The hESC line Envy expresses high levels of GFP in all differentiated progeny

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Human embryonic stem cells (hESCs) have been advanced as a potential source of cells for use in cell replacement therapies. The ability to identify hESCs and their differentiated progeny readily in transplantation experiments will facilitate the analysis of hESC potential and function *in vivo*. We have generated a hESC line designated 'Envy', in which robust levels of green fluorescent protein (GFP) are expressed in stem cells and all differentiated progeny.

The National Institutes of Health Human Embryonic Stem Cell Registry listed hESC line, hES 3 (ref. 1; http://www.escellinternational.com) was electroporated with a vector in which GFP was expressed under the control of the human β-actin (*ACTB*) promoter (**Fig. 1a**). A GFP-positive hES 3 clone identified 3 d after electroporation was isolated under epifluorescence illumination and expanded. Southern blot and PCR analysis suggested this clone harbored a single copy of the vector that had undergone substantial deletions upon integration into the genome (data not shown). Rapid amplification of 3′ cDNA ends (3′ RACE) experiments revealed that the vector had integrated into chromosome 12 at position q23.1 (**Fig. 1a**), an assignment confirmed by PCR using primers predicted to lie 5′ and 3′ of the proposed integration site (data not shown). Examination of this genomic region showed the GFP transgene (Genbank accession number AY952326) was located between the genes encoding thrombopoietin (*TPO*, also known as *THPO*) and solute carrier 25 (*SC25*) and had not disrupted any known genes (**Fig. 1a**). Envy cells could be maintained in bulk culture, and they expressed uniform, high levels of GFP, making them easily distinguishable from wild-type embryonic stem cells (**Fig. 1b–e**). Envy cells expressed the stem cell markers GCTM-2, E-cadherin and Oct4 in their undifferentiated state and retained a stable normal karyotype for more than 12 months or 116 passages (data not shown) (**Fig. 1f–j**). When injected into the testes capsule of SCID mice, passage 62 Envy cells formed teratomas containing a spectrum of different cell types including derivatives of ectoderm (neural rosettes), mesoderm (cartilage) and endoderm (glandular epithelia), confirming their pluripotentiality (**Fig. 1k–m**).

Envy cells retained uniform and robust GFP expression after *in vitro* differentiation into cells representative of the three embryonic germ layers. Differentiation of Envy cells toward the neuronal lineage showed that GFP expression was present at high levels

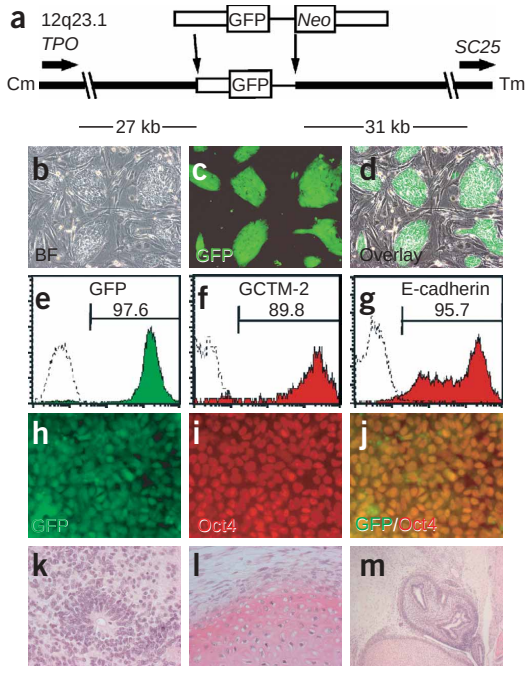

Figure 1 | Genetic and phenotypic analysis of Envy cells. (**a**) Structure of the ENVY locus showing the vector, locus and chromosomal location relative to neighboring genes, *TPO* and *SC25* (horizontal arrows indicate direction of transcription). Vertical arrows demarcate the 5′ and 3′ ends of the vector present in the Envy locus. Cm, centromere; Tm, telomere. (**b–e**) Envy cells grown in bulk culture had uniform high GFP expression clearly visible by fluorescence microscopy (**b–d**) and were thus easily distinguishable from unmanipulated hESCs by flow cytometry (**e**). (**f–j**) Flow cytometric experiments showed that Envy cells retained robust expression of the stem cell markers GCTM-2 (**f**) and E-cadherin (**g**); immunofluorescence microscopy experiments indicated uniform expression of Oct4 (**h–j**). (**k–m**) Xenotransplanted Envy cells formed teratomas containing a variety of different tissues including those reminiscent of neural rosettes (**k**), muscle and cartilage (**l**) and endoderm-derived secretory epithelia (**m**). See **Supplementary Methods** online.

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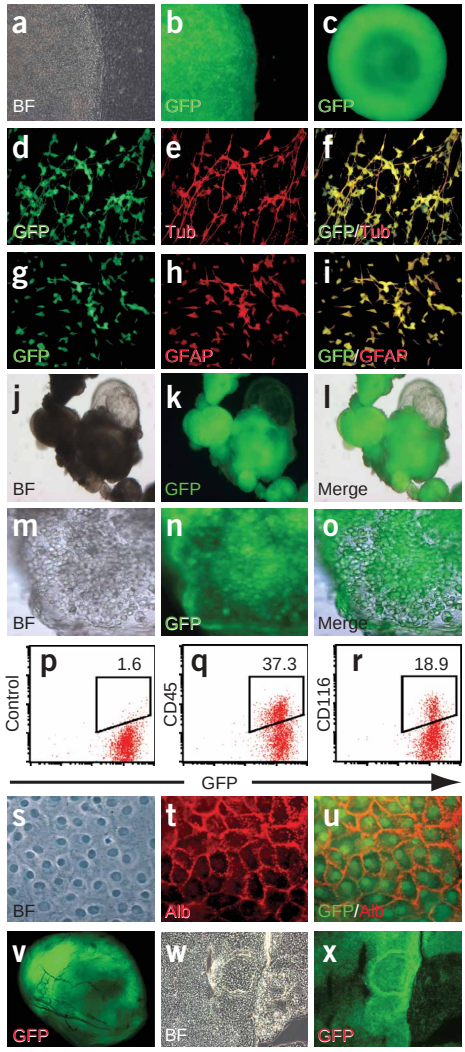

Figure 2 | Differentiated progeny of Envy cells retain robust GFP expression. (**a**–**i**) GFP expression was maintained during noggin-induced neural differentiation (**a**,**b**), neurosphere formation (**c**) and in differentiated neurons and astrocytes marked by expression of β-tubulin (Tub) (**d**–**f**) and GFAP (**g**–**i**), respectively. (**j**–**o**) In suspension culture, Envy cells formed GFP$^+$ embryoid bodies (**j**–**l**) that differentiated further to form GFP$^+$ blood cells (**m**–**o**). (**p**–**r**) FACS analysis indicated that all of the cell types within these cultures were GFP$^+$, including a proportion of cells that expressed the pan-hematopoietic marker CD45 (**q**) and the myeloid marker CD116 (GM-CSF receptor; **r**). (**s**–**u**) In cultures that favored the formation of endoderm, GFP expression was retained in cells expressing albumin (Alb). (**v**–**x**) GFP expression is highly visible in whole-mount Envy teratomas (**v**), reflecting the ubiquitous GFP expression detected with an anti-GFP antibody in histological (frozen) sections (**w**,**x**). BF, bright field. See **Supplementary Methods**.

during neurosphere formation and in differentiated cells expressing the neural and glial markers β-tubulin and GFAP, respectively (**Fig. 2a–i**). Envy embryoid bodies formed in suspension culture showed robust expression of GFP that was maintained in all of the differentiated progeny (**Fig. 2j–o**). Flow cytometric analysis indicated that in cultures of differentiated day 19 Envy cells containing blood cells, a mesoderm derivative, all of the cells expressed uniform high levels of GFP (**Fig. 2p–r**). In addition, GFP$^+$ beating cardiac muscle was also regularly observed (data not shown). When Envy cells differentiated into cells of the endoderm lineage, as determined by expression of α-fetoprotein, the hepatic stem cell marker GCTM5 (ref. 2; data not shown) or albumin (**Fig. 2s–u**), GFP expression was also retained at a high level. In teratomas, Envy cells retained robust GFP expression, clearly evident macroscopically and at the histological level, as revealed by immunohistochemical staining with an antibody against GFP (**Fig. 2v–x**).

Just as the ubiquitously expressed Rosa26 locus has been used in mouse embryonic stem cells to express exogenous genes in a reliable and predictable fashion[3,4], the ENVY locus may provide a similar conduit for the ubiquitous expression of exogenous genes in hESCs. Hitherto, identification of hESC-derived cells in animal grafting experiments has relied on the use of species-specific antibodies against ubiquitously expressed intracellular markers or *in situ* hybridization, two procedures that are cumbersome and require fixing the cells. Although other groups have generated GFP$^+$ hESCs[5–7], Envy cells have the major advantages of sustained, high GFP expression in all differentiated progeny in the absence of ongoing selection. Thus, although widespread variable expression of a reporter gene can be achieved with relative ease using specifically designed expression vectors[7], ubiquitous expression in differentiated progeny is a property only rarely attained. This uniformity and intensity of GFP expression should facilitate the analysis and enable the viable recovery of hESC-derived material from animal transplantation experiments[8] and recombinant tissue grafting experiments[9].

Note: Supplementary information is available on the Nature Methods website.

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COMPETING INTERESTS STATEMENT

The authors declare competing financial interests (see the *Nature Methods* website for details).

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