Cell

Capture of Authentic Embryonic Stem Cells from Rat Blastocysts

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SUMMARY

Embryonic stem (ES) cells have been available from inbred mice since 1981 but have not been validated for other rodents. Failure to establish ES cells from a range of mammals challenges the identity of cultivated stem cells and our understanding of the pluripotent state. Here we investigated derivation of ES cells from the rat. We applied molecularly defined conditions designed to shield the ground state of authentic pluripotency from inductive differentiation stimuli. Undifferentiated cell lines developed that exhibited diagnostic features of ES cells including colonization of multiple tissues in viable chimeras. Definitive ES cell status was established by transmission of the cell line genome to offspring. Derivation of germline-competent ES cells from the rat paves the way to targeted genetic manipulation in this valuable biomedical model species. Rat ES cells will also provide a refined test-bed for functional evaluation of pluripotent stem cell-derived tissue repair and regeneration.

INTRODUCTION

Authentic ES cells are defined by three cardinal properties: unlimited symmetrical self-renewal in vitro; comprehensive contribution to primary chimeras; and generation of functional gametes for genome transmission. ES cells are obtained from pluripotent epiblast cells of the mouse blastocyst extracted from the uterine environment and placed in the artificial context of laboratory cell culture (Buehr and Smith, 2003; Evans and Kaufman, 1981; Gardner and Brook, 1997; Martin, 1981). It is unclear whether ES cells themselves arise as a consequence of the synthetic culture milieu or represent a transient phase in ontogeny that is captured by arresting developmental progression (Buehr and Smith, 2003; Gardner and Brook, 1997; Niwa, 2007; Silva and Smith, 2008; Smith, 2001a).

Empirical evidence to date is that ES cells can reproducibly be derived from only a few inbred mouse strains. This is achieved using fibroblast feeders and/or the cytokine leukemia inhibitory factor (LIF) in combination with selected batches of fetal calf serum or the growth factor bone morphogenetic protein (Gardner and Brook, 1997; Ying et al., 2003a). The same conditions do not yield ES cells from most mouse strains and not at all from the rat (Brenin et al., 1997; Prelle et al., 1999). We (Buehr et al., 2003) and others (Fandrich et al., 2002; Vassilieva et al., 2000) have reported derivation of cell lines from preimplantation rat embryos that have superficial morphological resemblance to ES cells but do not express biologically relevant levels of the key transcription factor determinants of ES cell identity, Oct4 (Niwa et al., 2000) and Nanog (Chambers et al., 2003; Mitsui et al., 2003), and are not capable of germlayer differentiation in vitro, in tumors, or in chimeras. In our experience, such cells give rise only to extraembryonic trophoblast and hypoblast lineages, and we refer to them as extraembryonic stem (ExS) cells (Buehr et al., 2003).

Shortly after implantation the epiblast transforms into an epithelium and in rodents forms a cup-shaped structure termed the egg cylinder. Cell lines termed EpiSCs have recently been derived from the epithelialized epiblast of postimplantation mouse and rat egg cylinders (Brons et al., 2007; Tesar et al., 2007). These cells have fundamentally different growth factor requirements from ES cells. They are sustained by fibroblast growth factor (FGF) plus activin or nodal and not by LIF. In this respect they resemble primate embryo-derived stem cells (Thomson et al., 1998; Vallier et al., 2005; Xu et al., 2005). Like primate stem cells and unlike ES cells, EpiSCs are sensitive to single-cell dissociation and are generally passaged as clusters of cells rather than dispersed. They do exhibit capacity for multilineage differentiation and teratoma formation. Crucially, however, EpiSCs fail to incorporate properly into the inner cell mass (ICM) when injected into blastocysts and do not contribute significantly to chimeras (Tesar et al., 2007).

Although the tissue and stage of origin of ES cells and EpiSCs are defined, the identity between these cultured cell lines and resident cells in the embryo is uncertain (Rossant, 2008). The derivation of cell lines in culture may involve significant transcriptional and/or epigenetic reprogramming, particularly when potent stimuli such as FGF are involved. One example of this is the reprogramming of unipotent primordial germ cells to generate pluripotent EG cells (Durcova-Hills et al., 2006; Matsui et al., 1992; Resnick et al., 1992). Derivation of tripotent neural stem cells using FGF may be a similar instance (Gabay et al., 2003; Pollard et al., 2008). However, the key parameter determining derivation and propagation of mouse ES cells appears to be suppression or neutralization of extrinsic differentiation signals (Ying et al., 2003a, 2008), rather than provision of a self-renewal stimulus. Based on those findings we have postulated that ES cells represent a ground state in mammalian development and that this may be shared with preimplantation epiblast cells (Silva and Smith, 2008).

An empirically determined culture construction that is effective for derivation of ES cells only in specific inbred laboratory mouse strains may actually be counterproductive for maintaining ground state pluripotency more broadly. Serum and serum substitutes contain a variety of inductive stimuli that may activate commitment and differentiation programs. ES cells will continue to proliferate in the absence of serum. However, simple withdrawal of serum or other exogenous stimuli is not sufficient to prevent differentiation because of the autoinductive action of fibroblast growth factor 4 (FGF4) (Kunath et al., 2007; Stavridis et al., 2007; Ying et al., 2003b). FGF4 signaling through the MEK/ERK pathway drives ES cells into commitment. Genetic impairment or selective chemical blockade of this pathway can sustain self-renewal of mouse ES cells, even in the absence of LIF signaling (Chen et al., 2006; Ying et al., 2008). However, to suppress differentiation entirely and maintain high viability and growth rate when FGF/ERK signaling is reduced it is necessary either to provide LIF or to restrict activity of glycogen synthase kinase 3 (GSK3). GSK3 is a central node for negative modulation of a range of anabolic processes and generally acts to suppress cellular biosynthetic capacity (Frame and Cohen, 2001). GSK3 is inhibited by phosphorylation downstream of growth factors that activate phosphatidyl inositol 3 kinase and Akt. GSK3 is also a key component of the β-catenin destruction complex and pharmacological inhibition of GSK3 increases cytoplasmic and nuclear β-catenin, mimicking canonical Wnt signaling (Ding et al., 2000). The small molecule CHIR99021 selectively inhibits both GSK3α and GSK3β (Murray et al., 2004). The combination of three inhibitors (3i) that target FGF receptor, MEK, and GSK3 enables efficient derivation and propagation of germline-competent ES cells from a range of mouse strains (Ying et al., 2008) (J. Nichols and A.S., unpublished data). These findings suggest that the key to deriving and maintaining ES cells may be to shield the native epiblast ground state from activation of the ERK pathway by either exogenous or autocrine inductive stimuli that will normally drive developmental progression. Complementary inhibition of GSK3 stabilizes the ground state, likely via a combination of β-catenin-dependent action and β-catenin-independent anabolic effects (Silva and Smith, 2008) (J. Wray and A.S., unpublished data). To test the generality of this concept we have investigated the possibility of deriving true ES cells from the rat.

RESULTS

Sustained Expression of Pluripotent Markers in Rat Inner Cell Masses Explanted in 3i

We first examined the effect of the neutralizing 3i culture regime on ICMs explanted from rat blastocysts. In addition to 3i we provided LIF because although this is dispensible for mouse ES cell culture in 3i, we find that clonogenicity and ES cell derivation are invariably enhanced by addition of LIF (Ying et al., 2008) (J. Wray, J. Nichols, and A.S., unpublished data). As a source of LIF we used DIA-M feeders that have been genetically engineered to express the matrix-associated form of LIF (Buehr et al., 2003; Rathjen et al., 1990b). The feeders also support attachment of the ICMs.

Retained expression of the transcriptional determinant Oct4 can be used as a surrogate assay for presence of pluripotent cells (Buehr et al., 2003). In ICM explants in conventional culture Oct4 is rapidly extinguished (Buehr and Smith, 2003). ICMs were isolated from embryonic day (E) 4.5 rat blastocysts by immunosurgery and plated on DIA-M feeders. Explants were fixed at different time points and analyzed by immunostaining (Figure 1A). In standard culture with or without serum we observed loss of Oct4 by 3 days as previously described (Buehr et al., 2003). Concurrent with downregulation of Oct4 we noted ectopic appearance of Cdx2, the trophectoderm determinant and antagonist of Oct4 (Niwa et al., 2005). Expression of Oct4 and Cdx2 were generally mutually exclusive. In contrast, in the presence of 3i we found that Oct4 protein was maintained in the majority of cells after 3 days and expression of Cdx2 was suppressed. By 4 days the majority of cells in the core of the explants were Oct4 positive (Figure 1B). A second critical marker of pluripotent status, Nanog (Chambers et al., 2003; Mitsui et al., 2003), which is downregulated in similar fashion to Oct4 in conventional culture, was similarly maintained in serum-free medium supplemented with 3i, although with a more heterogeneous expression than Oct4. The effect of 3i is unlikely to be due to cell selection because the total number of ICM cells was actually higher in the 3i-treated cultures (Table S1 available online).

We then dissociated ICMs cultured in 3i for 3 days into small clumps and replated them in the same conditions. Cells remained viable and formed colonies of morphologically undifferentiated cells. These colonies continued to express Oct4 and Nanog (data not shown). In many previous experiments under various conditions (Buehr et al., 2003; Buehr and Smith, 2003), we have very rarely detected expression of Oct4 after replating and only confined to small clusters of cells, never throughout the outgrowths as seen in 3i. These observations suggest that 3i may suppress loss of pluripotency and differentiation of cultured rat epiblast.

Derivation of Continuous Cell Lines

We therefore investigated longer-term effects. ICMs from two different inbred strains, Dark Agouti (DA) and Fischer 344, were plated on DIA-M feeders in serum-free 3i. After 3–4 days, around one-third of the ICMs had attached and proliferated such that

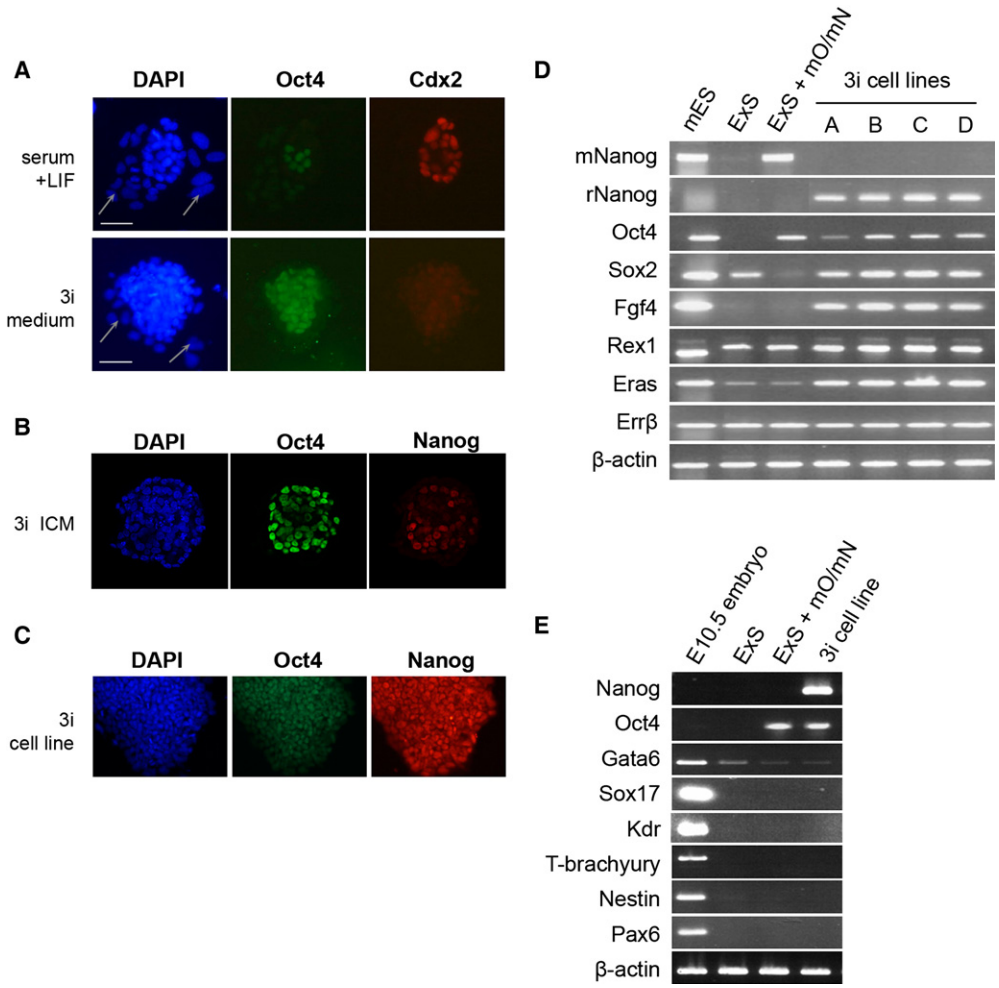

Figure 1. Inhibitors Sustain Expression of Pluripotency Markers in Rat ICM Primary Explants and Expanded Cell Lines

(A) Oct4 and Cdx2 immunofluorescence staining of ICM explant cultured in the presence of serum or in serum-free 3i for 3 days. Arrows indicate feeder cell nuclei.

(B) Oct4 and Nanog immunostaining of ICM after 4 day culture in 3i, detached from feeders for fixation and staining.

(C) Immunofluorescent staining of Oct4 and Nanog in established 3i cell line.

(D) RT-PCR analysis of pluripotent marker expression in expanded 3i cell lines compared with E14Tg2a mouse ES cells (mES), rat ExS cells (rExS), and rat ExS cells expressing mouse Oct and mouse Nanog (rExS + mO/mN) transgenes.

(E) RT-PCR analysis of differentiation marker expression in expanded 3i cell lines. cDNA from an E10.5 rat embryo provides a positive control.

they could be mechanically broken up into small clumps and replated in fresh wells. From one in three of these replated ICMs, small colonies of undifferentiated morphology developed. These colonies became large enough for manual transfer into new wells after 10 days. After a further few days they were dissociated manually and replated, invariably giving rise to multiple undifferentiated colonies. Thereafter cultures could be passaged repeatedly. We established twenty cell lines in this way from three independent experiments (Table 1A).

All lines were similar in morphology and growth characteristics. They proliferate as clumps of tightly packed cells, similar to mouse ES cells cultured in 3i (Ying et al., 2008). Individual cells have the high nucleus to cytoplasm ratio and prominent nucleoli characteristic of ES cells. They are apolar and lack processes, cytoarchitectural features, or other signs of specialization. Colonies have a tendency to detach from the feeder layer if they become too large, and cells were therefore routinely passaged every 3–4 days. The colonies can readily be dissociated by gentle trituration or enzymatically using Accutase. After dissociation, single cells isolated by micropipette readily give rise to undifferentiated colonies that can be serially passaged. Cells can be cryopreserved and recovered by conventional procedures using DMSO as cryoprotectant. Cultures have been expanded continuously for 6 months with no discernible change in growth rate, no indications of crisis or senescence, and little overt differentiation in presence of 3i.

Characterization of 3i Rat Cell Lines

To assess the identity of 3i rat cells we examined expression of key markers of pluripotency and lineage commitment. Nuclear localized Oct4 and Nanog proteins were detected by immunofluorescence microscopy (Figure 1C). RT-PCR analyses confirmed

Table 1. Derivations in 3i and 2i

(A) Derivation of Undifferentiated Cell Lines in 3i

	Strain	No. of ICMs	Outgrowths	Continuous
Expt 1	F	47	18	8
"	DA	29	6	2
Expt 2	F	30	11	6
"	DA	11	2	0
Expt 3	F	24	7	1
"	DA	24	16	3
Total	F + DA	165	60	20

(B) Derivation of Rat ES Cells in 2i

	Strain	No. of ICMs	Outgrowths	Continuous
Expt 1	F	47	34	28
Expt 2	DA	67	50	43
Expt 3	F + DA	114	84	71
Expt 4	SD	7	6	2
Total	F + DA + SD	235	174	144

Outgrowths refers to adherent primary explants. Continuous refers to proliferating cultures of undifferentiated cells after three passages. Many cultures were passaged further and in no case did a culture collapse to differentiation, growth arrest, or cell death if undifferentiated after three passages.

F, Fischer 344; DA, DA; SD, Sprague-Dawley.

expression of these genes along with other ES cell markers, *Rex-1*, *Errβ*, *Sox2*, *Eras*, *Stella*, and the Oct4/Sox2 target *Fgf4* (Figure 1D). To control for the possibility of crosscontamination with mouse ES cells or cDNA, primers for amplification of *Nanog* were designed against sequences specific for the rat gene. These primers consistently yielded products of anticipated size from rat cells but not from mouse ES cells. Conversely, mouse-specific primers did not yield any product from the rat cells. We also evaluated expression of differentiation markers. Transcripts for the hypoblast and definitive endoderm markers Gata6 and Sox17, the mesoderm markers brachyury and Flk1, or the neuroectoderm markers Pax6 and nestin were low or undetectable (Figure 1E).

We found that undifferentiated cells could not be maintained when withdrawn from 3i. They underwent cell death and surviving cells differentiated into heterogeneous morphologies accompanied by loss of expression of Oct4 and Nanog (Figures 2A and 2B). LIF showed little or no capacity to block this differentiation in either the presence or absence of feeders or serum. We tested whether 3i cells could be sustained in the specific growth conditions that are employed to propagate EpiSCs. Rat 3i cells differentiated when 3i was replaced with activin plus FGF2 (Brons et al., 2007), whether on feeders or fibronectin-coated dishes (Figure 2C). Furthermore, the activin receptor inhibitor SB431452, which induces differentiation of EpiSCs (Brons et al., 2007) but not of mouse ES cells, did not impede expansion of undifferentiated 3i rat cells. We also tested whether blastocyst-derived rat ExS cells (Buehr et al., 2003) could be maintained in 3i and found that they do not survive.

A hallmark of female ES cells and early epiblast is that both X chromosomes are active (Mak et al., 2004; Okamoto et al., 2004; Rastan and Robertson, 1985). Upon proper differentiation one X chromosome then becomes silenced (Wutz and Jaenisch, 2000). We stained XX 3i rat cells with an antibody against trimethylated histone 3 lysine 27 (H3K27), an epigenetic silencing mark that decorates the inactive X chromosome in interphase nuclei (Silva et al., 2003). Cells maintained in 3i showed diffuse immunoreactivity throughout the nucleus whereas cells differentiated by withdrawal of 3i in serum for 6 days exhibited a prominent focus of nuclear staining diagnostic of the inactive X (Figure 2D). These data indicate that in undifferentiated XX 3i rat cells both X chromosomes lack a global epigenetic silencing mark and that one X chromosome acquires this modification during differentiation. EpiSCs in contrast exhibit X inactivation (G. Guo and A.S., unpublished data), consistent with egg cylinder origin. Thus 3i rat cells exhibit a unique epigenetic feature of early epiblast and ES cells.

To investigate differentiation potential we injected both DA- and Fischer-derived lines under the kidney capsule of immunodeficient SCID mice. After 30–55 days, animals were sacrificed. Seven out of eleven exhibited a macroscopic tissue mass, varying in size from less than a gram to over 4 g, at the site of the injection. Histological analyses of two specimens, one from each line, revealed classical features of a teratoma, with multiple differentiated cell types and structures including striated muscle, bone, cartilage, keratinized epithelia, secretory epithelia of the gut, and others (Figure 2E and data not shown). We conclude that 3i rat cell lines are capable of producing teratomas and are competent for mature multilineage differentiation.

Rat 3i Cells Contribute to Chimeras

The above findings indicate that 3i cells are significantly different from previously described cell lines from rat embryos. However, the essential attribute of authentic ES cells is their capacity to colonize host embryos and contribute differentiated progeny to all germlayers plus the germlines of chimeric animals (Smith, 2001b). Following lipofection and selection in puromycin we derived a pool of stably transfected cells that expressed green fluorescent protein (GFP) (Figure S1). These cells were injected into blastocysts. Embryos harvested at E10.5 and at E13.5 showed widespread contribution of GFP-labeled cells (Figures 3A and 3B). Examination of vibratome sections confirmed distribution in all three germlayers. An E13.5 chimera was analyzed for contribution to primordial germ cells. Immunostaining of genital ridges for Oct4 and vasa homolog revealed coexpression with GFP, indicating that 3i cells can colonize the developing germline (Figure 3C).

We then examined potential to contribute to postnatal chimeras. Five overt coat color chimeras were born after injection of a GFP-labeled DA line into albino Fischer blastocysts. However, these animals either died perinatally or were euthanized prior to weaning due to jaw abnormalities (see below). Unfixed tissue squashes from one of the neonates showed GFP fluorescence in skin, lung, liver, and kidney (Figure 3D). Vibratome sections from a P25 chimera also revealed GFP-positive cells in a variety of tissues, including testis. Based on morphology and location, expression in the testis appeared to be in spermatogonial cells in addition to somatic sertoli and

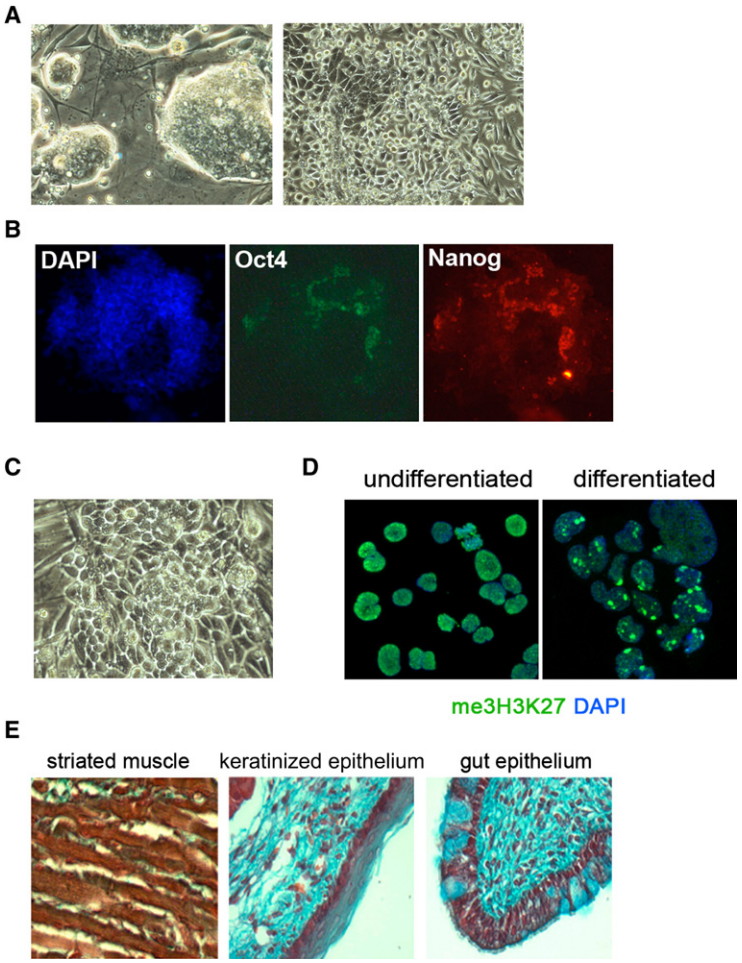

Figure 2. Differentiation of Rat 3i Cells In Vitro and in Teratomas

(A) Undifferentiated cells in 3i (left) and cells differentiated after withdrawal of inhibitors for 7 days on fibronectin in the absence of serum (right).

(B) Downregulation of Oct4 and Nanog immunostaining upon differentiation on feeders in serum.

(C) 3i cells differentiate in the presence of activin and FGF on feeders.

(D) Immunofluorescence for me3H3K27 (green) in undifferentiated cells and after differentiation for 6 days in serum. Undifferentiated cells show diffuse staining while differentiated cells exhibit a prominent H3K27me3 nuclear body. Cells were counterstained with DAPI (blue).

(E) Histological sections of teratomas derived from Fischer and DA 3i cell lines showing keratinized epithelium, striated muscle, and gut epithelium.

myoid cells (Figure 3E). Fluorescent spermatocytes were not evident, however (see below).

We also injected unmodified cells into blastocysts. Of 27 animals born after injection of Fischer cells into DA hosts, all were agouti with no detectable albino contribution. In contrast, from injections of DA cells into Fischer blastocysts two liveborn animals exhibited mixed agouti and albino coloring (Figure 3F). Both animals were heavily pigmented with the albino host contribution apparent only on the head. This skewed pattern is characteristic of rat chimeras between albino hooded and fully pigmented strains and does not correlate with overall contributions (Yamamura and Markert, 1981). Microsatellite analyses of tail, ear, and blood biopsies confirmed the chimeric nature of these animals (Figure 3G) and quantified the respective DA:Fischer contributions as approximately 75:25 and 25:75 in the two animals (Figure S2). Both chimeras developed into healthy adults.

Breeding from these DA:Fischer chimeras failed to yield transmission of the DA cell line genome, however. We then found in the course of cytogenetic characterization that all 3i lines analyzed were XX. Since both chimeras were male they could not support development of spermatocytes from the introduced cells, which would explain the absence of germline transmission and the restricted GFP staining in the chimeric testis (Figure 3E).

Of greater concern, however, was detection in two of the lines, including the GFP-labeled line that gave abnormalities in chimeras, of trisomy for chromosome 9. This was revealed by array-based comparative genome hybridization (aCGH) and confirmed by fluorescence in situ hybridization (FISH) (Figure S3 and data not shown). The sex bias and the chromosomal anomalies raised the possibility of inadvertent selection pressure during derivation and/or expansion of rat cells in 3i.

Derivation of Germline-Competent Rat Cells Using Two Inhibitors

We considered that application of the FGFR inhibitor SU5402 may not be optimal because it is less specific than the MEK and GSK inhibitors. An additional factor is that the effect of blocking FGFR is not restricted to the MEK/ERK cascade but will also reduce activation of phosphatidylinositol 3-kinase and other pathways downstream of FGFR that might be beneficial for ES cells. Accordingly we omitted SU5402 and switched to a two inhibitor (2i) formulation comprised of the more potent MEK inhibitor PD0325901 (Bain et al., 2007) with the GSK3 inhibitor. We have found that 2i supports clonogenic expansion of mouse ES cells more efficiently than 3i (Ying et al., 2008). Additionally, 2i facilitates isolation of induced pluripotent stem (iPS) cells during transfection-induced reprogramming (Silva et al., 2008).

Rat ICMs cultured in 2i on DIA-M feeders attached and yielded expandable undifferentiated cell lines at increased frequency compared with 3i cultures (Table 1B). The 2i cells were morphologically indistinguishable from lines derived in 3i (Figure 4A) and could similarly be expanded and cryopreserved. Consistent with the high frequency of derivation many of these 2i isolations gave positive PCR amplification of the Y chromosome-specific *Sry* locus (Figure S4). Metaphase spreads prepared from 12 lines all displayed a mixture of metacentric, telocentric, and acrocentric chromosomes characteristic of the rat and distinct from the telocentric chromosomes found in mice. Counts gave a modal chromosome number of 42. FISH analyses confirmed the absence of chromosome 9 amplifications in all 12 lines

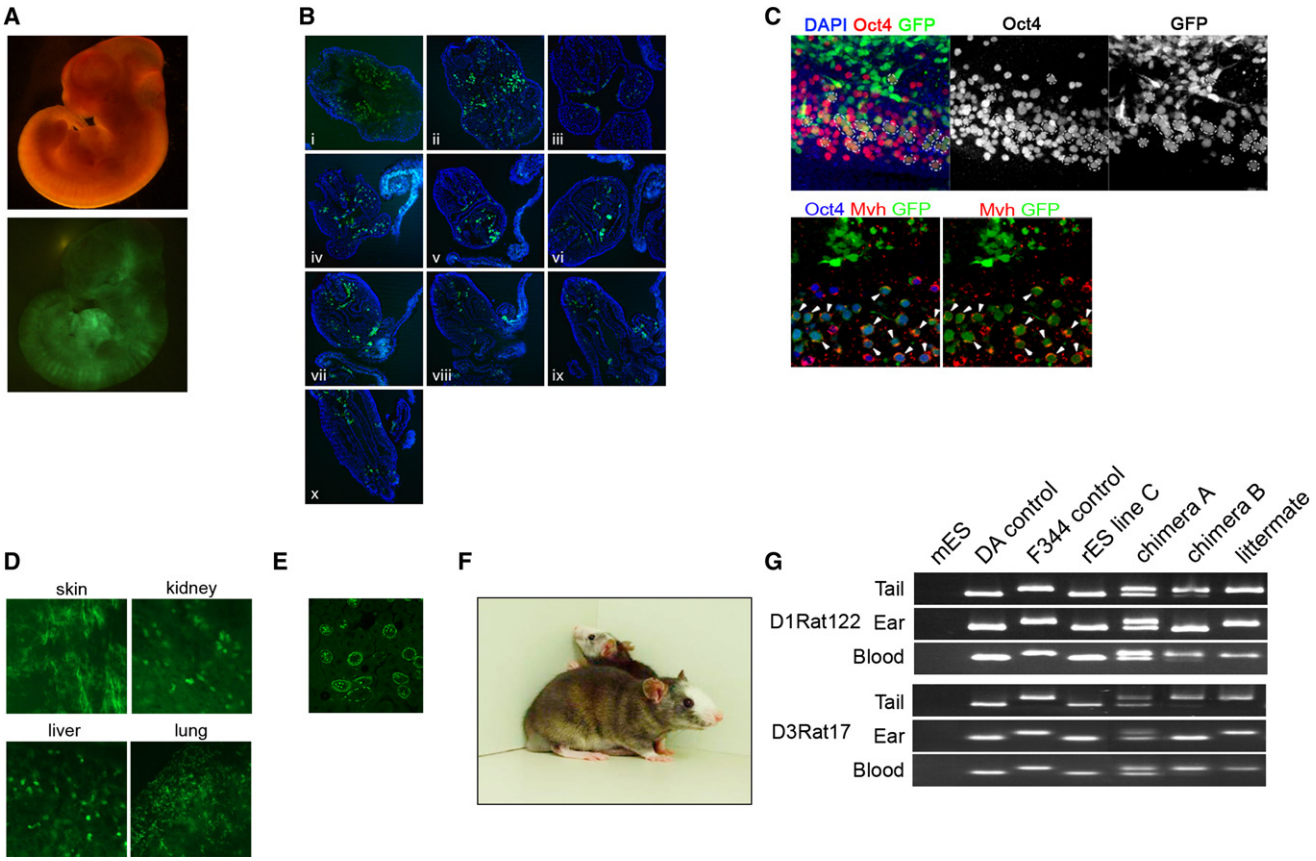

Figure 3. Contribution of 3i Cells to Chimeras and Colonization of Germline

(A) Bright-field and fluorescence images of E13.5 chimeric embryo.

(B) Vibratome slices through whole E10.5 chimera GFP fluorescence in green, DAPI nuclear staining in blue.

(C) GFP expression in Oct4 and vasa homolog positive primordial germ cells of E13.5 chimera. Arrowheads point to cells coexpressing GFP and vasa homolog.

(D) GFP expression in unfixed tissue squashes from neonatal chimera.

(E) GFP expression in vibratome section of testis from P25 chimera. GFP is apparent in Sertoli cells, myoid cells, and spermatogonia but absent from spermatocytes.

(F) Adult coat color chimeras derived from 3i cells. White snouts denote the recipient Fischer strain (hooded albino), body pigmentation the cell line DA strain.

(G) Microsatellite analyses of adult coat color chimeras. Polymorphic regions D1Rat122 and D3Rat17 were amplified by PCR from genomic DNA of tail, ear, and blood.

(Figure 4B). aCGH analysis substantiated rat identity and the absence of any major deletions or amplifications (Figure S3).

After expansion for five or six passages we attempted to generate chimeras from four of the 2i rat lines. Coat-color chimeras were obtained from injection of three DA lines into Fischer blastocysts (Table 2). All developed into healthy adults. We confirmed cell line contribution of approximately 50% by microsatellite analysis of one chimera (Figure S5). These lines all turned out to be female, however, and breeding from the female chimeras has so far failed to yield offspring with cell line genome. We also injected a male line, SD2, derived from the albino Sprague-Dawley strain, into blastocysts from the pigmented DA strain. We obtained a healthy male chimera (Figure 4C). This animal has been mated to Fischer females. To date he has sired 89 agouti pups and 13 healthy albino offspring (Figure 4D). Recessive albinos should only arise from SD2-derived sperm. Microsatellite analyses provide molecular validation that these animals are indeed Sprague-Dawley × Fischer hybrids (Figure 4E). We conclude that the cultured cell genome is transmitting reproducibly though the chimeric male germline.

2i Rat Cells Exhibit In Vitro Properties and Behavior of Embryonic Stem Cells

We then examined the properties of 2i rat cells relative to characteristics expected for embryonic stem cells based on the mouse. We first examined culture without feeders. Rat 2i cells did not adhere to uncoated or gelatin-coated plastic but did attach to fibronectin- or laminin-coated plates. They tended to detach from fibronectin as colonies expanded but could be reliably maintained on laminin in the presence of 2i plus LIF. Colonies adopted a more flattened morphology (Figure 5A) than on feeders, but they retained expression of nuclear Oct4 and Nanog proteins (Figure 5B). Furthermore, they rapidly reverted to tightly

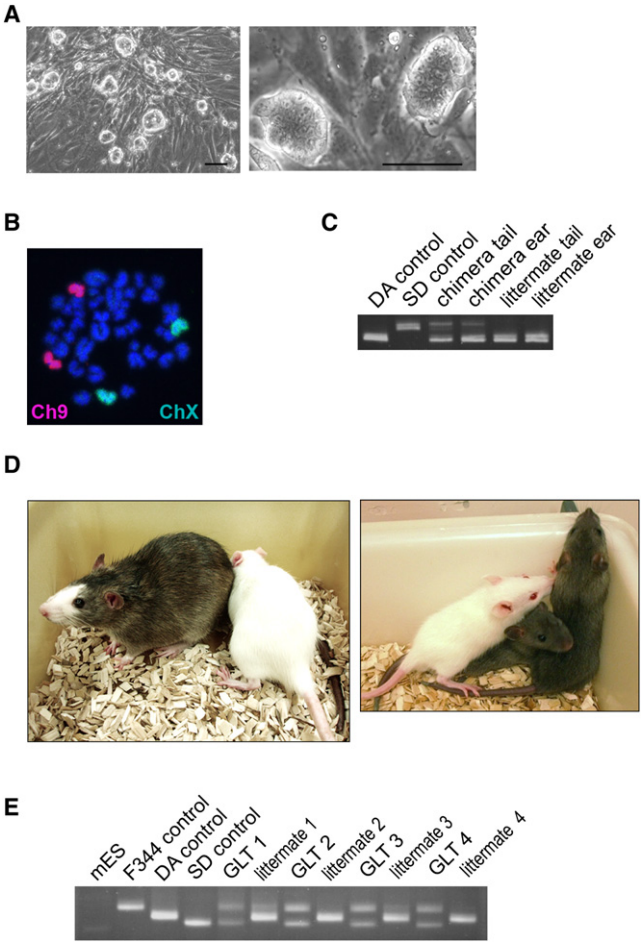

Figure 4. Rat Cells Derived in 2i Contribute to Chimeras and Give Germline Transmission

(A) Morphology of 2i colonies on feeders at low and high magnification.

(B) FISH for chromosomes 9 and X on metaphase spread from DA 2i cell line.

(C) Microsatellite D3Rat17 analysis of SD2 chimera and non-chimeric littermate.

(D) SD2 chimera and first albino offspring at 10 weeks of age (left), second albino germline pup and two littermates at weaning (right).

(E) Analysis of four albino SD2 offspring and agouti littermates for polymorphic region D2Rat250. The two albino germline pups exhibit both Fischer and Sprague-Dawley amplicons. Their agouti littermates show very weak signals for the Fischer D2Rat250 region because this amplification is suppressed in the presence of the DA amplicon (Figure S6). Agouti genotypes were independently confirmed as DA × Fischer by amplification of D1Rat122, which reliably amplifies and discriminates DA and Fischer, though not Sprague-Dawley (data not shown).

Table 2. Generation of Chimeras from 3i and 2i Rat ES Cells

Inhibitor Medium	Cell Line	Strain and Sex	Injected Embryos	Foetal Chimeras	Term Chimeras
3i	148C	DA, ♀	41	N/A	2♂
3i	148D-GFP	DA, ♀	46	5	N/A
3i	DA3-GFP	DA, ♀	80	1	5[a]
2i	DA8	DA, ♀	23	N/A	1♀, 2♂
2i	DA13	DA, ♀	21	N/A	1♀
2i	DA15	DA, ♀	22	N/A	1♀, 1♂
2i	SD2	SD, ♂	12	N/A	1♂

N/A, not applicable. DA (pigmented) cells were injected into Fischer (albino) host blastocysts; SD (albino) cells into DA hosts. For 148C injections naturally mated recipients were used for blastocyst transfer. In other cases pseudopregnant recipients were used.

[a] Three died neonatally, two sacrificed at 25 days due to jaw deformity.

packed three-dimensional colony growth when returned to feeders, even after multiple passages on laminin.

Cell-surface immunofluorescence staining revealed that 2i rat cells propagated on laminin show a SSEA-1 profile similar to mouse ES cells (Figure S7). Propidium iodide staining revealed that more than 50% of cells are in S phase, a cell-cycle profile shared with mouse ES cells (Figure 5C) and distinct from most somatic cells that tend to reside in G1 (Burdon et al., 2002).

We examined a range of transcripts associated with the pluripotent ES cell phenotype (Takahashi and Yamanaka, 2006) or with lineage commitment. Data in Figure 5D show that 2i rat cells passaged on laminin in 2i plus LIF express a repertoire of pluripotency markers similar to mouse ES cells and lack markers of differentiation, alpha-fetoprotein (*Afp*), and T-brachyury. As with 3i cells they express genes that discriminate between ES cells and EpiSCs, such as *Klf4*, *Rex1*, and *Stella*.

A characteristic of mouse ES cells is responsiveness to LIF acting via STAT3 (Burdon et al., 1999; Matsuda et al., 1999; Niwa et al., 1998). In contrast to its potent effect on strain 129 mouse ES cells, LIF did not prevent differentiation of 2i rat cells upon withdrawal of inhibitors, even in the presence of feeders or serum. However, 2i alone was also not sufficient to sustain self-renewal and cultures collapsed after the first passage without LIF unless cells were kept at very high density. Since autocrine LIF signaling can be anticipated at high cell densities (Rathjen et al., 1990a) we investigated colony formation from dissociated single cells. Very few colonies formed without LIF. In contrast numerous colonies grew up in the combination of 2i plus LIF (Figure 5E). When fixed and stained after 8 days, 50% of the colonies contained a central mass of alkaline phosphatase-positive morphologically undifferentiated cells (Figure 5F). We then examined induction of two well-characterized LIF target genes. *Egr1* is downstream of the Erk signaling arm, and *Socs3* is a direct target of STAT3 (Burdon et al., 1999). LIF was withdrawn for 6 hr then added back for 1 hr, with or without 2i. In rat cells and mouse ES cells, both genes were induced in the absence of inhibitors confirming LIF signal responsiveness (Figure 5G). In the presence of 2i, however, expression of *Egr1* was suppressed while *Socs3* induction was unaffected. This is consistent with inhibition of MEK/ERK signaling without affecting STAT3 activation by 2i (Ying et al., 2008) and indicates that STAT3 is the critical mediator of LIF action, as in mouse ES cells.

On exposure to serum in the absence of feeders, 2i, and LIF, cells differentiated (Figure 5H). This was accompanied by downregulation of *Nanog* and *Oct4* (Figure S8) and upregulation of *Gata6*, *Sox17*, and *Afp* (Figure 5I). These are markers of both hypoblast and definitive endoderm, though the nonepithelial

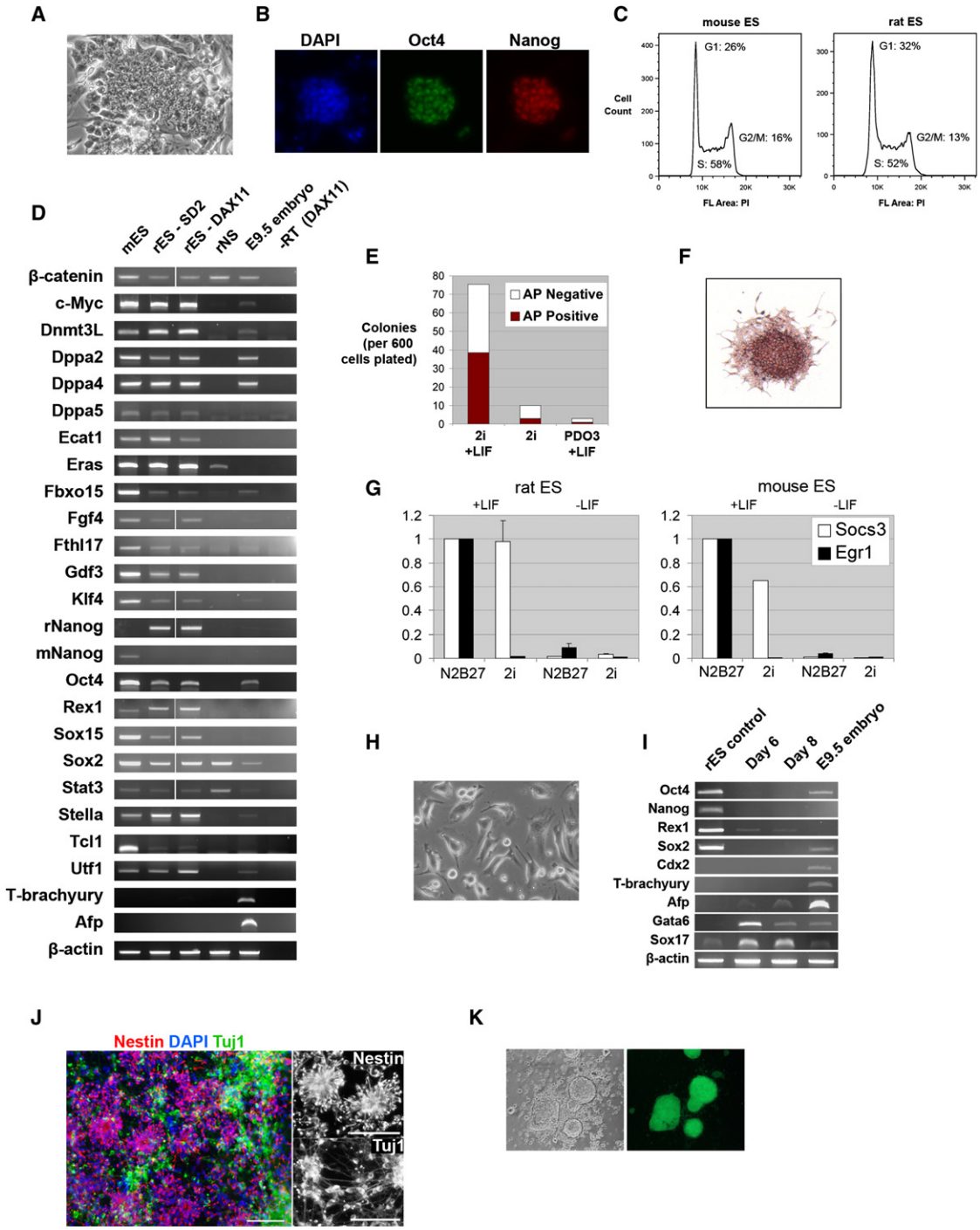

Figure 5. Rat 2i Cells Exhibit Properties of Embryonic Stem Cells

(A) Undifferentiated morphology on laminin in 2i plus LIF (passage 3).

(B) Oct4 and Nanog immunostaining in 2i plus LIF on laminin (passage 3).

(C) Cell-cycle profile determination by flow cytometric analysis of propidium iodide staining.

(D) RT-PCR analysis of pluripotent and germlayer markers in mouse ES cells and two independent rat cell lines after two (SD2) or three (DAX11) passages on laminin in 2i/LIF. Controls are rat neural stem (rNS) cells and whole E9.5 embryo. Except for Nanog, primers were selected for crossreaction on mouse and rat cDNA.

(E) Colony formation by dissociated single cells on laminin. Qualitatively similar results were obtained for several different cell lines.

(F) Typical alkaline phosphatase-positive colony showing undifferentiated core with differentiated cells on periphery.

(G) LIF induction of Stat3 target *Socs3* but not of Erk target *Egr1* in 2i for rat and mouse ES cells. Rat data are mean ± SD of two biological samples each assayed in duplicate; mouse data are averages of technical duplicates from one experiment.

morphology is suggestive of the former. Neither cell fate is readily obtained from mouse ES cells in adherent culture in serum, but it is noteworthy that in contrast to the mouse, epiblast cells in the rat have the ability to regenerate hypoblast (Nichols et al., 1998).

We investigated whether rat 2i cells may follow autoinductive differentiation into neuroectodermal lineages in serum-free culture (Kunath et al., 2007). Cells were plated overnight in 2i/LIF then cultured in unsupplemented N2B27 (Ying and Smith, 2003). Ten days later cultures were dominated by cells expressing the neural precursor marker nestin or immunoreactive for the early neuronal marker TuJ1 (Figure 5K). Nestin-positive cells were typically in rosette structures characteristic of neuroepithelial cells while TuJ1-positive cells generally exhibited extended processes expected for neuronal cells. Similar results were obtained for four independent 2i cell lines.

Finally, we tested whether rat 2i cells could be stably transfected by electroporation, the favored method of introducing DNA constructs for homologous recombination into mouse ES cells. Cells (1×10^6) were electroporated in the presence of 10% serum with 5, 20, or 50 μg linearized pPyCAGgfpIP plasmid (Chambers et al., 2003). At least 50% of cells survived electroporation and were plated overnight on DIA-M feeders in 2i medium plus 5% serum to aid viability. The following day the medium was changed to serum-free 2i. Puromycin (1 μg/ml) was added 72 hr after electroporation. The number of puromycin-resistant colonies increased with DNA concentration. With 50 μg of plasmid, 315 colonies were obtained after 8 days from 5×10^5 cells plated, an efficiency of 0.05%. Fluorescent colonies were picked into 96-well plates then expanded in dishes and successfully frozen/thawed without loss of GFP expression (Figure 5K). Chromosomal integration was confirmed by genomic PCR (Figure S9).

DISCUSSION

Collectively these findings demonstrate that application of 3i/2i plus LIF enables the derivation of rat cell lines with the expected properties of authentic ES cells: long-term self-renewal, pluripotency, teratoma formation, capacity for incorporation into the developing embryo, colonization of the germline, and generation of functional gametes. Although the incidence of germline transmission has been low to date, this is also typical of mouse ES cells unless C57BL/6 strain blastocysts are used as hosts for chimera production (Schwartzberg et al., 1989; Seong et al., 2004). Systematic screening of different donor and host strain combinations may give a preferred combination for production of germline chimeras in the rat.

The 3i and 2i rat ES cells express the key molecular markers found in mouse ES cells, Nanog, Oct4, Sox2, and Klf4. They are therefore potentially maintained by the same transcription factor circuitry (Chen et al., 2008; Kim et al., 2008). A recent report claims derivation of rat ES cells in conventional ES cell cultures with feeders, LIF, and serum (Ueda et al., 2008). However, consistent with many previous reports we find that these conditions are not adequate to derive or maintain rat ES cells. Furthermore the cell-surface and marker profiles reported by Ueda and colleagues are inconsistent with ES cell identity and suggest that their cells may be EpiSC like.

The 3i/2i rat ES cells express Klf4, Stella (*Pgc7, Dppa3*), and Rex1 (*Zfp42*), which discriminate ES cells from EpiSCs (Jiang et al., 2008; Tesar et al., 2007). They differentiate in the presence of serum and in the specific growth conditions used for EpiSCs. Conversely, EpiSCs are dependent on FGF/Erk signaling and rapidly die or differentiate in the presence of MEK inhibitor (G. Guo and A.S., unpublished data). A further distinction from EpiSCs is the absence of an X chromosome epigenetic silencing mark in female 2i/3i cells. Finally, and most importantly, the rat cells we have derived colonize the epiblast and contribute to chimeras.

Significantly, 2i/3i rat cells are responsive to LIF, although LIF alone is insufficient to sustain self-renewal. Mouse ES cells derived from non-129 genetic backgrounds are also dependent on factors in addition to LIF to sustain self-renewal, notably inhibition of MEK/ERK (Batlle-Morera et al., 2008; Buehr and Smith, 2003; Ying et al., 2008). This insufficiency is likely to be a key factor in previous failures to derive rat ES cells. Nonetheless, rat ES cells are dependent on LIF/Stat3 signaling, another clear distinction from EpiSCs. An interesting question for future investigation is whether there are qualitative or quantitative differences in LIF signaling between strain 129 mouse ES cells and ES cells from other strains and species. We speculate that LIF/Stat3 responsiveness may be a general feature of authentic ES cells.

Cell lines derived in 3i exhibited an unexpected sex bias. More worryingly, we detected in two independent lines trisomy for chromosome 9. In 2i derived lines, in contrast, we were able to derive equivalent numbers of male and female lines, and we have so far not observed any chromosomal anomalies. However, we have observed that both 3i and 2i rat ES cells are more sensitive than standard mouse ES cells, requiring fastidious quality control of tissue culture media for good viability and proliferation. Therefore factors other than the use of SU5402 may have contributed to either the sex bias or the trisomy. Furthermore, we suspect that male cell lines may take longer to emerge and could thus have been overlooked in our early studies. Nonetheless, it is advisable to dispense with an inhibitor such as SU5402 if it confers no obvious benefit.

The derivation of rat ES cells opens the door to application of gene targeting and related genome engineering technologies in the model species of choice for many aspects of biomedical research. Physiological interventions, toxicology studies, and evaluation of higher-order functions such as behavior and cognition are in general more sophisticated and informative in rats

(H) Differentiated morphology of 2i cells after 6 days culture in serum without 2i/LIF.

(I) RT-PCR analysis of marker expression during differentiation in serum.

(J) Immunostaining for nestin and TuJ1 after 10 days of adherent serum-free differentiation on laminin. Insets show higher-power images of nestin (upper) and TuJ1 (lower) staining.

(K) Expression of GFP in stable transfectant clone generated by electroporation and expanded after puromycin selection and freeze/thawing.

than in mice. The observation that rat ES cells can be stably transfected by electroporation is significant for future genetic modification studies. Availability of rat ES cells also increases the scope for testing the capacity of in vitro ES cell differentiation to produce cells that can integrate and function in adult tissue repair and regeneration.

Finally, effective preservation of ground state pluripotency in cultured rat embryo cells using neutralizing 3i/2i culture suggests that derivation of ES cells may indeed represent the capture of resident embryo cells rather than a tissue culture artifact. This points to an underlying conservation in the essential nature of the pluripotent state in rodent embryos and potentially in other mammals. We suggest that isolation of ES cells may represent a simple continuation of early epiblast replication in the absence of inductive signals (Silva and Smith, 2008) rather than require transcriptional or epigenetic reprogramming (Buehr and Smith, 2003; Chou et al., 2008). This raises the possibility that culture formulations based on the 3i/2i principle could facilitate derivation of ES cells from other mammals, including livestock species. It will also be of interest to investigate whether supernumerary human embryos cultured in 3i/2i may give rise to pluripotent cell lines that are qualitatively different from current human "embryonic stem" cells and closer to ground state rodent ES cells than to EpiSCs (Silva and Smith, 2008).

EXPERIMENTAL PROCEDURES

Details of RT-PCR, microsatellite analysis, cytogenetics, aCGH, transfections, and immunostaining are provided in the Supplemental Data.

Culture Procedure

Feeder cells were prepared from gamma-irradiated or mitomycin-C-treated DIA-M mouse fibroblasts that express matrix-associated LIF (Buehr et al., 2003; Rathjen et al., 1990b). ICMs and passaged cell lines were plated on DIA-M feeders in N2B27 medium (Ying and Smith, 2003) containing either 3i (FGF receptor inhibitor SU5402, 2 μM; inhibitor of MEK activation PD184352, 0.8 μM; GSK3 inhibitor CHIR99021, 3 μM) or 2i (MEK inhibitor PD0325901, 1 μM; GSK3 inhibitor CHIR99021, 3 μM) (Ying et al., 2008) plus human LIF (100 U/ml) prepared in-house (Smith, 1991). SU5402 was obtained from Calbiochem; other inhibitors were custom-synthesized by the Division of Signal Transduction Therapy, University of Dundee. Cell lines were routinely passaged by aspirating the colonies into fine pipettes and transferring the resultant disaggregated cells to fresh plates or by dissociation with Accutase. Feeder-free culture was performed on plates pre-coated with laminin, 10 μg/ml (Sigma). Monolayer neural differentiation was induced by adherent serum-free culture in N2B27 for 10 days without inhibitors or LIF, essentially as described for mouse ES cells (Ying et al., 2003b) except that laminin was used as a substrate for attachment.

Chimera Generation

Rat blastocysts at E4.5 days post coitum (dpc) were collected by noon on the day of injection and cultured for 2–3 hr in KSOM medium to ensure cavitation. Cells were disaggregated in Accutase and 10–12 cells injected into the blastocyst cavities. Injected embryos were transferred into uteri of naturally mated or pseudopregnant Fischer F344 females according to availability of vasectomized males.

Immunosurgery

Zonae were removed from E4.5 rat blastocysts with acid Tyrodes, and blastocysts incubated at 37°C in 20% anti-rat whole serum (Sigma) for 3 hr. Blastocysts were washed and incubated for 20 min in rat serum as a source of complement, and the lysed trophectoderm removed by pipetting.

Teratoma Generation

Approximately 200–400 cells were injected under kidney capsules of SCID mice (BALB/c JHan Hsd-Prkdc scid.). Tumors were collected at various times, and their weight determined by weighing tumor and kidney and subtracting the weight of the contralateral uninjected kidney. Tumors were embedded in paraffin wax, sectioned, and stained with Masson's trichrome.

Electroporation

1×10^6 2i rat ES cells were electroporated in N2B27 + 10% FCS with 5, 20, or 50 μg ScaI linearized pPCAGgfpIP plasmid using the Bio-Rad Genepulser apparatus (0.8 kV, 3 μF). 6×10^5 electroporated cells were plated into a 10 cm^2 well containing 2i medium + 5% FCS. The following day the medium was changed to serum-free 2i. 1 μg/ml puromycin selection was added 72 hr post-electroporation and the number of puromycin-resistant colonies counted 8 days post-electroporation. The electroporation efficiency increased as the DNA concentration increased from 5 to 50 μg. Electroporated clones could be picked into 96-well plates, expanded, and successfully frozen/thawed.

Cell-Cycle Profiling

5×10^5 cells were harvested 1 day after passaging by dissociation with Accutase (Sigma) and fixed in 70% ethanol at 4°C. Cells were stained in a solution of propidium iodide (10 μg/ml), RNase A (20 μg/ml) 0.1% BSA, and 0.1% Triton X-1 in PBS for 30 min at 37°C. Fluorescence was analyzed using a CyAn flow cytometer (Dako). Data were analyzed using FlowJo software (v.8.4.6). Cell-cycle distributions were calculated using a Watson pragmatic model.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures, one table, and nine figures and can be found with this article online at http://www.cell.com/supplemental/S0092-8674(08).

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