Changing potency by spontaneous fusion

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Recent reports have suggested that mammalian stem cells residing in one tissue may have the capacity to produce differentiated cell types for other tissues and organs1,2. Here we define a mechanism by which progenitor cells of the central nervous system can give rise to non-neural derivatives. Cells taken from mouse brain were co-cultured with pluripotent embryonic stem cells. Following selection for a transgenic marker carried only by the brain cells, undifferentiated stem cells are recovered in which the brain cell genome has undergone epigenetic reprogramming. However, these cells also carry a transgenic marker and chromosomes derived from the embryonic stem cells. Therefore the altered phenotype could be due to direct conversion of brain to embryonic stem cell but rather through spontaneous generation of hybrid cells. The tetraploid hybrids exhibit full pluripotent character, including multilineage contribution to chimaeras. We propose that transdifferentiation consequent to cell fusion10 could underlie many observations otherwise attributed to an intrinsic plasticity of tissue stem cells2.

The capacity to generate differentiated cell types representing all three definitive germ layers has traditionally been considered a property reserved to cells of the inner cell mass and epiblast in the early embryo and derivative embryonal carcinomas and embryonic stem (ES) cells in vitro11,12. Recently, however, remarkable potencies have been ascribed to stem cells isolated from various fetal and adult tissues including the central nervous system (CNS). CNS stem cells have been reported to contribute to haematopoietic lineages when injected into irradiated mice13, to produce muscle when co-cultured with skeletal myoblasts14, and to colonize multiple fetal lineages when introduced into pre-implantation embryos15. A further circumstance in which nervous system stem cells seem to change determination is during co-culturing with differentiating ES cells, when they form multineurulaic myotubules16. We investigated the basis of these phenomena and in particular whether during co-culture CNS cells may first convert to pluripotent ES cells as a route to generating other cell types.

We used distinct transgenic markers to isolate and identify descendants of both the ES cells and the CNS cells (Fig. 1). ZIF40 mice constitutively co-express resistance to the selection agent G418 and nuclear β-galactosidase activity14. Oct4-GFP mice express puromycin resistance and green fluorescent protein (GFP) exclusively in pluripotent and germline cells under direction of regulatory sequences of the mouse Oct4 gene15. Neurosphere cultures17 were initiated from dissociated forebrains of transgenic fetuses at embryonic day 14.5 carrying either of these markers. Neurospheres were initiated from dissociated forebrains of transgenic fetuses at embryonic day 14.5 carrying either of these markers. Neurospheres were recovered from each of 23 independent co-cultures. These included one experiment in which fetal telencephalic cells were combined directly with ES cells without prior preparation of

DNA ploidy and polymorphism

DNA contents per cell were determined by staining cells with propidium iodide and subsequent FACS analysis. Genomic DNA were extracted from embryonic stem cells, bone marrow cells, and BME3L cells. DNA was amplified using microsatellite primers (D9MIT48, Research Genetics) detecting polymorphisms between the bone marrow genome (mostly C57BL/6) and the embryonic stem cell genome (129/Sv), separated on 5% agarose gel and visualized by ethidium bromide staining.

Received 17 January; accepted 27 February 2002.

Published online 13 March 2002, DOI 10.1038/nature730.


Supplementary Information accompanies the paper on Nature’s website (http://www.nature.com).

Acknowledgements

The authors are indebted to A. Nagy for providing embryonic stem cell lines; Y. Yoneda for discussion; J. Crawford, D. Steinleider, M. Say and S. Sugrue for critical reading of the manuscript; and G. Brown, M. Jorgenson, A. Meacham, N. Devine, and Diagnostic Cytogenetics for technical assistance. This work was supported by grants from the National Institutes of Health (to N.T. and E.W.S.).

Competing interests statement

The authors declare that they have no competing financial interests.

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neurospheres. Cells surviving selection expressed nuclear β-galactosidase (ZIN40 cells) or cytoplasmic GFP (Oct4-GiP cells) as appropriate. Expression of the Oct4-GiP transgene (Fig. 1b) is striking because, like Oct4 itself, this transgene is expressed only in germline and pluripotent cells and is not active in CNS cells in vivo or in primary culture (ref. 15 and our unpublished observations). GFP activity is therefore indicative of at least partial epigenetic reprogramming of the somatic cell genome to a state of pluripotency. Thus it appeared that ES cells could be generated from fetal brain cells.

We then tested these cells for expression of the hygromycin resistance marker carried only by HT2 ES cells (Fig. 1a). The cultures were completely resistant to hygromycin. Furthermore, they were sensitive to gancyclovir, which kills only cells harbouring the HSV Tk transgene. To exclude the possibility that inefficient selection could have given rise to mixed cultures, we generated several clonal derivatives by limiting dilution. The clonal isolates all exhibited fetal and ES cell resistance/sensitivity phenotypes. Therefore transgenic selection markers of different origin are co-expressed in individual cells. We inferred that the selected cells were likely to be hybrids. Consistent with this, they had enlarged nuclei with multiple nucleoli (Fig. 2a, b). To test this interpretation directly, we prepared metaphase chromosome spreads (Fig. 2c). Analysis of 18 independent isolates revealed a tetraploid or near-tetraploid complement in all cases. The ES cells are male, and where a female fetus had been used as the source of brain cells, the sex chromosome complement was XXXY. The strain origin of chromosomes 8 and 14 can readily be discriminated owing to polymorphisms in centromeric heterochromatin\(^\text{19}\). Both strain 129 chromosomes of ES cell origin and non-strain 129 chromosomes of transgenic mouse origin were present in three separate isolates examined in this way (Fig. 2d).

These observations can be explained only by the formation of cell hybrids between CNS and ES cells. The hybrid cells expressed the ES cell markers\(^\text{12}\) alkaline phosphatase and stage-specific embryonic antigen (SSEA)-1 (data not shown). They expressed the essential pluripotent cell transcription factor Oct-4\(^\text{18,20}\) as well as the Oct4-GiP transgene. Like normal ES cells, they were dependent on the self-renewal factor leukaemia inhibitory factor (LIF) to suppress differentiation\(^\text{12}\). On aggregation they formed embryoid bodies\(^\text{22}\) containing both extra-embryonic endoderm and spontaneously contracting cardiomyocytes (Fig. 3). Embryoid bodies treated with retinoic acid\(^\text{22}\) gave rise to neurons. Thus the hybrids have in vitro self-renewal and differentiation properties similar to those of regular ES cells\(^\text{12}\). Hybrids produced by electrofusion of ES cells with thymocytes have similarly been found to exhibit ES cell characteristics\(^\text{23}\).

We examined the capacity for incorporation into embryonic development by blastocyst injection. Contributions to fetal tissues were detected in 8 of 23 transferred embryos by β-galactosidase staining for the ZIN40 marker (Fig. 4a). The contributions were modest by comparison with standard ES cells and uneven between tissues, but this is expected owing to competitive overgrowth of tetraploid hybrid cells by diploid host cells\(^\text{24}\). Interestingly, 1 of 14 live-born mice showed overt coat-colour chimaerism (Fig. 4b). This animal was euthanized and we analysed the internal organs for expression of nuclear β-galactosidase. Staining was detected in intestine, kidney, heart and most prominently in liver (Fig. 4c). A proportion of tetraploid hepatocytes is normally present in the liver, which may account for the persistent contribution of hybrid cells in this organ. These observations establish that the presence of a genome derived from a brain cell is not prohibitive for multilineage contribution of ES cell hybrids.

Finally we investigated whether similar fusion events could occur with cells isolated from adult brain. A modified schedule for co-culture and selection was adopted to allow for the comparatively slower proliferation rate of adult-derived neurospheres. Neurospheres were established from transgenic mice that express ubiqui-
Figure 3 Pluripotency of hybrid cells. Embryoid bodies were prepared from hybrid cells and outgrowths analysed by immunostaining for markers of cardiomyocytes (β-actin, a), or, if treated with retinoic acid, neurons (type III tubulin, b), or by in situ hybridization (c), which revealed Sparc messenger RNA in migratory cells of typical parietal endoderm morphology.

Figure 4 Contribution of ZIN40/HT2 hybrid cells to chimaeras. a, β-galactosidase staining of fetal chimaera at embryonic day 11.5, slightly retarded. b, Adult mouse chimaera. c, β-galactosidase staining of liver from an adult mouse chimaera.

Methods

Embryonic stem cells and hybrid cells were maintained in LIF-supplemented medium without feeders2. HT2 and 46C ES cells are of pure inbred 129/09a origin. Transgenic mice are on mixed 129 X MF1 (ZIN40 and Oct4-GiP) and 129 X C57Bl/6 (tauGFP) backgrounds. Neurosphere cultures were initiated and expanded in DMEM/F12 medium with N2 and B2 supplements plus fibroblast growth factor-2 (10 ng ml\(^{-1}\)). Dissociated fetal neural cells and ES cells were mixed in a 2:1 ratio and plated on dishes coated with poly-o-lysine and laminin at 1–1.5 x 10\(^5\) cells cm\(^{-2}\). Cultures were maintained in neural stem cell medium for 48 h, then changed to ES cell medium containing fetal calf serum and LIF (418; 200–400 μg ml\(^{-1}\)) was added after a further 2 days of co-culture, but puromycin (1 μg ml\(^{-1}\)) only after 6 days because the Oct4-GiP transgene is not expressed in brain cells. In the case of adult neural cells, the co-culture was initiated at a 1:10 ratio of brain cells to ES cells and maintained for 2 weeks before application of puromycin selection. ES cell differentiation and chimaera production followed standard protocols.

Received 28 November 2001; accepted 27 February 2002.

Shaggy/GSK3 antagonizes Hedgehog signalling by regulating Cubitus interruptus

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The Drosophila protein Shaggy (Sgg, also known as Zeste-white3, Zw3) and its vertebrate orthologue glycogen synthase kinase 3 (GSK3) are inhibitory components of the Wingless (Wg) and Wnt pathways. Here we show that Sgg is also a negative regulator in the Hedgehog (Hh) pathway. In Drosophila, Hh acts both by blocking the proteolytic processing of full-length Cubitus interruptus, Ci (G155), to generate a truncated repressor form (Ci75), and by stimulating the activity of accumulated G155 (refs 2–6).

Loss of sgg gene function results in a cell-autonomous accumulation of high levels of G155 and the ectopic expression of Hh-responsive genes including decapentaplegic (dpp) and Wg. Simultaneous removal of sgg and Suppressor of fused, Sufu, results in wing duplications similar to those caused by ectopic Hh signalling. Ci is phosphorylated by GSK3 after a primed phosphorylation by protein kinase A (PKA), and mutating GSK3-phosphorylation sites in Ci blocks its processing and prevents the production of the repressor form. We propose that Sgg/GSK3 acts in conjunction with PKA to cause hyperphosphorylation of Ci, which targets it for proteolytic processing, and that Hh opposes Ci proteolysis by promoting its dephosphorylation.

The Hh family of secreted proteins controls cell growth and patterning in many principal developmental processes in both vertebrates and invertebrates. Moreover, mutations in components of the Hh signalling pathway have been implicated in many human disorders, including cancer. During Drosophila limb development, posterior (P)-compartment cells express and secrete Hh which induces adjacent anterior (A)-compartment cells to express target genes including dpp, wg (leg only) and patched (ptc) by regulating the transcription factor Ci6,10,11. In A-compartment cells distant from the AP compartment boundary, Ci is processed to generate a truncated repressor form (Ci75) that represses a subset of Hh-responsive genes including dpp2,4. In A-compartment cells adjacent to the AP compartment border, Hh signalling blocks Ci processing to generate G175, and causes the accumulation of full-length Ci (G155)5. In addition, high levels of Hh stimulate a distinct transcriptional activation activity of Ci155, which is required for the expression of Hh-responsive genes such as ptc5,8,12.

In both wing and leg discs, loss of sgg function in the A compartment either by using sgg mutations or by overexpressing a dominant negative form of GSK3 (DN-GSK3)13 causes the accumulation of high levels of Ci155 in a cell-autonomous fashion without affecting ci-lacZ expression (Fig. 1a, b, c, h, j–l, and see Supplementary Information). In wing discs, anterior sgg cells or DN-GSK3-expressing cells located outside the wing pouch region ectopically express dpp, which is repressed by Ci75 (Fig. 1c, d). However, anterior sgg cells do not ectopically activate ptc, which is activated by Ci155 (Fig. 1f). In leg discs, anterodorsal sgg cells distant from the AP boundary ectopically express wg and low levels of dpp (Fig. 1g–i), a phenotype similar to that associated with sgg PKA double-mutant cells in which both Wg and Hh signalling pathways are ectopically activated14. As in the case of wing discs,

Figure 1 Ectopic Hh signalling activity in sgg−/− cells. a–c, A wing disc carrying sgg−/− clones and showing the expression of a marker gene (a), Ci155 (b) and dpp-lacZ (c). sgg−/− cells are recognized by strong Myc staining. Anterior sgg−/− cells accumulate high levels of Ci155 and ectopically express dpp-lacZ. The inset in b shows an enlarged view of the anterior sgg−/− clone (arrow in a). d, e, A wing disc containing clones of cells that express UAS-DN-GSK3 using actrindic > C202 > Gal4. Anterior DN-GSK3-expressing cells accumulate high levels of Ci155 (arrows in e) and ectopically express dpp-lacZ (arrows in d, f). A wing disc carrying sgg−/− mutants and exhibiting the expression of Ci155 and ptc-lacZ. Anterior sgg−/− mutant clones do not ectopically activate ptc-lacZ (arrow). g–i, j, k, l, m, n, o, p, q, r, s, t, u, v, w, x, y, z, AA, AB, AC, AD, AE, AF, AG, AH, AI, AJ, AK, AL, AM, AN, AO, AP, AQ, AR, AS, AT, AU, AV, AW, AX, AY, AZ, BA, BB, BC, BD, BE, BF, BG, BH, BI, BJ, BK, BL, BM, BN, BO, BP, BQ, BR, BS, BT, BU, BV, BW, BX, BY, BZ. Leg discs carrying sgg−/− clones and showing the expression of wg-lacZ (g), Ci155 (h) and dpp-lacZ (i). Anterodorsal sgg−/− cells accumulate high levels of Ci155 (h) and ectopically express low levels of wg-lacZ (g) and dpp-lacZ (i). j–l, A wing disc carrying sgg−/− clones and showing the expression of ci-lacZ and Ci155. sgg−/− cells (arrow) do not express ci-lacZ at higher levels.