup>/Lgr5<sup>EGFP-ERCre</sup>). Days of growth are shown above each panel. (B) Day 21 GFP and β-Gal expression. (C) Day 7 single-Lgr5<sup>hi</sup> cell organoid cultures. There are both LacZ-negative (top) and -positive organoids (bottom). (D) Percentage of organoids derived from single Lgr5<sup>hi</sup> cells (Hopx<sup>LacZ</sup>/Lgr5<sup>EGFP-ERCre</sup>) that are LacZ-positive. (Number of organoids analyzed is listed above each time point.) (E) (Left) Confocal image of LacZ and GFP double staining 5 months after a 5-day tamoxifen pulse to Hopx<sup>LacZ</sup>/Lgr5<sup>EGFP-ERCre</sup>/R26<sup>mT-mG</sup> mice (black arrowheads point to double-positive cells). (Right) Light microscope image of the same crypt demonstrating LacZ expression; arrowheads point to LacZ-positive, +4 cells. GFP expression (shown as a red membrane-bound signal) demarcates Lgr5 derivatives, and LacZ (blue) indicates Hopx expression. (F) Representative images of double-positive cells isolated after a single pulse of tamoxifen to Hopx<sup>LacZ</sup>/Lgr5<sup>EGFP-ERCre</sup>/R26<sup>Tom</sup> mice. The percentage of double-positive cells as compared to tdTomato cells is shown. At 18 hours after a single pulse, there are zero double-positive cells. White arrowhead points to double-positive cell in representative images. tdTomato (red) indicates Lgr5 derivatives; LacZ (blue) indicates Hopx expression. Scale bars: 25 μm (E and F) and 50 μm (A, B, C).
pattern distinct from that of GFP (Fig. 4B). At 3 days of culture, all organoids were LacZ negative, but by 7 days >20% of the organoids expressed β-Gal (Fig. 4, C and D), and by 21 days 100% (47/47) expressed β-Gal (Fig. 4D), demonstrating that Lgr5-positive cells can give rise to Hopx-expressing cells in vitro. In vivo, fate mapping of Lgr5 cells with HopxLacZ/+;Lgr5EGFP-ERCre/+;R26mTmG/+ mice, 5 months after a 5-day tamoxifen pulse, revealed entire crypt-villus structures that express membrane-bound GFP, including cells at the +4 position that simultaneously expressed β-Gal, indicating that they were derived from Lgr5-positive precursors (Fig. 4E). We also prepared near single-cell suspensions of crypts from HopxLacZ/++;Lgr5EGFP-ERCre/+;R26mTom/+ (HopxLacZ/+;Lgr5EGFP-ERCre/+;R26Tom/+) mice either 18 hours, 5 days, or 10 days after a single pulse of tamoxifen and analyzed the cells for LacZ and tdTomato expression. Eighteen hours after induction, we found no LacZ and tdTomato double-positive cells, consistent with Hopx-expressing cells being distinct from Lgr5-positive cells. However, over the ensuing 10 days, double-positive cells emerged, confirming that Lgr5-positive cells can give rise to Hopx-expressing, +4 cells (Fig. 4F).

Our results provide experimental evidence to support a proposed model (2) in which slowly cycling ISCs at the +4 position can give rise to Hopx-expressing cells and suggest that adult organ-specific stem cells in distinct niches can regenerate one another. Further elucidation of the unique properties of each stem cell population and the signals that regulate interconversion will be likely to inform gastrointestinal pathophysiology and stem cell biology in the future.

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Supporting Online Material

www.sciencemag.org/cgi/content/full/science.1213214/DC1
Materials and Methods
Figs. S1 to S7
References (25–29)

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Pluripotent Stem Cell–Based Cancer Therapy: Promise and Challenges

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The development of induced pluripotent stem cell (iPSC) technology has generated enthusiasm about the therapeutic potential of these cells for treating a variety of diseases. However, the evidence that they actually will be clinically useful is limited. Here, we discuss the potential therapeutic applications of iPSCs for treating cancer and other diseases and highlight the current barriers restricting their use.

Induced pluripotent stem cells (iPSCs) are a new type of stem cell that is generated by reprogramming the genome of an adult somatic cell, such as a skin fibroblast, to a pluripotent state. Such iPSCs share many similarities with embryonic stem cells (ESCs). Reprogramming of adult somatic cells to iPSCs requires certain pluripotency factors, including the transcription factors Oct4, Sox2, and Klf4 (1). These iPSCs are able to renew themselves indefinitely and to differentiate into many different cell types, including pancreatic-β cells, liver hepatocytes, cardiomyocytes, hematopoietic cells, and dopaminergic neurons. Consequently, there has been much enthusiasm about using iPSCs to generate tissues to treat a variety of diseases, including diabetes, liver cirrhosis, leukemia, and Parkinson’s disease, but ultimately how useful these cells will be for regenerative medicine is still not clear (2).

The first clinical trials to use human ESCs, which are closely related to human iPSCs, have just begun in patients with retinal disease and severe spinal cord injury (2). The goal of these early trials is to establish safety, hopefully with a hint of efficacy. If ESCs are established as a safe cellular therapy, then this will provide a foundation for clinical trials using human iPSCs. Unlike human ESCs, which must be prepared from human embryos, iPSCs can be reprogrammed from many (perhaps most) types of adult cells (3), thus avoiding the real and perceived ethical and pragmatic issues that arise with human ESCs. In addition, because iPSCs can be derived directly from the patient, iPSC-derived tissues would be genetically identical to tissues of the patient enabling autologous transplantation. In contrast, it would not be feasible to make ESCs for every patient, and so the use of ESCs in regenerative medicine would be limited to allogeneic transplantation.

Given that iPSCs do not have the ethical or immunogenic limitations associated with ESCs, they represent a stem cell technology that is more likely to be translatable to the clinic. One of the advantages of iPSCs is that they can be generated from diverse tissues of the human body and methods to improve human iPSC technology now allow the generation of iPSCs from various adult human tissues, including skin, hair, blood, and liver (3). Building on the knowledge obtained from human ESC studies, direct differentiation methods have been implemented to obtain highly specialized cell types from human iPSCs (3). The enthusiasm of scientists for using human iPSCs is due in part to the fact that nuclear reprogramming of adult human cells generates iPSCs endowed with the unlimited potential to reconstruct genetically identical tissues. This biomedical tool offers unprecedented opportunities to develop scalable, yet personalized cell-based therapy for patients with a variety of different diseases. Not only could a patient’s iPSCs be used to generate cells for transplantation to repair damaged tissue (for example, transplanting iPSC-derived liver cells to repair or regenerate injured liver), but also the differentiated progeny of such cells could be used to screen candidate drugs to treat the disease (Fig. 1). In addition, by focusing on diseases of

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unknown or unclear etiology, iPSC technology offers a robust platform to efficiently dissect molecular and cellular pathophysiology for the purposes of developing diagnostic, therapeutic, and preventive applications (Fig. 1).

**POTENTIAL APPLICATION OF iPSCS FOR CANCER TREATMENT**

Human iPSCs have been touted as the route to produce a variety of tissues that could be used in regenerative medicine. For example, iPSCs generated from the fibroblasts of type 1 diabetes patients have been induced to differentiate into insulin-producing pancreatic-β cells in vitro (4). In a mouse model of sickle cell anemia, transplantation of hematopoietic progenitor cells derived from autologous mouse iPSCs that had been genetically corrected resulted in rescue of the disease phenotype (5).

But how can the regenerative potential of iPSCs be commandeered to help treat cancer patients? There are two possible scenarios where iPSCs might be of value. First, one could envisage using iPSC-derived tissue to replace or repair tissues of cancer patients that have been injured by radiation, chemotherapy, or the surgical treatment necessary to eliminate the tumors. Because most cancers involve acquired genetic mutations in a specific tissue, iPSCs derived from other healthy tissues of the same patient theoretically could be used to regenerate those tissues damaged by the tumors themselves or subsequent treatments. However, human iPSC-mediated regenerative therapy requires that the iPSC-derived tissue shows robust engraftment in vivo. Unfortunately, only a few human ESC- or iPSC-derived cell types such as ESC-derived dopaminergic neurons or ESC- and iPSC-derived hepatocytes have been shown to engraft successfully in animal models of Parkinson’s disease and liver cirrhosis, respectively (6, 7). So far, many other cell types—such as hematopoietic cells derived from ESCs or iPSCs—that would have been predicted to engraft efficiently in vivo have proved surprisingly recalcitrant to engraftment. Recently, we have demonstrated that hepatic cells derived from iPSCs generated from healthy human donors or from patients with inherited liver disease, liver cirrhosis, or liver cancer were able to engraft the livers of mice treated with a chemical to induce liver injury (7, 8). Up to 30% of the mouse liver was composed of human hepatocytes after transplantation, and the engraftment levels were comparable with those observed after transplant of primary human hepatocytes (7, 8). Although this study is preliminary, it suggests that it may be possible to generate human hepatocytes from iPSCs derived from the nonliver tissue of patients with liver cirrhosis that could then be transplanted into these patients to promote liver regeneration and repair. Such a strategy might also have the potential to prevent patients with liver cirrhosis from progressing to hepatocellular carcinoma.

A second area in which patient-specific iPSCs may offer a distinct advantage for cancer treatment is immune therapy (9). It has been shown that human iPSCs derived from T lymphocytes retain the pre-rearranged T cell receptor (TCR) gene (10–13), suggesting that these iPSCs could be induced to differentiate into functionally active T cells. It will be interesting to see whether a large quantity of functional T lymphocytes carrying specificity against certain tumor antigens could be generated in vitro by reprogramming selected T cell clones into iPSCs and then subsequently differentiating them back into T lymphocytes that could then be infused into the patient. One of the concerns related to this approach, however, is safety. A mouse study has shown that mice derived from a mature T cell reprogrammed by somatic cell nuclear transfer (a procedure in use before the advent of iPSC technology) developed spontaneous T cell lymphomas at high frequency (14). It is currently unclear if this observed phenomenon is intrinsic to T cells bearing pre-rearranged TCRs. Future studies are needed to demonstrate the safety of human iPSCs derived from T cells.

One area where human iPSCs could be very useful for cancer treatment is for screening new drugs. Human iPSCs can be obtained from the cancer cells of patients, and these iPSCs and their progeny would provide cellular targets containing all of the mutations of the patient’s tumor on the genetic background of the patient. The cell types obtained by differentiating patient
iPSCs derived from cancer tissues may be more biologically relevant to human tumors than the traditional cancer cell lines, mouse tumors, or mouse xenograft models that are currently used in drug screening. In addition, hepatocytes generated from human iPSCs of different genetic backgrounds will also aid efforts to test the toxicity of candidate cancer drugs—hepatotoxicity is the most common reason that many promising cancer drugs never make it into the clinic (15).

CURRENT HURDLES TO iPSC-BASED CANCER THERAPY

There are many roadblocks that may hinder or delay clinical applications of human iPSCs for treating a variety of diseases, including cancer. Such roadblocks include (i) safety issues related to the generation of iPSCs, (ii) a lack of effective protocols for differentiating human iPSCs into different functional cell types, and (iii) a lack of GMP-compliant protocols for deriving, expanding, and differentiating human iPSCs.

A principal safety concern is insertional mutagenesis associated with the traditional derivation of iPSCs from adult human cells by use of a transcription factor transgene cocktail delivered by retrovirus or lentivirus vectors. This roadblock has been partly addressed by using virus-free and integration-free methods such as episomal vectors or mRNA- or protein-based strategies to reprogram adult human cells; these strategies work with varying efficiencies to generate iPSCs (16–21). Human iPSCs derived by these protocols are free of exogenous DNA sequences, thereby eliminating the potential for insertional mutagenesis and undesired cell behaviors caused by reactivation of reprogramming transgenes. However, just like other processes involving cell division and

Figure 1. Roadblocks to translating human iPSC technology to the clinic. Human iPSC technology potentially can be used for screening new cancer drugs (blue box) and ultimately for providing cells for transplant to treat a variety of diseases, including cancer (yellow box). Genetic mutations can be corrected in patient-derived iPSCs by gene targeting approaches. The main hurdles to using patient-specific iPSCs for disease modeling, drug screening, and transplantation purposes are (i) a lack of effective differentiation protocols, (ii) little or no engraftment capability for the majority of human iPSC-derived specialized cells, (iii) difficulties in modeling multifactorial diseases, (iv) the need for GMP-compliant conditions at each step, and (v) safety concerns regarding the potential tumorigenicity of iPSCs associated with their pluripotent state or with insertional- or culture-driven mutagenesis. Dotted arrow, not yet tested; solid arrow, only a few studies available; blue arrow, feasible but requires further study.
expansion, genomic mutations are inevitable during reprogramming and the subsequent expansion steps, as has been demonstrated in a recent whole-exome sequencing study (22). This study showed that human iPSC lines, regardless of the reprogramming methods used, contain approximately six protein-coding point mutations per exome. As whole-genome sequencing technology continues to develop and the costs for such studies continue to drop, it will become both feasible and necessary to examine individual human iPSC lines for their genomic integrity to ensure their safety for clinical applications.

Many cell types have been generated from human iPSCs; however, the functionality of most of the human iPSC-derivatives remains unknown and requires extensive testing. Although differentiation protocols have been established for obtaining hematopoietic progenitor-like cells as well as more specialized blood cell types, it has proved a daunting task to generate transplantable hematopoietic stem cells from human ESCs or iPSCs that can home to bone marrow after transplant and engraft successfully (23, 24). In contrast, human ESC- and iPSC-derived hepatic progenitors and mature hepatocytes have been shown to engraft mouse liver tissue after transplant and to be functional (7, 8, 25–28). The functionality of these iPSC-derived liver cells still needs to be improved for them to be comparable with their primary human hepatocyte counterparts. To achieve this goal will require more advanced knowledge about normal liver development. Studies using mouse iPSCs and more recently human iPSCs have suggested that there is epigenetic memory retained in the iPSCs reflecting their derivation from adult somatic (parental) cells and that such molecular memory may influence their potential to differentiate into certain tissues, including hematopoietic cells and pancreatic cells (7, 29–31). Understanding the mechanisms underlying the epigenetic memory (7, 29–31) retained by iPSCs will also benefit efforts to derive functional cell types that are safer and more suitable for therapy.

Currently, most of the reprogramming and differentiation experiments have been conducted as proof-of-principle studies. Translating this research into clinical therapies will require significant efforts to develop GMP-compliant conditions. Clinical grade iPSCs and differentiation reagents need to be developed. Given that it takes several months to generate, expand, and select human iPSCs even before the differentiation process begins, for clinical purposes it may be more practical to establish human iPSC banks that contain a wide range of iPSC lines derived from diverse human lymphocyte antigen (HLA) haplotypes. These banked iPSCs could then be used to produce HLA-compatible tissues and organs for allogeneic rather than autologous transplantation.

TREATING HEMATOLOGICAL MALIGNANCIES WITH ADULT STEM CELLS

Allogeneic blood or bone marrow transplantation (BMT) is the standard-of-care treatment for many hematological malignancies, such as leukemias, that cannot be cured with conventional-dose therapies. Histocompatibility barriers have historically limited the applicability of BMT to those patients who have HLA-matched donors. Transplants of HLA-mismatched bone marrow produce mortality rates in excess of 50%, mostly as a result of graft-versus-host disease (GVHD) (32, 33). Accordingly, upwards of 50% of patients, and the majority of some ethnic groups such as African-Americans (34), in need of allogeneic BMT lack appropriate donor options. The ability to generate hematopoietic stem cells from patient-derived iPSCs could theoretically provide a graft for all in need of BMT. However, the potent immunological allogeneic graft-versus-tumor effect, which is generally the most important antitumor activity of allogeneic BMT, would be lacking if the patient’s own iPSC-derived hematopoietic stem cells were used unless similar anticancer activity could be engineered into these cells. HLA-matched allogeneic iPSCs would be another alternative. On the other hand, recent data indicate that HLA barriers may no longer be a major impediment to the more widespread use of allogeneic BMT. Recent data show that allogeneic BMT from mismatched (haploidentical)–related BMT donors can be just as effective as that from matched siblings, with
similar rates of GVHD and survival (35, 36). Importantly, such an approach maintains the allogeneic graft-versus-tumor effect.

WHAT DOES THE FUTURE HOLD?
It is currently difficult to predict whether human iPSCs have real potential in regenerative medicine because in vivo studies reporting engraftment of cells derived from iPSCs are sparse. The two main uses of human iPSC technology would be for drug discovery and cell transplantation (Fig. 1). Using iPSCs for drug development would require establishing patient iPSC-based disease models for testing potential drugs. Pathogenesis research using patient-relevant models of complex diseases such as cancer would help in the discovery of better cellular and molecular targets for drug development. Regenerative therapy using iPSCs will require correction of the genetic defect (when necessary) and in vivo functional testing of these cells. Perhaps a more immediate use of human iPSC-derived cells will be for in vitro screening of candidate antitumor drugs given that improving and evaluating in vivo function for most iPSC-derived cell types is still in the future. Patient iPSC-based drug screening is likely to be particularly beneficial for cancer patients with late-stage inoperable tumors because conventional cancer cell lines and animal models have not been effective for developing drugs that are powerful enough to combat the intra- and intertumor heterogeneity of advanced cancers (37). Although there are many technical hurdles to overcome before establishing iPSC-based tailored therapy for cancer patients, the potential of pluripotent stem cell therapy is great. Together with continued improvements in current cancer therapies, iPSC technology provides an additional therapy to add to the antitumor armamentarium, but much more work needs to be done to demonstrate their true value in the clinic.

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The ability of transcription factors to directly reprogram the identity of cell types is usually restricted and is defined by cellular context. Through the ectopic expression of single *Caenorhabditis elegans* transcription factors, we found that the identity of mitotic germ cells can be directly converted into that of specific neuron types: glutamatergic, cholinergic, or GABAergic. This reprogramming event requires the removal of the histone chaperone LIN-53 (RbAp46/48 in humans), a component of several histone remodeling and modifying complexes, and this removal can be mimicked by chemical inhibition of histone deacetylases. Our findings illustrate the ability of germ cells to be directly converted into individual, terminally differentiated neuron types and demonstrate that a specific chromatin factor provides a barrier for cellular reprogramming.

**CONCEPT DEPENDENCY OF CHE-1 ACTIVITY**

We sought to establish a system in which we could study the mechanistic basis of the context dependency of transcription factor activity. To this end, we used a genetic approach in the nematode *C. elegans*, using the zinc finger transcription factor CHE-1, which is required to induce the identity of a specific class of gustatory neurons called ASE neurons (6, 7). CHE-1 exerts this activity through binding directly to a cis-regulatory motif (termed the “ASE motif”) present in many ASE-specific terminal differentiation genes, such as those encoding chemoreceptors, neurotransmitter receptors, signaling proteins, and neurotransmitter transporters (6). Like CHE-1, several other invertebrate and vertebrate transcription factors are also known to co-regulate many terminal features of differentiated neurons in such a manner and have been termed “terminal selectors” (8).

To test whether CHE-1 is not only required but also sufficient to induce ASE fate, we ectopically expressed CHE-1 throughout the entire animal in either larval or adult stages, using an inducible heat shock promoter. Such misexpression results in broad ectopic expression of an artificial reporter of CHE-1 transcription factor activity, which is composed of a multimerized ASE motif (“8x ASE motif reporter”) (6) (Fig. 1). This indicates that, in principle, CHE-1 can...
exert its biochemical activity of DNA binding and transcriptional activation without spatial or temporal constraints. In contrast, postembryonic ectopic expression of che-1 during larval or adult stages is able to induce markers for terminal ASE fate (the gcy-5 chemoreceptor and the ceh-36 homeobox gene; see table S1 for list of markers) only in one to three types of other sensory neurons (blue arrows), consistent with previous reports (6, 7). Heat shock induction of CHE-1 was done at late larval stages. Large boxes show magnified views of small boxes in the same panels. Table S1 contains details on transgenic reporters; table S2 shows data quantification. All images that show entire worms are at 160× magnification; all images showing parts of the worm are at 630× magnification.

Figure 1. Context dependency of CHE-1 induction of target genes. Ectopic expression of CHE-1 can induce an artificial reporter of CHE-1 activity (a multimerized binding site called the 8x ASE motif) throughout the animal (top row; observed in all 50 of the heat-shocked animals), but can induce ASE cell fate markers such as the gcy-5 chemoreceptor (exclusively expressed in ASER) or the ceh-36 homeobox gene (expressed in ASEU/R and AWCL/R) only in one to three types of other sensory neurons (blue arrows), consistent with previous reports (6, 7). Heat shock induction of CHE-1 was done at late larval stages. Large boxes show magnified views of small boxes in the same panels. Table S1 contains details on transgenic reporters; table S2 shows data quantification. All images that show entire worms are at 160× magnification; all images showing parts of the worm are at 630× magnification.

THE CHROMATIN FACTOR LIN-53 CONTROLS CONTEXT DEPENDENCY OF CHE-1

To test the hypothesis that the mechanistic basis for such context dependency lies in inhibitory, perhaps chromatin-based mechanisms that may prevent CHE-1 from reprogramming the identity of other cells, we established and screened through an RNA interference (RNAi) library that targets all genes in the C. elegans genome with predicted roles in chromatin regulation, based on the presence of characteristic protein domains (9) (table S3). We found that RNAi-mediated knockdown of lin-53, the C. elegans ortholog of the phylogenetically conserved, WD40 domain–containing retinoblastoma binding protein RbAp46/48 (10), permits ectopically expressed CHE-1 to induce the ASE neuronal fate markers gcy-5 and ceh-36 in a large number of normally non-neuronal cells in the midbody region of larval and adult animals (Fig. 2). Up to 52% of animals (n = 227) showed this effect (table S4), which could be observed using distinct, nonoverlapping double-stranded RNA clones that target lin-53 and all six of the tested transgenic che-1 lines (see supporting online material).
GERM CELLS ARE THE TARGET OF REPROGRAMMING

Closer examination of the animals revealed that the cells showing \( gcy-5 \) and \( ceh-36 \) induction are located within the gonad (Fig. 2). Differential interference contrast (DIC) microscopy showed that germ cells of \( che-1^{\text{heat-shock}}; lin-53(RNAi) \) animals lose their characteristic, fried egg–shaped nuclear and nucleolar morphology and adopt a speckled neuronal nuclear morphology (Fig. 2). Moreover, these cells grow cellular extensions resembling axo-dendritic projections (Fig. 2 and movies S1 to S3). These neuron-like cells indeed originate from the germ line, as they were not observed after ectopic \( che-1 \) expression and \( lin-53 \) removal in a \( glp-4 \) mutant background (fig. S1), in which no germ line is formed (11). Genetic removal of sperm \( [fem-3] \text{gf} \) mutants (12)) or oocyte \( [fem-3] \text{lf} \) mutants (13) or prevention of entry into meiosis \( [glp-1] \text{gf} \) mutants (14) does not affect neuron induction, whereas severe reduction of the mitotic pool \( [glp-1] \text{lf} \) mutants
which suggests that it is the mitotic germ cells that become neuron-like (fig. S1). This is further supported by the position of the converted cells relative to mitotic and meiotic markers (fig. S2) and the observation that animals in the second and third larval stages, which contain mostly mitotic but no meiotic cells, show germ cell–neuron conversion upon ectopic che-1 expression and lin-53 knockdown (table S4). The conversion to neuron-like cells is efficient and fast; as many as 60 germ cells (of a total of about 200 mitotic germ cells) undergo neuronal induction (fig. S3), and morphological changes and marker induction first occur 6 hours after che-1 induction (table S5). For comparison, the induction of the 8x ASE motif, an indicator of CHE-1 transcriptional activity occurs 4 hours after che-1 induction in the gonad. The induction of the 8x ASE motif in the gonad of wild-type animals, as well as antibody staining conducted in both wild-type and lin-53(RNAi) animals, rules out the possibility that lin-53(RNAi) merely results in germline derepression of the che-1 transgene. [We also note that lin-53(RNAi) does not result in germline derepression of a previously described transgenic array, let-858::gfp, known to be derepressed after loss of several different chromatin factors (15).]

**EXTENT OF GERM CELL–NEURON CONVERSION**

We assessed the nature of these che-1–induced, neuron-like cells with a number of fate markers. Through antibody staining of a marker that labels specific germ cell structures, the P-granules, we confirmed that this germ cell feature is indeed lost upon ectopic che-1 expression and lin-53 knockdown (fig. S4). Moreover, the reprogrammed cells express all five tested pan-neuronal reporter genes: the rab-3/Rab3 and snb-1/synaptobrevin genes, which encode presynaptic proteins normally exclusively expressed in all cells of the nervous system; the pan-neuronal axonal regulators unc-33/CRMP and unc-119; and the pan-neuronal signaling
factor rgef-1 (Fig. 3A; see table S1 for transgenic reporters). Antibody staining against endogenous proteins also shows ectopic expression of presynaptic synaptobrevin/ SNB-1 and RIM/UNC-10 proteins; these proteins appear to cluster in presynaptic specialization in the axonal extensions of the reprogrammed germ cells (Fig. 3B and fig. S5). The reprogrammed cells also express a ciliated marker gene, osm-6, a member of the intraflagellar transport particle, normally expressed exclusively in all ciliated sensory neurons, including ASE (Fig. 4A). Green fluorescent protein (GFP)–tagged OSM-6 protein also appears to cluster in particles in the induced neurons (Fig. 4A), which suggests that these cells express and cluster intraflagellar transport particles. All tested components of the gene battery that combinatorially define ASE identity are also expressed in the induced neurons. That is, aside from the above-mentioned chemoreceptor gcy-5 and the transcription factor ceh-36 (shown again in Fig. 4A), the putative chemoreceptor gcy-7, which is normally exclusively expressed in ASE, and the vesicular glutamate transporter eat-4, normally expressed in ASE and a restricted number of additional head ganglia neurons, are also expressed in the reprogrammed germ cells (Fig. 4A). Induction of eat-4 demonstrates that the reprogrammed cells are glutamatergic. The reprogrammed ASE-like cells do not express a battery of markers that are normally expressed in other neuron types, such as dopaminergic, serotonergic, cholinergic, and GABAergic markers, among others (Fig. 4B). This argues that the reprogrammed cells are not merely generic and/or misspecified neurons but closely resemble normally differentiated ASE neurons. Taken together, animals ectopically expressing che-1 and lacking lin-53 not only contain their normal set of two ASE gustatory neurons in the head, but contain a gonad filled with dozens of ASE-like neurons.

SPECIFICITY OF GERM CELL–NEURON CONVERSION

Removal of RNA-binding gene regulatory factors has been shown to result in the formation of teratomas (i.e., cells of various origins and types) in the germ line (16). The effects that we observed upon loss of lin-53 are not a reflection of teratoma formation, because we observed no expression of various neuronal fate markers other than those of ASE fate in the gonad of che-1\textsuperscript{heat-shock}; lin-53(RNAi) animals. However, the very same neuronal markers (GABA neuron marker, cholinergic neuron marker) were expressed in the gonad of animals in which the transcription regulator gld-1, a previously described repressor of teratoma formation (16), is knocked down (fig. S6). Therefore, removal of lin-53 does not by itself trigger alternative developmental programs but primes germ cells to be responsive to a neuronal fate inducer such as che-1.

GERM CELL CONVERSION TO OTHER NEURON TYPES

To test whether lin-53 removal also permits the conversion of germ cells into other neuron types, we tested two other terminal selector genes (8): the phylogenetically conserved Pitx-type homeobox gene unc-30, a terminal selector required for the generation of GABAergic motor neurons in the ventral nerve cord (17, 18), and the EBF-like transcription factor unc-3, required for the generation of two types (A- and B-type) of cholinergic motor neurons in the ventral nerve cord (19, 20). When ectopically misexpressed, neither unc-30 nor unc-3 was able to induce GABAergic or cholinergic neuron fate in the germ line, respectively. However, upon removal of lin-53, heat-shock induction of either unc-30 or unc-3 resulted, like che-1 induction, in germ cells losing their characteristic morphology and instead adopting neuron-like nuclear morphology and growing axonal projections (Fig. 5, A and B). In the case of unc-3, the ectopic neurons expressed a marker characteristic of cholinergic A/B-type ventral cord motor neurons (acr-2; Fig. 5B), whereas ectopic expression of unc-30 resulted in the expression of the GABAergic marker unc-47 (Fig. 5A). In neither case did we observe any ASE marker expression to be induced (>100 animals scored); also, neither cholinergic nor GABAergic markers were induced by che-1 (Fig. 4B). We conclude that upon loss of lin-53, germ cells acquire the ability to be reprogrammed into distinct neuron types through the activity of neuron type–specific terminal selector transcription factors.
Figure 4. Reprogrammed germ cells express markers for ASE, but not other neuronal fates. Experimental conditions and image labeling are as described in Fig. 3. (A) ASE-specific markers expressed in che-1 heat-shock; lin-53(RNAi) animals. osm-6::gfp is a full-length protein fusion. (B) Other neuronal fates are not expressed in che-1 heat-shock; lin-53(RNAi) animals. The left column shows an overall view of the entire animal to illustrate the expression of non-ASE markers (all mCherry-based reporters) in other neuron types, and the right column shows an enlarged view (white box) of the gonad region, illustrating that these markers do not become induced in the che-1 reprogrammed germ cells, labeled with gcy-5::gfp.
Does lin-53 removal prime germ cells to respond only to factors that induce neuronal fates, or can they now respond to other factors as well? To address this question, we ectopically expressed a selector gene, the *C. elegans* MyoD homolog *hlh-1* that was previously shown to be able to ectopically induce muscle fate in early embryos (21). We found that *hlh-1* is unable to convert the germ cells of *lin-53(RNAi)* animals into muscle cells (fig. S7). These negative results need to be cautiously interpreted, but may represent a first hint toward a target selectivity of *lin-53*. That is, *lin-53* may only restrict the developmental potential specifically toward a neuronal developmental program, whereas other factors may serve to prevent the induction of other, non-neuronal differentiation programs.

LIN-53, which is ubiquitously expressed (10), is one of several phylogenetically conserved histone chaperones that are thought to assist in the recruitment of various distinct types of histone modifiers or remodelers (including histone methyltransferases, histone acetylases, and histone deacetylases) to histone H3 and histone H4 (22, 23). LIN-53 orthologs in various species have been found to be integral components of at least six different protein complexes, each displaying diverse biochemical and biological roles: the NURD and NURF nucleosome remodeling complexes, the CAF-1 chromatin assembly factor complex, the Sin3A transcriptional repressor complex, the PRC2 histone methyltransferase complex, and the HAT1 histone acetyltransferase complex (22, 23) (table S6). RNAi of representative members of each of these complexes either did not phenocopy the *lin-53(RNAi)*–induced reprogramming ability of ectopic CHE-1 expression or could not be interpreted because of early lethality induced by RNAi (table S6). However, two of the LIN-53–associated complexes (NURD and Sin3a) each contain at least two histone deacetylase (HDAC)
components and the reprogramming role of *lin-53* may involve several complexes; for these reasons, we sought to broadly inhibit HDAC function by using two distinct chemical inhibitors, valproic acid and trichostatin A (24). At sublethal doses, animals treated with either drug survived and permitted heat shock–induced *che-1* to induce ASE fate in the germ line (and no other cell type) even in the presence of functional *lin-53* (*Fig. 6*). Even though these drug effects may be unrelated to normal *lin-53* function, these results nonetheless provide a strong indication that histone modifications are key players in restricting the ability of a transcription factor to reprogram cellular identity.

**CONCLUSIONS**

Our finding that the removal of a single chromatin factor, together with the induction of single transcription factors, can produce distinct and specific neuron types in a heterologous cellular context is a testament to the simplicity of programs that control neuronal differentiation. The role of complex, multistage neuronal developmental programs may mainly lie in orchestrating the activation of a terminal regulatory routine—that is, one or more terminal selectors that directly control terminal differentiation genes. This notion may apply to more complex systems as well, because recent work has shown that it takes as few as two transcription factors to drive a differentiated fibroblast toward a specific neuronal fate (25). Our findings are also a testament to the totipotency of germ cells (26). This totipotency is normally kept in check by a variety of transcriptional and posttranscriptional mechanisms (26), but is unleashed either spontaneously in pathological situations (germ cell tumors) (27) or upon culturing germ stem cells under specific conditions, which transform these cells into cells indistinguishable from pluripotent embryonic stem cells (28).

We have shown that the ability of germ cells to be directly reprogrammed into neurons can be unleashed, even in the adult animal, through removal of a single gene that...
we speculate to be involved in rendering neuronal differentiation genes inaccessible to transcriptional induction by, for example, contributing to the formation of facultative (i.e., conditional and developmentally regulated) heterochromatin. Seen in a broader context, our results indicate that the reprogramming of cellular identity may critically depend not just on providing the correct transcription factor(s) that induce a specific fate, but also on the removal of inhibitory mechanisms that restrict transcription factor activity. We anticipate that the disablement of such inhibitory mechanisms may provide an efficient strategy to ectopically generate neuron types in other organisms, or perhaps even in cell culture, using isolated germ cells.

References and Notes
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Supporting Online Material
www.sciencemag.org/cgi/content/full/science.1199082/DC1
Materials and Methods
Figs. S1 to S7
Tables S1 to S6
Movies S1 to S3
References
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10.1126/science.1199082
Born in 1564, Galileo Galilei once contemplated a career in the priesthood. It’s perhaps fortunate for science that upon the urging of his father, he instead decided to enroll at the University of Pisa. His career in science began with medicine and from there he subsequently went on to become a philosopher, physicist, mathematician, and astronomer, for which he is perhaps best known. His astronomical observations and subsequent improvements to telescopes built his reputation as a leading scientist of his time, but also led him to probe subject matter counter to prevailing dogma. His expressed views on the Earth’s movement around the sun caused him to be declared suspect of heresy, which for some time led to a ban on the reprinting of his works.

Galileo’s career changed science for all of us and he was without doubt a leading light in the scientific revolution, which is perhaps why Albert Einstein called him the father of modern science.

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I. H1 cells (passage 6) were seeded in 96 well plates (Matrigel-coated) in the various media. Media were changed every 24 hours. The number of cells was determined using a CyQuant cell proliferation assay kit.

II. Evaluation of human embryonic stem cells (H9.2 cells) cultured in NutriStem® hESC XF using Matrigel. Growth of hESCs cultured in NutriStem® hESC XF was compared to growth using competitor A. Cell counts are reported for days 2, 4 and 7.

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NutriStem® MSC XF Basal Medium
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NutriStem® MSC XF Supplement Mix
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