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Emergence of undifferentiated colonies from mouse embryonic stem cells undergoing differentiation by retinoic acid treatment

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Abstract

Retinoic acid (RA) is one of the most potent inducers of differentiation of mouse embryonic stem cells (ESCs). However, previous studies show that RA treatment of cells cultured in the presence of a leukemia inhibitory factor (LIF) also result in the upregulation of a gene called *Zscan4*, whose transient expression is a marker for undifferentiated ESCs. We explored the balance between these two seemingly antagonistic effects of RA. ESCs indeed differentiated in the presence of LIF after RA treatment, but colonies of undifferentiated ESCs eventually emerged from these differentiated cells — even in the presence of RA. These colonies, named secondary colonies, consist of three cell types: typical undifferentiated ESCs expressing pluripotency genes such as *Pou5f1*, *Sox2*, and *Nanog*; cells expressing *Zscan4*; and endodermal-like cells located at the periphery of the colony. The capacity to form secondary colonies was confirmed for all eight tested ESC lines. Cells from the secondary colonies — after transfer to the standard ESC medium — retained pluripotency, judged by their strong alkaline phosphatase (ALP) staining, typical colony morphology, gene expression profile, stable karyotype, capacity to differentiate into all three germ layers in embryoid body formation assays, and successful contribution to chimeras after injection into blastocysts. Based on flow cytometry analysis (FACS), the proportion of *Zscan4*-positive cells in secondary colonies was higher than in standard ESC colonies, which may explain the capacity of ESCs to resist the differentiating effects of RA and instead form secondary colonies of undifferentiated ESCs. This hypothesis is supported by cell-lineage tracing analysis, which showed that most cells in the secondary colonies were descendants of cells transiently expressing *Zscan4*.

Keywords

retinoic acid; *Zscan4*; mouse embryonic stem cells; pluripotency

Introduction

Mouse embryonic stem cells (ESCs) are derived from the inner cell mass (ICM) of blastocysts and retain their pluripotent state in culture, with the capacity to differentiate into derivatives of all three embryonic germ layers in vitro and to contribute to chimeras after

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injection into blastocysts (Smith et al. 1988, Williams et al. 1988). ESCs readily differentiate after either removal of the leukemia inhibitory factor (LIF) or treatment with differentiation-inducing factors such as retinoic acid (RA), which induces differentiation of ESCs towards neural, endoderm, and mesoderm lineages (Colleoni et al. 2011, Edwards and McBurney 1983, Johannesson et al. 2009, Rohwedel et al. 1999).

Our previous studies further showed that the RA treatment of mouse ESCs resulted in six-fold upregulation of *Zscan4* (a.k.a., *Gm397*) on the third day in RA conditions (Sharova et al. 2007). *Zscan4* was originally identified for its specific expression in two-cell mouse embryos and in 1–5% of undifferentiated ESCs (Falco et al. 2007). It has also been shown that *Zscan4* is a marker of undifferentiated ESCs and plays critical roles for telomere elongation and genome stability of ESCs (Amano et al. 2013, Zalzman et al. 2010). Therefore, its upregulation in RA conditions seemed contrary to the well-known differentiation-inducing effect of RA. The existence of alternative actions of RA is supported by findings that the pulse exposure to RA counteracts differentiation of ESCs even in the absence of LIF (Wang et al. 2008). Also, RA was shown to induce the development of pluripotent primordial germ cells (PGCs) and male gametes in 3D culture (Geijsen et al. 2004, Kerkis et al. 2007).

In this paper, we explore the balance of differentiating and counter-differentiating effects of RA in mouse ESCs and show that pluripotent colonies emerge in ESC cultures despite the continuous presence of RA. Besides standard pluripotent cells expressing *Nanog* and *Pou5f1*, these colonies (named secondary colonies) include an unusually high proportion of cells expressing *Zscan4*, as well as some endodermal-like cells on the periphery. Cell-lineage tracing analysis shows that most cells in the secondary colonies are descendents of cells transiently expressing *Zscan4*. We hypothesize that refraction of some ESCs to the differentiation-inducing effects of RA and to form secondary colonies could be related to the action of *Zscan4*.

Materials and Methods

Original MC1 (129.3) and MC2 (C57BL/6) ESC lines were obtained from the expanded frozen stock (vials with passages 5 and 3, respectively) at Johns Hopkins University, where they were derived; line dsRed was provided by Dr. Soriano. These cells were then expanded at NIA, frozen and later used to derive *Zscan4*-related clones: MC1-ZE-3 (Falco et al. 2007), MC2-ZE-18, and ZE-dsRed with Emerald reporter under control of *Zscan4* promoter stably transfected in MC1, MC2, and dsRed ESCs, correspondingly. ESC line R1-Oct4-GFP with GFP-*Oct4* reporter was provided by Dr. A. Nagy; C57BL/6 v26.2 was purchased from Open Biosystems (cat. #MES1393, Pittsburgh, PA); ES-D3-GL was purchased from ATCC (cat. #SCRC-1003, Manassas, VA); iPS-Ng-20D was provided by Dr. S. Yamanaka; and TGC 8-8 was provided by Dr. Hogan. *Zscan4*-cre-ERT2 was developed at NIA for lineage tracing of cells expressing *Zscan4* (Zalzman et al. 2010). Standard ESC culture was done as described (Sharova et al. 2007). To generate secondary colonies, ESCs were seeded in six-well plates at clonal density $10^2/\text{cm}^2$ in standard ESC medium (DMEM, 15% FBS; 1000 u/ml LIF, ESGRO; 1mM sodium pyruvate; 0.1 mM NEAA, 2 mM glutamate, 0.1 mM beta-mercapto ethanol, and 50U/50µg per ml penicillin/streptomycin) supplemented with 50nM all trans

retinoic acid (RA, Sigma, cat# R2625, St. Louis, MO) and cultured for 7 d with daily medium change. ALP staining was performed with 85R Sigma kit according to manufacturer protocol for microscopy. For flow cytometry analysis (FACS) of Emerald(+) cells, MC1-ZE-3 and MC2-ZE-18 cells were harvested and analyzed by Guava easyCyteTM Mini System flow cytometer (Guava Technologies, Millipore, Billerica, MA).

For global gene expression profiling with microarrays, individual secondary colonies were manually picked on day 7, RNA extracted and processed as described (Sharova et al. 2007). For comparison, we used ESCs in standard culture conditions as well as cells derived from secondary colonies and then cultured 10 passages in standard conditions. Cy3-CTP-labeled sample targets (in two biological replications) were prepared with total RNA by Low RNA Input Fluorescent Linear Amplification Kit (Agilent, Santa Clara, CA) and hybridized to NIA Mouse 44K Microarray v3.0 (Agilent, design ID 015087) (Carter et al. 2005) together with Cy5-CTP labeled reference target, which was produced from mixture of Stratagene Universal Mouse Reference RNA and RNA from MC1 cells. For statistical analysis, we used NIA Array Analysis, which estimates the False Discovery Rate (FDR) to account for multiple hypothesis testing (Sharov et al. 2005). Microarray data are submitted to GEO/NCBI database, accession number GSE40495.

To visualize the expression of *Pou5f1* and *Nanog* in relation to *Zscan4*-positive [*Zscan4*(+)] cells, MC1-ZE-3 ESCs were cultured in the presence of 50 nM RA for several days, fixed in 4% PFA and stained with POU5F1 and NANOG antibodies. For tracking *Pou5f1* and *Nanog* expression in ESCs in the lineage of cells originated from *Zscan4*(+) cells, *Zscan4*-cre-ERT2 ESCs were cultured in the medium supplemented with RA and tamoxifen for 3 d and then in the RA medium without tamoxifen for four more days. ESCs were fixed on day 7 in 4% PFA and stained with lacZ, *Pou5f1* and *Nanog* antibodies. Cre-recombinase in *Zscan4*-cre-ERT2 ESCs is under the control of the *Zscan4* promoter. It translocates from the cytoplasm into the nucleus only in the presence of tamoxifen and excises a neomycin cassette from the LacZ ORF (Soriano 1999), leading to heritable constitutive lacZ expression in all cells that expressed *Zscan4* at the time of exposure to tamoxifen. *Zscan4*-cre-ERT2 cells were fixed 4 min in tissue fixative (Millipore, Cat. #BG-5-C), washed with PBS and incubated 2 h at 37°C with X-Gal substrate (X-Gal Stock Solution, Millipore, Cat. #BG-3-G) diluted 1:40 in Tissue Stain Base Solution (Millipore, Cat. #BG-8-C). Immunohistochemistry was performed as described (Zalzman et al. 2010). Antibodies used: *Oct3/4* – SantaCruz, Dallas, TX, mouse, 1:250 (sc5279), secondary – donkey-antiMouse 568(A10037, 1:800); *Nanog* – BD Biosciences, East Rutherford, NJ, mouse, 1:500 (560259), secondary – same as for *Oct3/4*; *Gata4* – SantaCruz, goat, 1:1000 (sc1237), secondary – donkey-antiGoat 647(A21447, 1:400); β -gal – Abcam, Cambridge, MA, chicken, 1:500 or 1:1000 (ab9361), secondary – goat-antiChicken 488(A11039, 1:400); *Zscan4* – GenScript, Piscataway, NJ, rabbit; 1:2000 (antiserum made for sequence CSTYHRHLRNYHRSD), secondary – donkey-antiRabbit 568 (A10042, 1:800). For flow cytometry analysis (FACS), MC1-ZE-3 cells were fixed in BD Cytofix/CytopermTM solution (BD Biosciences, cat #554722), permeabilized with BD Perm/Wash buffer (BD Biosciences, cat #554723), stained with specific antibodies (see above) and analyzed by Guava EasyCyte Mini System flow cytometer (Guava Technologies, Millipore).

For EB assay, ESCs were harvested on day 3 of culture, resuspended in EB medium (DMEM, 10% FBS; 1mM sodium pyruvate; 0.1 mM NEAA, 2 mM glutamate, 0.1 mM beta-mercapto ethanol, and 50U/50µg per ml penicillin/streptomycin) and plated in AggreWell™ 400 (Stemcell Technologies, Vancouver, BC), 1.2×10^6 cells/well to produce EB with ~1000 cells. On day 3 formed EB were transferred in Corning® Ultra-low attachment culture dishes (cat #3262, Corning, NY) and cultured for an additional four days. On day 7, floating EB were transferred to cell culture treated plates for attachment. The beating muscles and other differentiation types were scored on day 3 of attached EB culture. To assess the capacity to contribute to chimeras, ESCs were injected into blastocysts obtained from CD1 females (Charles River, Wilmington, MA, 8–12 wk old) which were superovulated by PMSG followed by hCG (Sigma) administration and mated with CD1 males. Two-cell embryos were collected by flushing oviducts using M2 medium (Millipore) and were cultured in KSOM (Millipore) drop for 3 d until they reached blastocyst stage at 37°C in 5% CO₂. After cell injection, blastocysts were transferred to the uterus of recipient mice. Chimeras were evaluated by their coat color contribution. For karyotyping, ESCs were cultured in the standard medium with 30 ng/ml of colcemid (Gibco, Carlsbad, CA, #15212-012) for 4 h, treated with 0.55% KCl for 8 min at 37°C, and fixed in glacial acetic acid methanol (2:5). Prepared slides were stained with Giemsa (Gibco #10092-013) and analyzed under x100 magnification using Axiovert 200 (Zeiss, Oberkochen, Germany). Telomere length was estimated by qPCR as described earlier (Callicott and Womack 2006).

Results

Undifferentiated secondary colonies of ESCs emerged during retinoic acid supplementation

To explore the balance of opposite effects of RA – induction of differentiation and upregulation of undifferentiation-specific *Zscan4* – on mouse ESCs, we first used cell line MC1-ZE-3 (Falco et al. 2007) containing an Emerald-*Zscan4* reporter (*Zscan4* promoter followed by Emerald ORF) to simplify the visualization of *Zscan4*-positive [*Zscan4*(+)] cells. Cells were plated at clonal density (at which growing colonies do not tend to coalesce with each other), and were then cultured in standard ESC medium supplemented with a low concentration of RA (50 nM, which is two-fold higher than the physiological plasma level) (Tzimas et al. 1996). We avoided higher concentrations of the RA, because they caused irreversible differentiation of ESCs. Initially ESCs showed signs of differentiation, such as flattening of colonies and reduction of both ALP staining and immunostaining for pluripotency-related transcription factors *Pou5f1* and *Nanog* (Supplementary Figure S1A). However, by day 6–7, dome-shaped colonies similar to those in undifferentiated ESCs began to emerge among the seemingly differentiated cells (Fig. 1, Supplementary Figure S1A, B). These colonies were termed “secondary colonies” to distinguish them from untreated standard ESC colonies. The secondary colonies included many *Zscan4*(+) cells, detected by activation of the Emerald-*Zscan4* reporter (green cells in Fig. 1). The majority of secondary colonies (~70%) also showed high expression of ALP, *Pou5f1*, and *Nanog*, comparable to control cells cultured without RA (Supplementary Figure S1A, B).

When ESCs were cultured in medium with the same low concentration of RA (50 nM) but in the absence of LIF, no dome-shaped secondary colonies appeared within 8 d of culture, although a few groups of ALP-positive cells were present (Supplementary Figure S2A). The expression of pluripotency marker *Pou5f1* also declined in these cells (not shown). This indicated that LIF was necessary for the formation of secondary colonies. Cells cultured in RA and in the absence of LIF had no self-renewal capacity, as they differentiated completely after replating in standard culture medium (Supplementary Figure S2B). Secondary colonies were also not observed when ESC cultures were plated at high density, even in the presence of LIF.

To test whether secondary colonies can form in other pluripotent cell lines, we used the same culturing method (i.e., clonal density, 50 nM RA, and LIF) for eight other pluripotent cell lines: six ESC lines (C57BL/6 V26.2, R1-Oct4-GFP, ES-D3-GL, MC2-ZE-18, ZE-dsRed, MC2); one embryonic germ cell (EGC) line (TGC 8-8); and one induced pluripotent stem cell (iPSC) line (iPS-Ng-20D). All cell lines tested formed secondary colonies by day 7. Strong ALP-staining is shown for cell lines C57BL/6 V26.2 and iPS-Ng-20D (Supplementary Figure S3A, B). In addition, R1-Oct4-GFP, which bears a GFP reporter under the control of the *Pou5f1* promoter, showed a high expression of this pluripotency-related transcription factor, judged by the presence of GFP fluorescence (Supplementary Figure S3C). Expression of *Zscan4* in the secondary colonies was confirmed in two cell lines with Emerald reporter under control of the *Zscan4* promoter: MC2-ZE-18, ZE-dsRed (Supplementary Figure S3E, F).

To further test self-renewal capacity of cells derived from the secondary colonies, we replated them 10 times in the standard conditions (i.e., standard plating density, standard ES culture medium, 3 d in culture). After replating, these cells formed standard dome-shaped colonies with strong ALP-staining (Supplementary Figure S4). Secondary colonies can also be replated in RA conditions for at least three passages. However, continuous passaging of cells in RA conditions was not sustainable, as the proportion of differentiating cells increased progressively.

Secondary colonies included three sub-populations of cells: standard ESCs, *Zscan4*-positive cells, and endodermal-like cells

To explore molecular mechanisms involved in the formation of the secondary colonies, we used microarrays to measure gene expression in manually picked secondary colonies compared to standard ESCs. Expression of pluripotency-related genes in the secondary colonies could be either unchanged (e.g., *Sox2*, *Esrrb*, *Zfp42*); upregulated (e.g., *Nanog*, *Klf4*, *Tbx3*); or downregulated (e.g., *Pou5f1*, *Eed*, *Phc1*, *Myc*, *Fgf4*, *Lefty1*, *Nr0b1*, and *Dnmt3b*) compared to standard ESCs (Fig. 2A).

Although *Pou5f1* was downregulated ~2-fold in secondary colonies, its expression level still appeared sufficient to prevent the differentiation of cells. As expected, *Zscan4* expression in secondary colonies was increased — by eight-fold compared to standard ESCs (Fig. 2B). Other genes that are known to be co-regulated with *Zscan4* (*Arg2*, *Dub2*, *Eif1a*, *Gm428*, *Gm5*, *Lmx1a*, *Pramel6*, *Tcstv1*, *Tcstv3*, *Tdpz4*, *Tmem92*, and *Zfp352*) (Amano et al. 2013, Macfarlan et al. 2012), also were increased in expression in the secondary colonies by 3- to

12-fold compared to standard ESCs (Fig. 2B). The secondary colonies also showed high expression of endoderm- and endothelial-related genes (e.g., *Gata6*, *Gata4*, *Sox7*, *Sox17*, *Foxa2*, *Sox7*, *Sox17*, *Pdgfra*, *Isx*, and *Dkk1*) (Fig. 2C), consistent with published reports on endoderm induction by RA (Johannesson et al. 2009). By contrast, genes associated with extra-embryonic lineages (*Esx1*, *Plac1*, *Cited1*, *Cited2*, and *Hand1*) were only slightly overexpressed in the secondary colonies. The gene expression changes were all reversed after passaging of cells derived from secondary colonies in standard culture condition (Fig. 2B, C purple bars).

Because expression of genes associated with the standard ESCs, *Zscan4*(+) cells, and cells differentiated from ESCs are known to be incompatible (Hamazaki et al. 2004, Macfarlan et al. 2012, Singh et al. 2007), we used immunocytochemistry to assess whether the secondary colonies were heterogeneous. Staining for *Nanog* (antibody, marker of standard ESCs), *Zscan4* (Emerald reporter), and *Gata4* (antibody, endoderm marker), showed that these three genes were indeed expressed in different subpopulations of cells (Fig. 2D–G). Early secondary colonies (day 3) showed a clear separation of *Zscan4*(+) cells (Emerald reporter, MC1-ZE-3 cell line) and *Pou5f1*(+) and *Nanog*(+) cells (Fig. 2H,I), in line with the characteristic findings that *Zscan4*(+) cells do not produce the POU5F1 or NANOG protein (Macfarlan et al. 2012).

FACS experiments with cells comprising secondary colonies stained for both *Zscan4* and *Gata4* confirmed that different subpopulations of cells express these genes, apart from a small proportion of cells (1.2–2.5%) that showed double staining. Cells with *Gata4* expression (ca. 7%) were located mostly at the edges of colonies (Fig. 2F), similar to the pattern of cell segregation in ESCs (Niakan et al. 2010), whereas pluripotent cells expressing *Nanog* and *Pou5f1* were located closer to the center of colonies (Fig. 2D, H, I). Cells with *Zscan4* expression were scattered in a colony, although more cells seemed near the edges (Fig. 2E). Thus, secondary colonies included three subpopulations of cells: standard ESCs, *Zscan4*(+) cells, and endodermal-like cells.

Secondary colony-derived ESCs were pluripotent

The results thus far suggested that the secondary colonies that emerged from the seemingly differentiated cells contain pluripotent ESCs. To test this notion, we manually picked the secondary colonies with a micropipette and cultured them in the standard condition for 10 passages without RA. Morphologically, these ESCs became more like the standard ESCs. We then carried out in vitro cell differentiation assays. ESCs derived from the secondary colonies formed normal embryoid bodies in LIF(–) conditions and differentiated into derivatives of endoderm, ectoderm (neurons), and mesoderm (muscles, blood vessels) (Supplementary Figure S5). We observed no bias in the direction of differentiation compared to standard ESCs. Thus, although cells were exposed to the low dose of RA during the formation of the secondary colonies and showed a reduction of pluripotency markers *Pou5f1* and *Nanog*, they regained their pluripotent phenotype after the removal of RA.

To further examine pluripotency in vivo, we injected the ESCs, derived from the secondary colonies and cultured for 10 passages in standard medium, into blastocysts and observed chimeras in the progeny from three tested cell lines: MC1-ZE-3, ES-D3-GL, and iPS-

Ng-20D (Fig. 3A). Chimeras were formed in all three lines, which confirmed the pluripotent state of cells passaged through secondary colonies. In one cell line (MC1-ZE-3), chimerism was significantly higher compared to control ESCs (i.e., parental ESCs that underwent the same number of passages) (88.1% vs. 38.3%; $\chi^2 = 31.7$ $p = 2 \times 10^{-8}$) (Fig. 3A). However, in ES-D3-GL and iPS-Ng-20D cell lines, any change of chimerism was not significant ($\chi^2 = 0.09$, $p = 0.76$; $\chi^2 = 0.43$, $p = 0.51$, respectively). Known factors affecting the contribution of cells to chimeras include the length of telomeres and karyotype, both of which tend to deteriorate in ESCs after ca. 20 passages (Longo et al. 1997, Rebuzzini et al. 2008). Telomere length measured by qPCR appeared unchanged between MC1-ZE-3 cells derived from the secondary colonies and control ESCs (Fig. 3B), whereas the proportion of cells with normal karyotype was higher in MC1-ZE-3 cells exposed to RA than in control cells ($\chi^2 = 6.248$; $p = 0.0124$) (Fig. 3C). These results may explain their increased contribution to chimeras. In contrast, no difference in karyotype stability was observed in ZE-dsRed and ES-D3-GL cell lines.

Capacity of secondary colonies to resist the differentiation effects of RA is likely associated with the increased proportion of *Zscan4*-positive cells

The most unusual feature of secondary colonies is the upregulation of *Zscan4*, which is inferred from the intensive Emerald staining of secondary colonies formed by cell lines with Emerald-*Zscan4* reporter (MC1-ZE-3, MC2-ZE-18, and ZE-dsRed, Fig. 1, Supplementary Figure S3) and from the results of microarray analysis (Fig. 2B). This motivated the study of whether the expression of *Zscan4* was related to the capacity of secondary colonies to resist the differentiation effects of RA. First, we used FACS to check if the proportion of *Zscan4*(+) cells in secondary colonies was greater than in standard ESC culture (because this information cannot be inferred from microarrays or images of colonies). The proportion of *Zscan4*(+) (i.e., Emerald-positive) cells from line MC1-ZE-3 measured by FACS (Guava EasyCyte flow cytometer) was ~2-fold higher in RA conditions (i.e., in secondary colonies) than in controls without RA addition (Supplementary Figure S6A). Similar experiments with MC2-ZE-18 cells showed a >10-fold difference in *Zscan4*(+) cells between RA and control conditions (Supplementary Figure S6B). Stronger response of MC2-ZE-18 cells to RA treatment may result from a lower initial level of *Zscan4* expression in this cell line. In both cell lines, the RA treatment resulted in the increase of the proportion of *Zscan4*(+) cells to 15–20% as compared to 1–5% in standard ESC cultures.

Although the proportion of *Zscan4*(+) cells has increased in secondary colonies, it did not approach 100%, and it is not clear how *Zscan4*(–) cells remained pluripotent. To analyze their individual capacity, we used lineage tracing to show that nearly all pluripotent cells without *Zscan4* expression originated from cells that had transient expression of *Zscan4* earlier. We used *Zscan4*-CreERT2 cells (Zalzman et al. 2010), which become permanently lacZ-positive after they express *Zscan4* during treatment with tamoxifen. When tamoxifen was added to the medium with RA, the majority of cells in the secondary colonies showed strong lacZ staining, indicating that these cells were *Zscan4*(+) at some point during the tamoxifen/RA treatment (Supplementary Figure S6C).

To test whether *Zscan4*(+) cells later regained the expression of pluripotency-related transcription factors *Pou5f1* and *Nanog*, we combined staining for lacZ with immunostaining for the transcription factors. In mature secondary colonies (on day 7), *Pou5f1* and *Nanog* expression in *Zscan4*-cre-ERT2 ESCs widely overlapped with lacZ staining, which marked the cells that were *Zscan4*(+) 1–3 d earlier (Fig. 2J, K). This indicated reactivation of POU5F1 and NANOG proteins several cell cycles after *Zscan4* was expressed, and confirmed that transiently *Zscan4*(+) cells remained pluripotent. When the secondary colonies were transferred to standard ESC medium, cells appeared mostly lacZ-positive (Supplementary Figure S6D), indicating that these cells are indeed descendants of *Zscan4*(+) cells in the secondary colonies.

Discussion

The major result of this study is that a low-dose (50 nM) of RA added to the standard medium did not cause total differentiation of mouse ESCs within 7 d. Instead, undifferentiated colonies (which we call “secondary”) appeared and grew, surrounded by differentiated cells. These colonies had typical undifferentiated morphology, ALP staining, and expressed pluripotency-related transcription factors. Formation of secondary colonies required both RA and LIF in the medium. In this respect our findings differ from a previous report that pulse-treatment with RA can support self-renewal of ESCs in medium without LIF (Wang et al. 2008). Cells derived from the secondary colonies had normal capacity for differentiation and showed a high contribution to chimeras after injection into blastocysts. The proportion of chimeras was not reduced compared to control cells of the same passage number and, in one cell line, it was even statistically higher than controls.

The peculiar feature of secondary colonies was the increased proportion of *Zscan4*(+) cells (15–20% compared to 1–5% in ESCs). *Zscan4* is expressed in early preimplantation embryos, with a maximum at the late two-cell stage (Falco et al. 2007). In ESCs, it is co-expressed with several hundred other genes, the majority of which are also expressed in two-cell embryos (Akiyama et al. 2015, Amano et al. 2013, Macfarlan et al. 2012). *Zscan4*(+) cells show transient derepression of heterochromatin, which becomes actively transcribed (Akiyama et al. 2015). This can rationalize the upregulation of certain transposable elements (Macfarlan et al. 2012) and expression of the group of genes typical for the *Zscan4*(+) cells, because the majority of them (including *Zscan4* itself) are ordinarily located in heterochromatin. Our previous experiments with induction and knockdown of *Zscan4* showed that the gene is required to support the expression of many other genes typical of the *Zscan4*(+) cells (Nishiyama et al. 2012, Nishiyama et al. 2009). Thus, *Zscan4* seems to play a key role in the activation of coregulated genes in heterochromatin.

The cell-lineage tracing experiments clearly showed that all pluripotent cells in secondary colonies once were *Zscan4*(+) during RA treatment. Thus, we think that *Zscan4*-related cell changes (e.g., derepression of heterochromatin and elongation of telomeres) may contribute to the resistance of secondary colonies to the differentiation-inducing effects of RA.

Regarding the increase of *Zscan4*(+) cells by RA, it is worth mentioning a possible involvement of *Nr0b1*. It has been shown that *Nr0b1* is downregulated in RA conditions

(Hosler et al. 1993) (see also Fig. 2C), and the downregulation of *Nr0b1* results in a substantial increase of *Zscan4c* expression as well as in the upregulation of endoderm-related genes in ESCs (Fujii et al. 2015). Thus, *Nr0b1*($-/-$) cells described in the previous report (Fujii et al. 2015) are possibly similar to the secondary colonies described in our study. However, it is not clear whether this is the only or the main mechanism that increases the proportion of *Zscan4*(+) cells in secondary colonies. For example, in our previous study, the knockdown of *Nr0b1* via shRNA (90–95% reduction after 72 hr) did not result in the increase of *Zscan4* expression (Nishiyama et al. 2012). Interestingly, the knockdown of *Nr5a2* – an upstream gene of *Nr0b1*, did cause an increase in *Zscan4* expression (Nishiyama et al. 2012), which seems to be consistent with experiments of Fujii et al.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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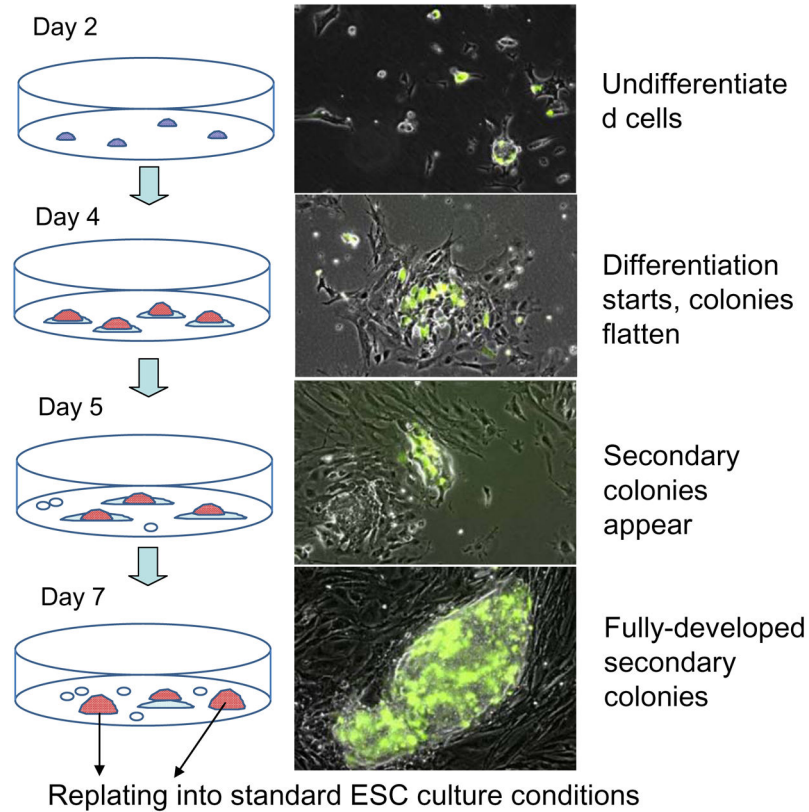
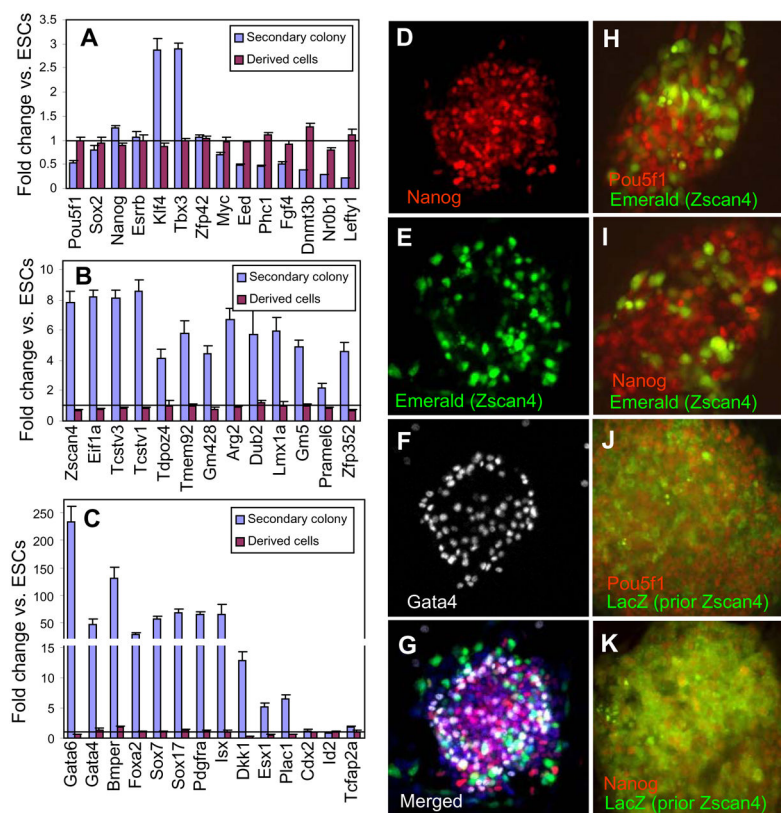
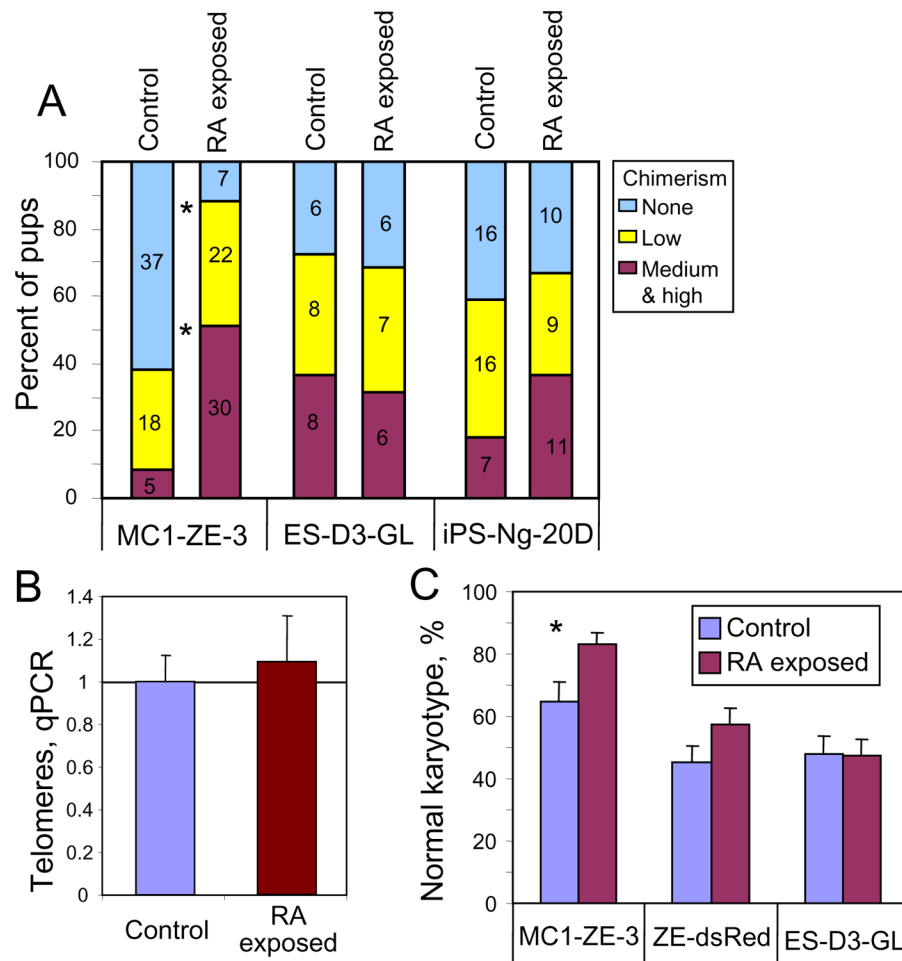


Figure 1.

Emergence of undifferentiated ESC colonies in retinoic acid conditions. Left panel: the experiment design, red cells are ALP-positive. Right panel: MC1-ZE-3 cells with Emerald-*Zscan4* reporter (green) plated at clonal density show the emergence of secondary colonies.

**Figure 2.**

Expression of genes in secondary colonies. Expression of (A) pluripotency related genes, (B) genes associated with *Zscan4* expression in ESCs, and (C) differentiation-related genes in manually picked secondary colonies (MC1 cells in 50 nM retinoic acid on day 7) and in descendants of these cells cultured in standard ESC conditions for 10 passages (derived cells), based on microarray data; expression is normalized to undifferentiated standard ESCs (line = 1). Heterogeneity of gene expression: (D-G) expression of *Nanog* (immunostaining), *Zscan4* (Emerald reporter), and *Gata4* (immunostaining). Expression of *Zscan4* in early secondary colonies (day 3, Emerald reporter) (H, I) and cumulative expression in late secondary colonies (day 7, lacZ staining in *Zscan4*-CreERT2) (J, K) combined with immunostaining for pluripotency-related factors *Pou5f1* (H, J) and *Nanog* (I, K); lacZ staining in *Zscan4*-CreERT2 cells shows cells that previously expressed *Zscan4*.

**Figure 3.**

Contribution to chimeras and genome integrity of cells exposed to retinoic acid (RA) (i.e., from secondary colonies) and then cultured in standard conditions for 10 passages, as compared to control cells (i.e., parental ESCs that underwent the same number of passages). (A) Proportion of chimeric mice obtained from cell injection into blastocysts (number of pups shown); (B) telomere length assessed with qPCR; (C) karyotype of cells exposed to RA and in control cells; asterisk (*) indicates significant difference ($p < 0.05$).