Embryonic Stem Cells Contribute to Mouse Chimeras in the Absence of Detectable Cell Fusion

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ABSTRACT

Embryonic stem (ES) cells are capable of differentiating into all embryonic and adult cell types following mouse chimera production. Although injection of diploid ES cells into tetraploid blastocysts suggests that tetraploid cells have a selective disadvantage in the developing embryo, tetraploid hybrid cells, formed by cell fusion between ES cells and somatic cells, have been reported to contribute to mouse chimeras. In addition, other examples of apparent stem cell plasticity have recently been shown to be the result of cell fusion. Here we investigate whether ES cells contribute to mouse chimeras through a cell fusion mechanism. Fluorescence *in situ* hybridization (FISH) analysis for X and Y chromosomes was performed on dissociated tissues from embryonic, neonatal, and adult wild-type, and chimeric mice to follow the ploidy distributions of cells from various tissues. FISH analysis showed that the ploidy distributions in dissociated tissues, notably the tetraploid cell number, did not differ between chimeric and wild-type tissues. To address the possibility that early cell fusion events are hidden by subsequent reductive divisions or other changes in cell ploidy, we injected Z/EG (lacZ/EGFP) ES cells into ACTB-cre blastocysts. Recombination can only occur as the result of cell fusion, and the recombined allele should persist through any subsequent changes in cell ploidy. We did not detect evidence of fusion in embryonic chimeras either by direct fluorescence microscopy for GFP or by PCR amplification of the recombined Z/EG locus on genomic DNA from ACTB-cre::Z/EG chimeric embryos. Our results argue strongly against cell fusion as a mechanism by which ES cells contribute to chimeras.

INTRODUCTION

PLURIPOTENT ES CELLS HAVE THE ABILITY to differentiate into cells of the three germ layers—ectoderm, mesoderm, and endoderm—following mouse chimera production, teratoma formation, and embryoid body formation. The generation of mouse chimeras through blastocyst injection has

been used extensively to generate knock-out mice, where gene targeted ES cells function in transmitting a manipulated genome through the germline of chimeric mice. A number of recent reports have identified cell fusion as the explanation for apparent cell plasticity in a variety of cell types *in vivo* (Alvarez-Dolado et al., 2003; Gibson et al., 1995; Gussoni et al., 2002; Nygren et al.,

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TABLE 1. FISH ANALYSIS OF WILD-TYPE AND CHIMERIC TISSUES*

	% chimerism	No signal	Y	X	XY	XX	XYX	XXY	XXYY	XXXY	XXX YYY	XXXX YYYY	Cells scored
Wild-type													
E10.5 embryo	0	0	1	0	99	0	0	0	0	0	0	0	200
E13.5 embryo	0	0	1.5	0	98.5	0	0	0	0	0	0	0	200
P3 blood	0	0	1.5	0.5	96.5	0	0	0	1.5	0	0	0	200
P3 liver	0	0	2	0	96	0	1	0	1	0	0	0	200
P3 lung	0	0	0.5	1	98.5	0	0	0	0	0	0	0	200
P3 brain	0	0	3	0	96.5	0	0	0	0.5	0	0	0	200
24 mo liver	0	0	0	0	10	0	0	0	42.5	0	1.5	46	200
24 mo brain	0	0	1.5	0.5	97	0	0	0	1	0	0	0	200
24 mo lung	0	0	2	0	96.5	0	0	0	1.5	0	0	0	200
24 mo intestine	0	0	0	0	100	0	0	0	0	0	0	0	200
24 mo spleen	0	0	0	0	100	0	0	0	0	0	0	0	200
24 mo bone marrow	0	0	0	0	99.5	0	0	0	0.5	0	0	0	200
24 mo blood	0	0	0.5	0.5	99	0	0	0	0	0	0	0	200
24 mo heart	0	0	2	0	97	0	0	1	1	0	0	0	200
Chimeras													
E10.5 embryo	45	0	0	4	94.5	0	0.5	1	0	0	0	0	200
E13.5 embryo 1	29	0	1	2.5	96.5	0	0	0	0	0	0	0	200
E13.5 liver 1	43	0	2	2.5	95	0	0	0	0.5	0	0	0	200
E13.5 embryo 2	78	0	0.5	0.5	97.5	0	1	0.5	0	0	0	0	200
P3 liver	27.9	1	2	0	95	0	0.5	0.5	1	0	0	0	200
P3 brain	5	0	1.5	0	98	0	0	0.5	0	0	0	0	200
P3 lung	16	1.8	2.7	0	92.9	0	0	0.9	1.7	0	0	0	112
P3 intestine	12	0	1	1	96	1	0.5	0.5	0	0	0	0	200
P3 spleen	7	0	3.5	0.5	93.5	0	0	0	2.5	0	0	0	200
P3 bone marrow	1.2	0	1.5	0.5	95	0.5	0	0.5	1.5	0.5	0.5	0	200
P3 blood	1	0	2	0.5	95	0	0	1	1	0.5	0.5	0	200
P3 heart	9	0	0.5	1	98.5	0	0	0	1	0	0	0	200
24 mo liver	19	0	0	0	9.5	0	0	0	29	0	4.5	57	200
24 mo brain	7	0	1.5	0	93	0	0	0	5.5	0	0	0	200
24 mo lung	46	0	1.5	0.5	98	0	0	0	0	0	0	0	200
24 mo intestine	3	0	1	0	98.5	0	0	0	0.5	0	0	0	200
24 mo spleen	42	0	0.5	3	96.5	0	0	0	0	0	0	0	200
24 mo bone marrow	9	0	1.5	0	97.5	0	0	0	1	0	0	0	200
24 mo blood	2	0	0	3.5	96.5	0	0	0	0	0	0	0	200
24 mo heart	16	0	1	1	98	0	0	0	0	0	0	0	200

*Data from male mice only are presented.

2004; Oh et al., 2003, 2004; Spees et al., 2003; Terada et al., 2002; Vassilopoulos et al., 2003; Wang et al., 2003; Weimann et al., 2003). Consequently, it is important to rule out a cell fusion mechanism when assessing the stem cell characteristics of a given cell type. Although ES cell pluripotency as assayed by chimera production following blastocyst injection has been documented for decades, to our knowledge, data addressing the possibility that cell fusion plays a role in ES cell pluripotency in this context have not been reported.

Experiments in which embryos at the two-cell stage are fused into a single tetraploid embryo suggest that tetraploid cells have a selective disadvantage in the developing mouse embryo. Diploid ES cells injected into such tetraploid blastocysts out-compete the tetraploid cells, and the resulting embryo is comprised solely of the in-

jected ES cells (Nagy et al., 1993). However, this evidence is countered by cases in which cell fusion between ES cells and somatic cells produced tetraploid hybrid cells, which were shown to be pluripotent through their ability to contribute to mouse chimeras after blastocyst injection (Tada et al., 2001; Ying et al., 2002).

These observations led us to investigate whether ES cells contribute to mouse chimeras through a cell fusion mechanism. Two methods were used in our studies. First, X and Y chromosome FISH analysis was performed to follow the ploidy distributions of cells isolated from embryonic, neonatal, and adult wild-type and chimeric mice. Second, we used the Cre/LoxP system to track the fusion history of cells *in vivo* by injecting Z/EG (lacZ/EGFP) ES cells into ACTB-cre blastocysts. The results of these two sets of

FIG. 1. Interphase FISH of wild-type and chimera-derived cells. Examples of FISH analysis showing XY diploid (a, d), XXXYY tetraploid (b, e), and XXXXXYYYYY 8n (c, f) interphase nuclei. (a–c) Cells isolated from a 24-month wild-type male liver; (d–f) cells from the liver of a 24-month chimeric mouse. A Y whole chromosome paint (green) and an X telomere probe (red) were used in the FISH analysis. Cells were counterstained with DAPI.

studies were inconsistent with a cell fusion mechanism and argue strongly against cell fusion as the mechanism by which ES cells contribute to chimeras.

RESULTS

No difference in cell ploidy distributions in wild-type versus chimeric embryos and mice

To determine whether ES cell chimeras contain significant numbers of tetraploid cells due to early cell fusion events, we microinjected ES cells into blastocysts and evaluated ploidy levels in cells from these chimeras through fluorescence *in situ* hybridization (FISH) analysis. Male ES cells derived from a 129-ROSA26 transgenic mouse (Shawlot et al., 1999; Soriano, 1999) were microinjected into C57BL/6 blastocysts and transferred to pseudopregnant female mice. Chimeric embryos were allowed to develop to E10.5, E13.5, postnatal day 3, and 24 months. For simplicity, data from male mice only are presented. Wild-

type C57BL/6 mice aged E10.5, E13.5, postnatal day 3 (P3), and 24 months served as controls.

For both chimeric and wild-type mice, whole E10.5 embryos were mechanically dissociated into single-cell suspensions, while E13.5 embryonic liver, brain, and bulk embryos were isolated and dissociated mechanically and enzymatically (collagenase). Brain, lung, intestine, spleen, and hearts were isolated from P3 and 24-month-old mice and enzymatically dissociated into single-cell suspensions, while bone marrow and peripheral blood were prepared separately. Liver cells were isolated by perfusing the portal vein with collagenase. In the case of chimeric embryos and mice, a fraction of each of the single cell suspensions was used to quantitate percent chimerism through X-gal staining or quantitative real-time PCR (qPCR) analysis using primers designed to amplify β -galactosidase DNA. The remainder of cells were used for X and Y chromosome FISH analysis to quantitate ploidy levels in chimeric tissues. An X-telomere probe and a Y whole chromosome paint (WCP) were used to visualize X and Y chromosomes in cells in inter-

phase. The number of X and Y chromosomes was counted for each cell analyzed, and 200 individual cells were scored per sample. Cells were designated as diploid (1X, 1Y), aneuploid (0X, 0Y; 1X, 0Y; 0X, 1Y; 1X, 2Y; 2X, 1Y), tetraploid (2X, 2Y; 3X, 1Y; or 4X), 6N (3X, 3Y), or 8N (4X, 4Y; see Table 1). Examples of cells scored as diploid, tetraploid, and 8N are shown in Figure 1. We then compared the number of aneuploid, diploid, tetraploid, 6n, and 8n cells between wild-type and chimeric samples. The data are summarized in Figure 2.

The presence of tetraploid or other aneuploid cells would be one indication that cell fusion is partly or totally responsible for the contribution of ES cells to chimeric mice. For example, if ES cells contribute solely through a cell fusion mechanism to mouse chimeras, the percentage of chimerism might correspond to a tetraploid or higher ploidy population of cells.

X and Y chromosome FISH analysis on an E10.5 control embryo revealed 99% XY diploid cells, and 1% aneuploid cells containing only a Y chromosome. FISH analysis on a wild-type E13.5 embryo showed that 98.5% of the cells in the embryo were XY diploid. Aneuploid cells, containing an individual Y chromosome, were limited to 1.5%. Tetraploid (XXYY), or higher ploidy cell populations were not found in wild-type E10.5 and E13.5 embryos. The absence of polyploidy in E10.5 and E13.5 embryos is in agreement with studies showing that naturally occurring tetraploidy and polyploidy are not significantly evident until later in postnatal development (Gandillet et al., 2003; Guidotti et al., 2003; Gupta, 2000). FISH analysis on tissues from wild-type P3 mice revealed at least 96% XY diploid cells, with 1.5% or fewer XXYY tetraploid cells. Aneuploid levels of singlet X or Y chromosomes or XYY distributions did not exceed 3% in these cases. Similar results were observed from XY FISH analysis on a male 24-month wild-type mouse in all tissues except the liver. In nonliver tissues, diploid XY cell distributions ranged from 96.5–100%, while tetraploid XXYY populations did not exceed 1.5%. Aneuploid cells containing a single X or Y chromosome were limited to 2%. One percent or fewer showed an XYY profile. Liver cells contained 10% diploid XY cells, 42.5% tetraploid XXYY cells, 1.5% 6n cells, and 46% 8n cells. These observations are consistent with studies in rats showing that hepatocyte polyploidy is first evident at 3 weeks of age. By 6 weeks, polyploid cells are more prevalent than diploid, and at 10–12 weeks, 20% of he-

patocytes are diploid and 75% are polyploid (Gandillet et al., 2003; Guidotti et al., 2003).

Ploidy distributions of cells isolated from chimeric embryos did not differ significantly from those seen for wild-type controls. In E10.5 and E13.5 samples, the percentage of XY cells was slightly lower, from 94.5–97.5%. Among these early time point samples, 2% or fewer had a single Y, and 0.5–4% had a single X. 1.5% or fewer showed a XXY or XYY distribution, and 0.5% were scored as tetraploid. In the P3 samples, from 92.9–98% of cells were XY diploid, with 2.5% or fewer tetraploid. Similarly, in nonliver tissues isolated from 24-month-old chimeras, 92.9–98% of cells were XY diploid, 2.5% or fewer tetraploid. Liver cells contained 9.5% diploid XY cells, 29% tetraploid XXYY cells, 4.5% 6n cells, and 57% 8n cells. In addition to the embryos and mice for which data are shown in Figure 2 and Table 1, four additional E10.5 chimeras and one additional E13.5 chimera were analyzed by FISH with similar results. Importantly, there was no correlation between percent chimerism and percent of tetraploid or aneuploid cells in any of the chimera samples.

Use of the Z/EG transgene to detect cell fusion masked by subsequent change in cell ploidy

It is possible that evidence of early cell fusion events is subsequently lost. This might occur by a mechanism such as reductive divisions, as seen in the liver (Wang et al., 2003), or by some as yet undescribed mechanism. If this were to occur prior to E10.5 during development, using X and Y chromosome FISH analysis would be ineffective in detecting cell fusion. Although reductive divisions have not been found in studies such as those focusing on associations between cell fusion and bone marrow transplantation (Harris et al., 2004), evidence of reductive divisions was seen by Wang et al. (2003) in the liver. To address this issue, we utilized the Cre/LoxP recombination system. By creating chimeras between ES cells carrying a floxed target reporter gene and host blastocysts carrying a ubiquitously expressed Cre transgene, we used recombination of the target gene as a heritable marker of past cell fusion events. In our system, recombination of the Z/EG sequence can only occur if Cre recombinase is introduced into recombination target-bearing Z/EG cells through cell fusion. Z/EG recombination permanently marks the locus, even if reductive

FIG. 2. No difference in cell ploidy distributions in wild-type versus chimeric mice. Wild-type C57BL/6 male mice (a) and ROSA26::C57BL/6 chimeric mice (b–d) aged E10.5, E13.5, postnatal day 3 (P3) and 24 months were analyzed for ploidy distribution of various cell types. For all graphs, diploid (2n), tetraploid (4n), 6n, 8n, and aneuploid (3n, <2n) are represented as a percentage of total cells analyzed. For data from chimeric tissues, the percentage of chimerism is indicated as a second bar. Tissues analyzed by FISH include whole embryos (wh), liver (LI), brain (BR), lung (LU), intestine (IN), spleen (SP), bone marrow (BM), blood (BL), and heart (HE).

divisions, or other resorting of genetic material occurs. Only in the events of cell death or decreased proliferation will these Z/EG genomic recombination marks be lost or underrepresented

in later stage embryos. Our strategy is outlined in Figure 3.

The Z/EG double-reporter transgene consists of two reporter genes under the transcriptional

control of the ubiquitously expressed CAGGS promoter, and mice carrying this transgene were generated and characterized by Novak et al. (2000; see diagram in Fig. 3). In the absence of Cre recombinase activity, lacZ is expressed ubiquitously and EGFP is not expressed. In the presence of Cre recombinase, the lacZ coding sequence is excised leading to enhanced green fluorescent protein (EGFP) expression. This change can be detected both by visualization of EGFP fluorescence and by PCR of the Z/EG locus. Z/EG mice were crossed to strain 129 females, and blastocyst stage embryos were isolated and used to generate ES cell lines. The presence of the Z/EG transgene in the resulting ES cell clones was assessed by β -galactosidase staining and PCR on genomic DNA. Clones were analyzed for chromosome number via Giemsa staining of metaphase nuclear spreads. A Z/EG ES cell clone, Z/EG 1.5.2, with 70% diploid cells (40 chromosomes) was used for subsequent experiments. To determine developmental potential, Z/EG 1.5.2 cells were microinjected into C57BL/6 blastocysts. Injected blastocysts were transferred to pseudopregnant foster female mice and allowed to develop to E11.5. E11.5 chimeric embryos were dissected and whole-mount β -galactosidase staining was performed to observe chimerism. The level of contribution observed ranged from undetectable to high, confirming the ability of the ZEG 1.5.2 cells to contribute efficiently to chimeras (data not shown).

The Z/EG reporter transgene is functional in Z/EG 1.5.2 ES cells

Activity of the Z/EG transgene was confirmed *in vivo* by studies in which Z/EG transgenic mice were crossed to Cre recombinase-expressing transgenic mice (pCX-NLS-Cre (Novak et al., 2000). To test the Z/EG construct in our Z/EG 1.5.2 ES cells, we introduced Cre recombinase under the control of the CAGGS promoter by nucleofection of a mammalian expression vector carrying the Cre gene (pCre) (Score et al., 2005). EGFP expression was visible in cells 24 h post-Cre nucleofection, indicating the ability of Cre to excise the lacZ coding sequence leading to EGFP expression. Nucleofected Z/EG 1.5.2 ES cells continued to divide as evidenced by an increased number of EGFP positive cells at 48 h relative to 24 h, indicating viability and proliferation are maintained in Z/EG ES cells nucleofected with

Cre (Fig. 4a,b). To mimic Cre/LoxP interaction in a cell fusion context, analogous to what could occur between Z/EG 1.5.2 ES cells and ICM cells following Z/EG cell injection into Cre-expressing blastocysts, we fused Z/EG 1.5.2 ES cells with ACTB-cre expressing neural stem cells (NSCs) through polyethylene glycol (PEG)-induced cell fusion. Similar results were seen compared with Cre nucleofection in that EGFP-positive cells were visible 24 h following cell fusion between Z/EG 1.5.2 ES cells and ACTB-cre NSCs (Fig. 4c,d). The number of EGFP expressing cells increased 72 h post-cell fusion, indicating the ability of fused cells to proliferate (Fig. 4e,f). No EGFP was detected in unmanipulated cells (data not shown).

No detectable recombination in ACTB-cre::Z/EG chimeric embryos

Z/EG 1.5.2 ES cells were microinjected into ACTB-cre blastocysts and the blastocysts were transferred to pseudopregnant female mice. Chimeric embryos were isolated at E9.5. Because ACTB-cre heterozygous mice were used to produce the host blastocysts, each embryo was assayed for the presence of ACTB-cre recombinase by performing PCR on placental genomic DNA using primers designed to amplify the Cre recombinase sequence. Among ACTB-cre-positive embryos, whole-mount β -galactosidase staining and PCR were performed to confirm the presence of Z/EG DNA. Chimeric embryos positive for ACTB-cre and Z/EG sequences were used in subsequent experiments. We used two approaches to assess whether fusion had occurred in these embryos: fluorescence microscopy to detect EGFP expression, and a PCR assay in which genomic DNA was isolated from whole ACTB-cre::Z/EG E9.5 chimeric embryos and amplified using primers designed to distinguish between nonrecombined Z/EG (N) and Cre mediated recombined Z/EG (R) genomic DNA sequences (Fig. 5a).

If cell fusion does not take place between Z/EG ES cells and ACTB-cre cells in chimeric embryos, any ZEG 1.5.2-derived cells will stain positively for β -galactosidase, no EGFP should be detected, and nonrecombined Z/EG PCR products, but not recombined products, should be amplified. If chimeric embryos have some amount of cell fusion between Z/EG and ACTB-cre cells, both β -galactosidase and EGFP should be detectable, and both

FIG. 3. Use of the Z/EG transgene to detect cell fusion in chimeric embryos. Schematic illustrating use of the Cre–LoxP system to detect rare cell fusion events. In the absence of cell fusion β -galactosidase expression persists, while cell fusion events lead to Z/EG recombination, deletion of the β -galactosidase sequence, and EGFP expression.

nonrecombined and recombined ZEG sequences should be present. Finally, if ES cells contribute to mouse chimeras solely through a cell fusion mechanism, chimeric embryos should express EGFP but not β -galactosidase and contain predominantly the recombined form of the Z/EG sequence.

We analyzed a total of 4 E9.5 ACTB-cre::Z/EG chimeric embryos with levels of chimerism (as determined by PCR for the β -galactosidase sequence) ranging from 14–41% (Fig. 5b). We did not detect any EGFP in these embryos by fluorescence microscopy (data not shown). Although the pattern of β -galactosidase and EGFP expression noted above suggests that fusion did not occur, rare cell fusion events are unlikely to be detected by this method. Therefore, we also used a PCR-based method to detect recombination of the Z/EG transgene. PCR primers designed to amplify nonrecombined and recombined Z/EG DNA contain a common forward primer (promoter) and reverse primers specific to β -galactosidase and EGFP sequences. Regardless of the cell in which it ultimately resides, the recombined Z/EG locus indicates that cell fusion occurred at some point during development, because cre is only present in host blastocysts and the target Z/EG locus is only present in the injected ES cells. Figure 5c shows PCR performed on nonrecom-

bined Z/EG DNA using CAGGS-LacZ primers and PCR performed on recombined Z/EG DNA using CAGGS-EGFP primers. Recombined ZEG DNA was generated by mating ACTB-cre mice to ZEG mice and isolating DNA from doubly transgenic offspring. In these mice, both transgenes should be expressed in every cell, resulting in complete recombination in all tissues.

To test the sensitivity of our assay to detect recombined ZEG DNA, we performed PCR on nonrecombined Z/EG DNA spiked with serial dilutions of recombined Z/EG DNA. Using standard PCR, we found we could detect Z/EG recombination down to the level of 0.1% (Fig. 5c). We used qPCR to confirm this level of detection (data not shown).

We performed PCR on the four ACTB-cre::ZEG chimeric embryos described above. PCR detected nonrecombined Z/EG DNA, but not recombined Z/EG genomic DNA from ACTB-cre::Z/EG E9.5 chimeric embryos (Fig. 5d). Similarly, qPCR on DNA from these ACTB-cre::Z/EG E9.5 chimeric embryos did not amplify detectable levels of recombined Z/EG DNA (data not shown). The high level of chimerism (14–41%) in our ACTB-cre::Z/EG E9.5 chimeric embryos combined with the absence of detectable recombined Z/EG DNA strongly suggests that ES cells do not contribute to mouse chimeras through a cell fusion mechanism.

Cell fusion is evident in skeletal muscle of postnatal chimeras

As an additional test of the ability of our assays to detect cell fusion, we produced a second set of chimeras that were allowed to reach 3 weeks of age. We assayed skeletal muscle from these mice, a tissue in which cell fusion has been documented as a normal part of development (Huppertz et al., 1998). Because myoblast fusion is reported to begin between embryonic day 10 and 11 (Brand-Saberi, 2002), we would not expect to find evidence of cell fusion in the E9.5 embryos shown in Figure 5. However, in 3-week old ACTB-cre::Z/EG chimeras, some muscle fibers formed by fusion of adjacent cells would be expected to be GFP positive.

Skeletal muscle was harvested from a 3-week-old ACTB-cre::Z/EG chimeric mouse and cryo-sectioned to detect EGFP-positive cells resulting from cell fusion between Cre-expressing cells and Z/EG-expressing cells. EGFP-positive cells were evident in cross-sections of skeletal muscle from an ACTB-cre::Z/EG chimeric mouse and an ACTB-cre;Z/EG doubly transgenic mouse (Fig. 6a–b,e–f), while EGFP-positive cells were not detected in wild-type skeletal muscle (Fig. 6c–d). PCR performed on ACTB-cre::Z/EG chimeric skeletal muscle also confirmed Z/EG recombination (Fig. 6g). The presence of nonrecombined and recombined Z/EG PCR products indicates mosaicism in ACTB-cre::Z/EG chimeric skeletal muscle resulting from partial contribution of Z/EG ES cells to skeletal muscle development. Non-EGFP-positive skeletal muscle cells arise from fibers formed by fusion of cells with the same genotype, as opposed to ACTB-Cre cells fusing with Z/EG cells. These results confirm the ability of our transgenic assay to detect EGFP expression resulting from cell fusion in chimeric mice.

DISCUSSION

ES cells have the ability to differentiate into cell types of the three germ layers *in vitro* as observed through embryoid body formation and directed differentiations, and *in vivo* through teratoma formation and chimera production. Although the pluripotent status of ES cells has been well accepted, the possibility that ES cells might contribute to chimeric embryos and mice by a cell fu-

sion mechanism has not been rigorously tested. Our results rule out a major role for cell fusion in ES cell chimera contribution, and argue strongly against any role for fusion between ES and ICM cells during chimera production.

Through FISH analysis comparing the percentage of tetraploid and higher ploidy cells in wild-type and chimeric tissues, we did detect a low level of aneuploid cells in all our samples. There are several possible explanations for this observation. It may be that our results are an accurate reflection of a small number of aneuploid cells resident in these tissues. It is also possible that these data represent artifacts due to technical issues. For example, in cases of singlet Y chromosome interphase nuclei, the X telomere probe is smaller than the Y whole chromosome paint, so while the X chromosome probe is more localized than the Y WCP, the Y WCP is brighter than the X telomere probe, perhaps causing us to fail to detect the X chromosome signal. The X telomere probe was chosen over an X whole chromosome paint because two whole chromosome paints (X and Y) would likely result in overlapping fluorescence using interphase FISH analysis. A slight increase in singlet X chromosome detection efficiency would come at the expense of diploid, tetraploid, and higher ploidy distribution detection.

Cell fusion has been associated with the development of normal tissues such as the placenta (Huppertz et al., 1998, 2001), muscle (Taylor, 2000), macrophages (Vignery, 2000), and bone (Kahn et al., 1982; Loutit and Nisbet, 1982). Our FISH analysis on postnatal mice avoided tissues such as skeletal muscle and osteoclasts where cell fusion is known to occur naturally during development. However, we did observe EGFP-positive cells in skeletal muscle derived from ACTB-cre::Z/EG chimeras, indicating the ability of our cre/lox assay to detect cell fusion *in vivo*. Tissues such as liver have been shown to have populations of tetraploid or higher ploidy cells that are thought to arise from endoreduplication (Dudas et al., 2003; Guidotti et al., 2003; Gupta, 2000). Although it has not been determined precisely when polyploidy arises in mouse tissues, it has been postulated that liver cell polyploidy begins in rats around 3 weeks of age (Gandillet et al., 2003; Guidotti et al., 2003; Gupta, 2000). The difference in liver cell polyploidy we observed between wild-type and chimeric mice is likely to reflect natural polyploid variability, as it has been

FIG. 4. The Z/EG reporter transgene is functional in Z/EG 1.5.2 ES cells. Bright-field and fluorescence overlay (a, c, e) and EGFP fluorescence (b, d, f) 48 h posttransfection of Z/EG 1.5.2 ES cells with a pCre plasmid (a and b) and 24 hours (c and d) or 72 hours (e and f) after cell fusion between Z/EG 1.5.2 ES cells and ACTB-cre NSCs.

shown that hepatocyte polyploidy (4n) in rats can range between 10–20% (Gandillet et al., 2003; Guidotti et al., 2003).

There are several reports of cell fusion occurring in the context of tissue repair and regeneration. Most reports wherein fusion was detected were studies in which bone marrow cells were

grafted in the setting of total body irradiation. Although cell fusion events in the absence of additional tissue repair or injury are rare following bone marrow transplantation (1 in 100,000 cells) (Ogle et al., 2005), the main avenue for repopulation of damaged cardiac muscle and liver tissues in mouse ischemia and liver disease models

is through cell fusion between donor bone marrow derived cells and host cardiac and liver cells (Nygren et al., 2004; Ogle et al., 2005). Nygren et al. (2004) showed the presence of multinucleated cells in cardiac tissue with nuclei showing characteristics of both donor and recipient cells following transplantation of bone marrow cells into ischemic myocardium. Two studies showed that in an FAH^{-/-} liver disease model the apparent plasticity or transdifferentiation of bone marrow-derived hepatocytes was explained by cell fusion events between bone marrow macrophages and hepatocytes (Wang et al., 2003; Willenbring et al., 2004). Although ES cell blastocyst injection is an invasive procedure where injection needles puncture cell-cell junctions between trophectodermal cells during the transfer of ES cells to the blastocoel cavity, cellular damage is minimal and cannot be compared with larger scale cellular damage observed in cardiac ischemia and FAH^{-/-} liver disease. In addition, macrophages are not present in the preimplantation-stage embryo, so therefore a macrophage-induced cell fusion mechanism is not plausible.

The relatively low sensitivity of our recombinant Z/EG PCR assay (1:1000 cells) means that we might fail to detect very rare fusion events. However, because of the very low numbers of ICM cells present at the blastocyst stage (21–22 cells at 96 h) (Watkins et al., 2007) even a single fusion event between an ES cell and an ICM cell would comprise approximately 5% of cells in the embryo. From this point, two possibilities can be envisioned (1) the fused cell divides at a similar rate as nonfused cells, and its progeny continue to comprise 5% of the embryo. In this case, our assay would only need to detect one fusion event in 20 cells, well within the sensitivity we see. (2) The fused cell dies or fails to proliferate. In this case, ES cell-derived genetic material such as the Z/EG locus will not be present at significant levels in the resulting chimera at later stages of development. We would argue that by definition such fused cells are not “contributing” to chimeras.

Although we have provided evidence that ES cells do not contribute to mouse chimeras through cell fusion events, the precise mechanism of ES contribution to chimeras is not known. Although they share many characteristics including gene expression patterns, ES cells and cells of the early embryo are not identical. For example, ES cells have a normal cell doubling time of 12 h *in vitro*, but cells of E6.5 and E7.5 embryonic ectoderm have doubling times of 5 and 8 h, respec-

tively (Lawson and Pedersen, 1992; Snow and Bennett, 1978). Thus, to contribute at significant levels, ES cells must increase the speed of their cell cycle. Insight into how ES cell cycling is modified in the blastocyst environment, and what cell surface and extracellular signaling molecules are involved should lead to a better understanding of the mechanism by which ES cells contribute to mouse chimeras.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's media (DMEM) high glucose, DMEM/F-12, fetal calf serum (FCS), L-glutamine, streptomycin/penicillin, nonessential amino acids, sodium pyruvate, KaryoMax/GURR tablets, and KCl were obtained from Invitrogen (Carlsbad, CA). Trypsin and PBS were obtained from Cellgro (Herndon, VA). Fetal calf serum (FCS) was obtained from HyClone (Logan, UT). EGF, bFGF, and N-2 plus supplement were obtained from R&D Systems (Minneapolis, MN). LIF was obtained from Chemicon Inc. (Temecula, CA). β -Mercaptoethanol, gelatin, NaCO₃, BSA, HCl, and glucose were obtained from Sigma Chemical Co. (St. Louis, MO). Nucleofector solutions (mES kit) were obtained from Amaxa Inc. (Gaithersburg, MD). Taqman SYBR green universal mix PCR reaction buffer was obtained from Perkin-Elmer Applied Biosystems (Boston, MA). The QIAamp DNA micro kit was obtained from Qiagen Inc. (Valencia, CA). Methanol and glacial acetic acid were obtained from Fisher Scientific Company L.L.C. (Pittsburgh, PA). HTF and mHTF were obtained from Irvine Scientific (Santa Ana, CA).

Mice

C57BL/6, 129, Z/EG (Tg(ACTB-Bgeo/GFP)21Lbe/J) (Novak et al., 2000), and ACTB-Cre (Tgn(ACTB-cre)2Mrt) (Lewandoski et al., 1997) mice were obtained from Jackson Laboratory (Bar Harbor, ME). Z/EG mice were backcrossed onto C57BL/6. CD1 mice were obtained from Charles River (Wilmington, MA). Timed matings between male and female C57BL/6 mice produced blastocysts used for ES cell blastocyst injection to generate mouse chimeras for FISH analysis. Injected blastocysts were transferred into the uteri of pseudopregnant female mice and allowed to develop. Mouse embryonic fibroblasts (MEFs)

FIG. 5. No detectable recombination in ACTB-cre::Z/EG chimeric embryos. (a) Diagram illustrating PCR primers designed to amplify nonrecombined or recombined Z/EG DNA. The forward primer is specific to the CAGGS promoter and reverse primers are specific to β -galactosidase (CAGGS-LacZ, nonrecombined) or EGFP (CAGGS-EGFP, recombined) sequences. (b) Four X-gal-stained ACTB-cre::Z/EG E9.5 chimeras with chimerism levels ranging from 14–41% (as determined by PCR for the β -galactosidase sequence). (c) PCR performed on nonrecombined Z/EG (N), recombined Z/EG (R), and nonrecombined Z/EG DNA spiked with serial dilutions (1:10, 1:100, 1:1000, and 1:10,000) of recombined Z/EG DNA using GAPDH (left panel), CAGGS-LacZ (middle panel), and CAGGS-EGFP (right panel) primer pairs. Note that recombined DNA can be detected at the level of 1:1000 dilution. (d) PCR performed on genomic DNA from the four chimeric embryos shown in b using GAPDH (left panel), CAGGS-LacZ (middle panel) and CAGGS-EGFP (right panel) primer pairs. No PCR product corresponding to the recombined sequence is detected in the chimera samples.

used as a feeder layer for ES cell culture were derived from C57BL/6 E13.5 embryos as described (Hogan et al., 1994). Timed matings between heterozygous ACTB-cre male and female mice produced blastocysts used in generating ACTB-cre::Z/EG chimeras. ACTB-cre; Z/EG doubly transgenic mice were produced by mating ACTB-cre male mice to Z/EG female mice. Neural stem cells (NSCs) were isolated from ACTB-cre expressing male mice mated with C57BL/6 female mice. All care and treatment of mice was in accordance with University of Minnesota IACUC guidelines.

Z/EG ES cell derivation

Z/EG male mice were mated to 129 females and resulting blastocysts were flushed from the uterus and transferred to 48-well tissue culture dishes containing MEFs and DMEM media supplemented with 30% FCS, 2000 U/mL LIF, penicillin/streptomycin, L-glutamine, nonessential amino acids, and 50 μ M β -mercaptoethanol (Auerbach et al., 2000). ICM outgrowths were trypsinized and replated in 48-well plates containing MEFs and ES derivation media. ES cell colonies were passaged onto larger tissue culture dishes and gradually transitioned to standard ES culture media. Chromosome counting of metaphase spreads was performed to quantitate the percentage of euploid cells for each line derived. The Z/EG 1.5.2 line had 70% of cells with 40 chromosomes, and was used in these studies.

ES cell culture

Mouse ES cells were cultured on irradiated MEFs. ES culture media contained high glucose DMEM supplemented with 15% FCS, 10 ng/mL recombinant leukemia inhibitory factor (LIF), penicillin/streptomycin, L-glutamine, nonessential amino acids, sodium pyruvate, and 50 μ M β -mercaptoethanol. ES cells were cultured for less than 25 passages to maintain the integrity of chromosome number. It has been noted that low passage ES cells have fewer cytogenetic abnormalities than high passage ES cells (Auerbach et al., 2000). ES10 ES cells used for these studies were >50% euploid as assessed by chromosome counting of Giemsa stained metaphase spreads. ES10 cells, derived from Gt(ROSA)26Sor/J mice (ROSA26) (Shawlot et al., 1999; Soriano, 1999), were used to generate mouse chimeras for detecting cell fusion through FISH analysis. Z/EG 1.5.2 ES cells were used to generate ACTB-

cre::Z/EG mouse chimeras to detect cell fusion using the Cre/LoxP system.

Chromosome counting

Chromosome counting was performed by seeding ES cells on tissue culture dishes precoated with 0.1% gelatin overnight in ES culture media. Colcemid (0.06 μ g/mL) was added to the dishes and ES cells were incubated at 37°C for 2–3 h. ES cell pellets were incubated with KCl at room temperature and then incubated with a cold fixative solution (3 methanol:1 glacial acetic acid). Fixed cells were dropped on slides and allowed to dry. The next day slides were stained with Giemsa dye (KaryoMax/GURR tablets), and chromosomes were counted using standard microscopy.

Neurosphere culture

Neuroepithelial cells were isolated from the forebrains of E10.5 ACTB-cre embryos by dissecting telencephalons and dissociating them into a near single cell suspension in DMEM media. Approximately $1\text{--}2 \times 10^5$ neural stem cells (NSCs) were plated into six-well plates containing N-2 plus medium supplemented with 20 ng/mL EGF and bFGF. Neurospheres were passaged every 4 days by centrifuging for 5 min at 40 g and partially dissociating the neurospheres by pipetting up and down. For cell fusion studies, neurospheres were dissociated into a single cell suspension by centrifugation for 5 min at 40 g, trypsinization for 5 min, and passage of dissociated cells through a 70 μ m cell strainer (BD Falcon, BD Biosciences, Bedford, MA) into media containing 10% FCS to inactivate trypsin. Cell suspensions were then centrifuged at 1800 rpm for 5 min and washed with PBS. Single cell NSCs were then used in the cell fusion assay with Z/EG ES cells.

Generation of ES chimeras

E3.5 blastocysts were flushed from the uteri of C57BL/6 females using DMEM media. Blastocysts were incubated at 37°C with 5% CO₂ in HTF media supplemented with 0.04 g BSA/10 mL HTF until the blastocysts were well expanded. ES chimeras were generated by microinjecting 10–15 ES10 or Z/EG ES cells into E3.5 C57BL/6 blastocysts in mHTF media supplemented with 0.04 g BSA/10 mL mHTF. Injected blastocysts were allowed to expand for up to 2 h in a 37°C incubator.

FIG. 6. Cell fusion is evident in postnatal ACTB-cre::Z/EG chimeric skeletal muscle. Cross sections of skeletal muscle from ACTB-cre::Z/EG chimeric mice (a, b), wild-type C57BL/6 mice (c, d), and ACTB-cre::Z/EG doubly transgenic mice (e, f). Green and blue indicate EGFP fluorescence and Hoechst stain respectively. (g) PCR performed on nonrecombined (N) and recombined (R) Z/EG DNA, and ACTB-cre::Z/EG chimeric skeletal muscle DNA (1) using GAPDH (left panel), CAGGS-LacZ (middle panel) and CAGGS-EGFP (right panel) primer pairs.

tor (5% CO₂) in HTF media until they were transferred to pseudopregnant foster CD1 female mice.

Transfection

Z/EG ES cells were harvested through trypsinization, pelleted, and resuspended in 100 μ L mouse ES nucleofector solution. Program A-23 on the Amaxa Nucleofector was used to transfect pCre plasmid into Z/EG ES cells (Score et al., 2005). Transfected cells were immediately placed in ES culture media and added to tissue culture plates preincubated with 0.1% gelatin for 5 min at room temperature and 30 min with ES media in a 5% CO₂ 37°C incubator. Fluorescence microscopy was used to detect EGFP 24 h post-transfection.

Skeletal muscle isolation

Perfusion fixation. Wild-type and ACTB-cre::Z/EG chimeric mice were perfused with PBS for 3 min and with zinc formalin (Z-Fix concentrate—Anatech LTP, Battle Creek, MI) for 8 min. Gastrocnemius muscles were dissected, postfixed overnight in zinc formalin, treated with 30% sucrose, and washed three times with H₂O.

Cryosectioning. Muscles were imbedded in Tissue-Tek O.C.T. compound (Sakura, Tokyo, Japan), and cryosectioned into 10- μ sections. Cryosections were allowed to dry and fixed for 5 min in zinc formalin, washed three times in PBS, stained with Hoechst for 8 min, washed three times in PBS, and mounted in IMMU-MOUNT (Thermo Electron Corp., Pittsburgh, PA). Fluorescence microscopy was used to detect EGFP and Hoechst.

Cell fusion. Cell fusion was performed as described (Barch et al., 1991). Z/EG ES cells and ACTB-cre NSCs were mixed in a 1:1 ratio, washed with PBS, and centrifuged at 855 rpm for 5 min in a 50-mL conical tube (BD Falcon). The supernatant was removed and 1 mL of 50% polyethylene glycol (PEG) 1500 (Roche, Mannheim, Germany) was added to the dish over 1 min. While stirring, 20 mL of DMEM media was added to the cells over 5 min. The cells were centrifuged at 855 rpm for 5 min, washed with DMEM, and cultured in ES media. Fluorescence microscopy was used to detect EGFP 24-h post-cell fusion.

DNA isolation. Genomic DNA was isolated from E9.5 ACTB-cre::Z/EG chimeric embryos us-

ing a QIAamp DNA micro kit. Genomic DNA was isolated from the tails of ACTB-cre; Z/EG double transgenic mice by incubating mouse tails overnight at 55°C in a lysis buffer containing 10 mM Tris-Cl, 100 mM NaCl, 1 mM EDTA, 2% SDS, and 200 μ g proteinase K in H₂O. Isopropanol was added to the supernatants and the samples were thoroughly mixed. Precipitated DNA was centrifuged and supernatants were discarded. Seventy percent ethanol was added to the DNA pellet and the samples were centrifuged again. DNA pellets were dried and dissolved in TE (pH 8.0).

PCR. Primers used to detect nonrecombined Z/EG sequences include (CAGGS-LacZ): forward 5'-GTTTCGGCTTCTGGCGTGT -3' and reverse 5'-GTTGCACCACAGATGAAACG-3'. Primers used to detect recombined Z/EG sequences include (CAGGS-EGFP): forward 5'-GTTTCGGCTTCTGGCGTGT -3' and reverse 5'-GTAG-GTCAGGGTGGTCACGA-3'. Primers used to amplify cre recombinase, β -galactosidase, and GAPDH sequences are available upon request. PCR reactions were performed in a total volume of 25 μ L using a MasterTaq kit (Eppendorf, Hamburg, Germany). PCR cycling conditions are also available upon request. PCR products were analyzed on 2% gels using a BioRad Molecular Imager Gel Doc XR System and Quantity One® software.

Real time quantitative PCR (qPCR). qPCR was completed with SYBR Green PCR Master Mix reagents using an ABI PRISM 7700 (Perkin-Elmer Applied Biosystems) thermocycler. Cycling conditions are available upon request. PCR was analyzed using ABI Sequence Detection Systems software.

β -Galactosidase staining. Single cell suspensions and embryonic tissues were washed in PBS and fixed in 2% formaldehyde and 0.2% glutaraldehyde in PBS at room temperature for 5 min. The fixative was removed and the cells and embryos were washed three times for 5 min in PBS. PBS was removed and an X-gal staining solution (potassium ferricyanide, potassium ferrocyanide, MgCl₂, Np40, X-gal) was added to the cells. Cells and embryos were incubated with the X-gal staining solution overnight in a 37°C incubator.

Dissociation of tissues for FISH analysis. E10.5 wild-type and chimeric embryos were dissoci-

ated into single-cell suspensions by pipetting up and down with a 1000 μ L pipette tip in DMEM media. E13.5 wild-type and chimeric embryos were dissociated first mechanically by cutting the embryo into small pieces using a razor blade or forcing the tissue through a Cell Dissociation Sieve–Tissue Grinder Kit (Sigma). Embryonic tissues were then treated with 0.08% collagenase B (Sigma) and incubated at 37°C for 30 min with gentle rocking. Cell suspensions were washed three times in DMEM and fixed shortly thereafter. Tissues from wild-type and chimeric postnatal day 3 and 24-month-old mice were dissociated using the same techniques. Brain, lung, intestine, spleen, and heart cells were dissected and subjected to mechanical and enzymatic dissociation protocols to produce single-cell suspensions from these tissues. Bone marrow was harvested by flushing the femur with PBS and pipetting up and down to create a single-cell suspension. Peripheral blood was isolated, pelleted, and treated with ammonium chloride to lyse red blood cells. Liver cells from 24-month-old wild-type and chimeric mice were isolated by perfusion. Liver perfusion through the portal vein consisted of 3 min with EBSS (Earles Basic Salt Solution) without Ca/Mg, 3 min with EBSS with Ca/Mg, and 10 min with 0.5 mg/mL collagenase D (Roche) in EBSS with Ca/Mg. This method is consistent with standard liver perfusion protocols for isolating rat hepatocytes, where hepatocyte polyploidy was assessed using FISH analysis or FACS analysis for DNA content (Gandillet et al., 2003; Gupta, 2000; Wang et al., 2003). Although Wang et al. (2003) performed FISH analysis on hepatocytes cultured *in vitro* for up to 40 h, our results indicate that FISH analysis performed directly after liver perfusion best represents hepatocyte polyploidy, where *in vitro* hepatocyte culture for 24 and 40 h results in decreased numbers of 6n and 8n hepatocytes (data not shown).

Fluorescence in situ hybridization (FISH)

Mouse sample preparation. Cellular pellets from mouse embryonic, postnatal day 3, and 24-month-old mouse blood, bone marrow, and organ tissue were incubated in 0.075 M KCl for 15 min at 37°C and fixed with 3:1 methanol/acetic acid (Barch et al., 1991).

Mouse X probe. A mouse BAC clone for X chromosome telomere was identified using the Na-

tional Center for Biotechnology Information (NCBI) Website and obtained from the Korenberg mouse library (Research Genetics, Invitrogen, Carlsbad, CA). Isolation of the BAC clone DNA was performed with a plasmid DNA midi kit (Roche). The isolated DNA was labeled by nick translation reaction (Invitrogen Nick Translation System) using digoxigenin-11-dUTP (Roche). Sizes of the nick translated fragments were confirmed by electrophoresis on a 1% TBE gel. The labeled DNA was precipitated in COT-1 DNA, sodium acetate, and 95% ethanol, then dried and resuspended in 50% formamide hybridization buffer.

Mouse Y probe. A commercial FITC-labeled mouse Y chromosome paint probe was obtained from Cambio (Dry Drayton, Cambs, UK).

FISH analysis. Probe cocktails were prepared containing the digoxigenin-labeled X chromosome BAC probe along with the FITC-labeled Y chromosome paint probe. The probe mix was hybridized to freshly prepared slides from methanol/acetic acid fixed pellets. The probes were denatured for 8 min at 72°C and preannealed at 37°C for 60 min. Slides were denatured in 70% formamide/2 $\times$ SSC for 1.5 min at 72°C, hybridized with probes for 16 h at 37°C, and washed in 2 $\times$ SSC at 72°C for 1 min. The digoxigenin labeled probes were then detected by incubation with antidigoxigenin-rhodamine conjugated antibody and counterstained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI). The interphase cells and probe signals were visualized under an Olympus BX51 microscope outfitted with a 175W Zenon lamp and DAPI, FITC, and Texas red filters. FISH images were captured and analyzed using an interferometer-based cooled coupled device (CCD) camera and FISHview software (Applied Spectral Imaging, Vista, CA).

FISH signal scoring. For each mouse sample, a total of 200 interphase cells were examined and scored for the presence of FITC and rhodamine signals. For one sample, postnatal day 3 chimera lung, only 112 cells could be scored.

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