Differentiation (2003) 71:135–141

ORIGINAL ARTICLE

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Developmental fate of embryonic germ cells (EGCs), *in vivo* and *in vitro*

Accepted in revised form: 21 October 2002

Abstract Embryonic germ cells (EGCs) derived from mouse primordial germ cells (PGCs) are known both to colonize all cell lineages of the fetus and to make tumors *in vivo*. When aggregated with eight-cell embryos, EGCs from a new EGC line expressing green fluorescent protein (GFP) were found to contribute preferentially to the epiblast but unexpectedly were also capable of colonizing primary endoderm. When injected under the kidney capsule, EGCs derived from 12.5 days *post coitum* (dpc) PGCs formed differentiated tumors. The ability of EGCs to differentiate in an organ culture system depends upon their partners in cell culture. When EGCs, marked with a LacZ transgene, were mixed with disaggregated and reaggregated mouse fetal lung in an organ culture system, they remained undifferentiated. In urogenital ridge reaggregates on the other hand, some EGCs were capable of differentiating to form small epithelial cysts.

Key words primordial germ cells · embryonic germ cells · chimera · green fluorescence protein · aggregates · differentiation

Introduction

The mouse germ cell lineage is established during gastrulation, in the extra-embryonic region posterior to the primitive streak (Lawson et al., 1999). Primordial germ cells (PGCs) then migrate along the hindgut and into the genital ridges, the site of future gonads. PGCs in the female genital ridge enter the prophase of meiosis from about 13.5 days *post coitum* (dpc). At the same time, PGCs in the male genital ridge undergo mitotic arrest. However, PGCs taken from the genital ridges of either female or male mouse embryos 11.5 dpc will enter the prophase of meiosis in a fetal lung reaggregate maintained in an organ culture system (McLaren and Southee, 1997).

Under appropriate culture conditions (Matsui et al., 1992; Resnick et al., 1992), pluripotent stem cell lines capable of indefinite proliferation *in vitro* can be derived from PGCs either at 8.0–8.5 dpc before migration (Matsui et al., 1992; Resnick et al., 1992; Stewart et al., 1994; Labosky et al., 1994), at 9.5 dpc during migration (Durcova-Hills et al., 2001) or at 11.5 or 12.5 dpc, after entry into the genital ridge (Matsui et al., 1992; Labosky et al., 1994; Tada et al., 1998). Termed embryonic germ cell (EGC) lines, these resemble embryonic stem cell (ESC) lines closely in the morphology of colonies and the expression of cell surface antigens (Matsui et al., 1992). The stage-specific embryonic antigen 1 (SSEA1), germ cell nuclear antigen 1 (GCNA1), non-tissue specific alkaline phosphatase (AP) activity, and Oct-3/4 are markers used to characterize their undifferentiated status (Matsui et al., 1992; Enders and May II, 1994; Durcova-Hills et al., 2001). EGCs derived from PGCs in the genital ridge have, however, been shown to differ from ESCs with respect to the methylation patterns of their DNA (Labosky et al., 1994; Tada et al., 1998), reflecting the erasure of genomic imprints from certain imprinted genes. EGCs reintroduced to the early embryo are capable, like ESCs, of extensive colonization of fetal cell

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lineages (Tada et al., 1998; Durcova-Hills et al., 2000; 2001) including the germ line (Labosky et al., 1994; Stewart et al., 1994; Tada et al., 1998). The differentiation potential of EGCs to give rise to a variety of cell types has been shown *in vitro* (Rohwedel et al., 1996; Ohta et al., 1999). EGCs have been derived in non-murine species (Pain et al., 1996; Shim et al., 1997; Park et al., 2000) including human (Shamblott et al., 1998). Recently, it has been demonstrated that the microenvironment is important for the self-renewal and cell fate decisions of stem cells (Heissig et al., 2002). Here we investigate whether a microenvironment made from either lung or genital ridge cells can support the proliferation and/or differentiation of EGCs.

The developmental potential of mouse ESCs in chimeras has been extensively studied, using both biochemical markers such as glucose phosphate isomerase (GPI) (Beddington and Robertson, 1989) and also cell markers such as LacZ (Lallemand and Brûlet, 1990). More recently, it has been shown that a modified form of the Green Fluorescent Protein (MmGFP) could be used to trace the fate of such GFP-expressing ESCs in living pre- and post-implantation embryos (Zernicka-Goetz et al., 1997; Hadjantonakis et al., 1998). Mouse EGCs have been less extensively studied. We have therefore made a number of new EGC lines from gonadal (11.5 and 12.5 dpc) germ cells carrying LacZ or GFP. To confirm and extend previous observations on the developmental fate of EGCs reintroduced into early embryos, we have used an EGC line expressing MmGFP so that we could examine the resulting chimeras by confocal microscopy. To study the fate of EGCs in organ culture, we have co-cultured EGCs marked with GFP or LacZ with either embryonic lung cells or genital ridge cells. We have also injected EGCs under the kidney capsule, to form teratomas containing differentiated tissues.

Methods

Mice

An MmGFP-expressing EGC line was derived from transgenic embryos obtained after mating between F1 (CBA/H × C57BL/10) females and transgenic F1 males expressing MmGFP from a ubiquitous EF1 alpha promoter. EGCs (11.5 and 12.5) marked with LacZ were derived from embryos obtained after mating MF1 females with ROSA 26 males carrying a LacZ transgene. 12.5 dpc 129 SvEv EGCs were derived from male embryos obtained after mating 129 SvEv females with 129 SvEv males. For the chimera experiments eight-cell embryos were recovered from F1 females, which were also used as foster mothers. 129 SvEv males were used for the teratoma studies. Lung tissue and genital ridges were derived from fetuses of randomly bred MF1 strain mice. The morning the vaginal plug was observed was considered as 0.5 days *post coitum* (dpc).

Antibodies

Anti-SSEA1 (TG1, anti-mouse IgM) and anti-mouse vasa homolog (MVH, anti-rabbit IgG) were a kind gift from Dr. Peter Beverly and Dr. Toshiaki Noce, respectively. Anti-Oct-4 (anti-mouse IgG) antibody was purchased from BD Transduction Laboratories. The appropriate secondary antibodies conjugated either with FITC or Texas Red were purchased from Santa Cruz Biotechnology Inc.

Cell sorting and derivation of EGC lines

An MmGFP-expressing EGC line, called 11.5 EGCs-GFP4, was derived and maintained as described by Durcova-Hills et al. (2001) with minor modifications. Briefly, genital ridges were isolated from MmGFP transgenic fetuses at 11.5 dpc. A cell suspension, enriched for PGCs by the immunomagnetic cell sorting method (Pesce and De Felici, 1995), was seeded onto mitotically arrested STO feeder cells in DMEM culture medium (Dulbecco's Modified Eagle medium, Gibco) containing 20% FCS (fetal calf serum, Sigma), 0.1 mM 2-mercaptoethanol, 2 mM L-glutamine, 0.5mM pyruvate, penicillin/streptomycin, and supplemented with LIF (leukaemia inhibitory factor) and bFGF (basic fibroblast growth factor, human recombinant, Gibco). After 10 days of culture, colonies with EGC-like morphology were transferred onto fresh feeders in culture medium supplemented only with LIF. Some colonies were observed under a confocal microscope (Biorad 1024 attached to a Nikon Eclipse 800 microscope) for MmGFP expression in primary culture and stained for alkaline phosphatase (AP) activity.

LacZ-expressing EGCs for the aggregates were derived from either male or female ROSA 26 embryos carrying a LacZ transgene (11. 5 or 12.5 dpc). For injections under the kidney capsule, a new 129 SvEv EGC line was prepared from 12.5 dpc male PGCs.

Characterisation of EGCs

Alkaline phosphatase (AP) activity, SSEA1 staining, and sexing were performed as described by Durcova-Hills et al. (2001).

Chimeras

MmGFP-expressing EGCs, used between passages 6 and 15, were selected under an inverted fluorescence microscope (Nikon). Chimeras obtained by aggregating 8–16 cell zona-free embryos and 10–15 MmGFP-expressing EGCs were cultured in KSOM (Speciality Media, Inc. Lavaltte, N.J.) supplemented with BSA (4 mg/ml, Sigma) at 37°C under paraffin oil for 24 hours. Chimeric blastocysts, expressing MmGFP, were transferred into recipient females (2.5 dpc). Embryos were recovered at 6.5 or 8.5 dpc, counting noon of the plug day of the pseudopregnant recipient as 0.5 dpc. They were positioned between two coverslips, observed by confocal microscopy, and scanned with 30–100% laser power to determine the localization of MmGFP-expressing EGCs.

In vitro differentiation

Lung aggregates were prepared by trypsinizing one half to two-thirds of a 13.5 dpc MF1 lung, adding a known number of EGCs (10^3, 10^5, 10^6), mixing by pipetting, then centrifuging to form a pellet and culturing for 4–6 days on agar blocks as described by McLaren and Southee (1997). The lung cells in the aggregates were derived from both epithelial and mesodermal tissue.

Urogenital ridge aggregates were prepared and cultured in the same way, using 4–6 ridges per aggregate, from 11.5 or 12.5 dpc fetuses. No difference was detected between aggregates made with male and female EGCs, so the results have been combined. In some experiments, the genital ridge and the mesonephric region were aggregated separately. At the end of the culture period, the pellets were fixed, processed for β-galactosidase activity, sectioned, and stained with hematoxylin. The 11.5 dpc fetuses were sexed by staining amnion cells for sex chromatin (Palmer and Burgoyne, 1991).

Aggregates for immunofluorescence staining with anti-SSEA 1,

anti-Oct-4, anti-MVH, and anti-α fetoprotein were fixed with 4% paraformaldehyde, permeabilised with 0.1% Triton X-100, and incubated with appropriate primary antibodies overnight at 4°C. After washing, aggregates were incubated with appropriate secondary antibodies overnight at 4°C, washed with PBS, and counterstained with TOTO-3 (Molecular Probe). Samples were mounted and observed by confocal microscopy.

Teratomas

12.5 dpc PGCs (129×129) were cultured for 23 days. Intact EGC colonies recovered after 2 sub-cultures were injected under a kidney capsule of 129 SvEv males. After 6 weeks, teratomas were recovered, weighed, fixed in 4% paraformaldehyde in PBS, embedded in paraffin, and sectioned. Hematoxilin and eosin staining was used to observe different type of tissues. Van Giesen and alcian blue staining were used to identify connective tissue.

Immunohistochemistry

After deparaffinizing, the teratoma sections were blocked in 10% goat serum with BSA in PBS for 1 hour at room temperature and then incubated with GCNA1 antibody (1:1) at 4°C overnight. After three washes with 1% goat serum with BSA in PBS, sections were incubated with horseradish peroxidase (HPR)-conjugated anti rat-IgG (1:100, Jackson Laboratories) for 1 hour at room temperature and washed three times with PBS. The sections were stained with 3,3-diaminobenzidine (DAB, Sigma) for 5 minutes at room temperature, lightly counter-stained with hematoxilin and mounted in DPX.

Results

EGCs can contribute to embryonic and extraembryonic lineages when aggregated with 8–16 cell embryos

To enable us to follow the fate of EGCs in living embryos, we used our new male EGC line that expresses MmGFP. The undifferentiated status of these EGCs was confirmed by detection of alkaline phosphatase activity and expression of SSEA 1 and GCNA 1 antigens (data not shown).

In five separate experiments, we produced chimeras by aggregating MmGFP-expressing EGCs with 8–16 cell embryos. MmGFP expression was variable in the EGC line, with some strongly expressing, some weakly expressing, and some negative cells (Fig. 1A). For the aggregations, colonies with strongly expressing cells were used. We followed the marked cells during preimplantation and then postimplantation development. When embryos after aggregation with MmEGCs were cultured until the blastocyst stage, we found that the green EGCs colonized both inner cell mass and trophectoderm. Some chimeric blastocysts were transplanted to the uteri of pseudopregnant females and 45% of the transferred embryos implanted (Table 1). We first examined the localization of the EGCs in gastrulating embryos at 6.5 dpc. In 89% of the chimeras, the green EGCs showed an extensive colonization of the epiblast (Table 1, Fig. 1C). An unexpected finding, however, was that EGCs were also able to contribute to the extraembryonic lineages in 20% of chimeric embryos. Of those, 87.5% contained green cells in the extra-embryonic endoderm rather than in the extra-embryonic mesoderm (Fig. 1D). Normal development was confirmed when chimeric embryos developed to the early somite stages (8.5 dpc), to term, and subsequently into healthy fertile adult mice (Table 1). Male chimeras were mated with F1 females. We found green cells in different tissues of the progeny, including liver, heart, ovary, and testis, demonstrating that EGCs were able to colonize the germ line in the host embryos (data not shown).

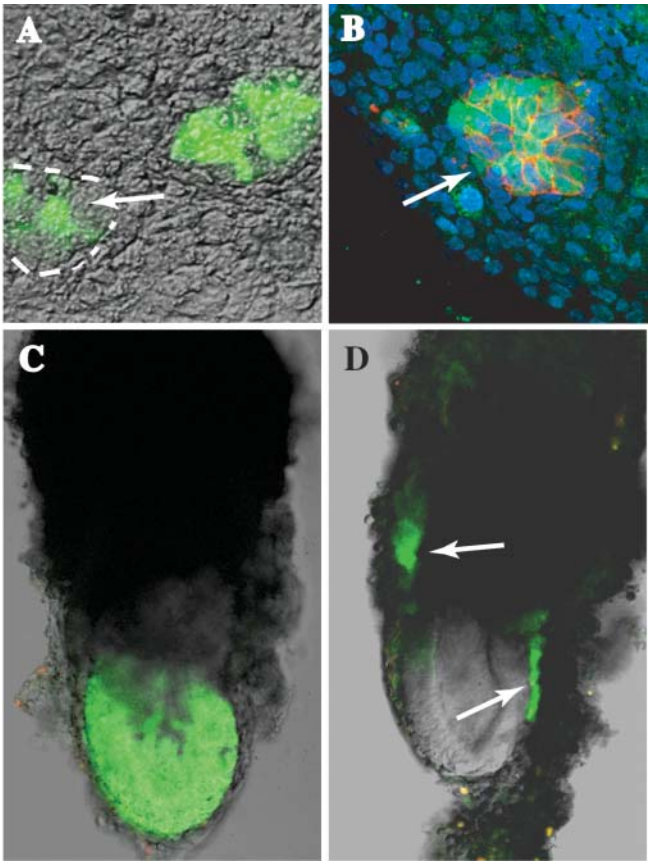

Fig. 1 Developmental potential of EGCs marked with GFP *in vitro* and *in vivo*. Undifferentiated MmGFP-EGCs showed variable expression of GFP. (**A**) In one of the colonies shown (surrounded by *dotted line*), one of the EGCs (*white arrow*) is negative for GFP. The background cells are feeder cells. (**B**) MmGFP-EGCs co-cultured with lung cells formed a large colony (*arrow*). The undifferentiated status of the colony (*green*) was confirmed by SSEA1 staining (*red*). MmGFP-EGCs were aggregated with morulae and transferred to the recipients. Embryos were recovered at 6.5 dpc. Chimeric embryos were identified by the presence of GFP when viewed under a confocal microscope. (**C**) Representative 6.5 dpc chimeric embryo showing colonization by green EGCs in the epiblast. (**D**) EGC descendants identified in the extra-embryonic endoderm (*arrows*). Fluorescent and transmitted light images have been combined. Original magnification A and B ×200, C and D ×100.

Table 1 Contribution of EGCs marked with GFP to the development of chimeric embryos

No. of embryos implanted (% of embryos transferred)	Stage of recovery	No. of chimeric embryos	Embryonic contribution	Extra-embryonic contribution
103/227 (45%)	6.5 dpc	46/95 (48%)	41/46 (89%)	9*/46 (20%)
	8.5 dpc	1/4 (25%)	1/1 (100%)	
	New-borns	2/9 (22%)	Tail/coat colour	N.D.

* 4 contributed to both lineages; N.D. – not determined

EGCs can give rise to teratomas containing many different cell types

EGCs are capable of forming tumors after subcutaneous injection into nude mice (Matsui et al., 1992). We tested the ability of newly derived 12.5 dpc male EGC lines to make tumors. We injected between 5 to 7 intact colonies under a kidney capsule of six adult 129 males. After 6 weeks, the teratomas were recovered, weighed, and processed for histological analysis. Both EGC lines (called 12.5 Sv3 and 12.5 Sv6–1) gave rise to tumors. The weight of tumors induced was in the range 0.05–1.10 g. The amount of differentiated tissues varied among individual tumors. Each tumor contained a broad variety of tissues, including keratinized, simple and secretory epithelia, adipose cells, collagen, cartilage, forming bone, striated muscle, and neuron ganglia (Fig. 2). Nests of undifferentiated cells were confirmed by GCNA1 expression (Fig. 2F).

The ability of EGCs to differentiate *in vitro* depends upon the cell type of the co-aggregate

Lung aggregates: In order to test the ability of EGCs to undergo differentiation *in vitro*, EGCs marked with LacZ were added to aggregates of embryonic lung cells at concentrations of 10^7 or 10^6 cells per aggregate (4 aggregates analysed), 10^5 cells per aggregate (12 aggregates analysed), or 10^3 cells per aggregate (30 aggregates analysed). After culture for 4–6 days, the aggregates were serially sectioned and processed for β-galactosidase activity. The status of differentiation was assessed by the morphological appearance of LacZ-positive colonies. Most EGCs in aggregates formed small or large colonies resembling undifferentiated EGCs. At the higher concentrations, EGCs were distributed mostly around the periphery of the aggregate, and extensive necrosis was seen in the center of the aggregate. Necrosis was much less apparent in control lung aggregates (data not shown). At lower concentrations, EGCs tended to be distributed throughout the aggregate, and necrosis was less apparent. No histological evidence of EGC differentiation was seen in any of the lung aggregates processed for β-galactosidase activity, but to confirm the undifferentiated state of the EGCs, a further series of aggregates was made using EGCs carrying either a GFP or LacZ transgene. Some aggregates were examined by confocal microscopy as whole mounts or as single cell suspensions, obtained by trypsinisation. Small or large colonies of MmGFP EGCs were positive for SSEA1 staining (Fig. 1B). Some cells within SSEA1 positive colonies exhibited variable or no expression of GFP. Aggregates made with EGCs marked with LacZ also formed colonies expressing SSEA1 antigen (data not shown). When aggregates were disaggregated into single cell suspensions, we found cells positive for SSEA1 and Oct-4 (data not shown) but no cells expressing either MVH or α-fetoprotein. In negative controls, lungs disaggregated and re-aggregated without added EGCs never gave positive signals for SSEA1, Oct-4, MVH or α-fetoprotein.

Urogenital ridge aggregates: Urogenital ridge aggregates seeded with EGCs at either 10^5or 10^3 EGCs per aggregate presented a somewhat different appearance. Most EGC colonies again appeared to consist of undifferentiated cells, confirmed by their positive SSEA1 and Oct4 staining (Fig. 3D, E). Most aggregates, however, also contained some EGC derivatives resembling epithelia (Table 2), often forming circular structures (Fig. 3A, B). These structures were more often seen in the larger aggregates, which were easier to process histologically. (This may explain the apparent difference between aggregates from female and male urogenital ridges, since female urogenital ridges are smaller than male). The structures appeared very different to testis cords or tubules but bore a superficial resemblance to sections of mesonephric ducts. Aggregates were therefore made using only the genital ridge or only the mesonephric portion of the urogenital ridge, as well as only from female or only from male fetuses. The epithelialized EGC formations were seen in all. Examination of serial sections suggested that they were sections of cysts rather than tubules.

Discussion

Any study of cell fate during embryonic development relies upon the stable and ubiquitous expression of a marker gene. Therefore, to be able to follow the fate of EGCs we have used either LacZ (Lallemand and Brûlet 1990; Suemori et al., 1990) or MmGFP (Zernicka-Goetz et al., 1997; Hadjantonakis et al., 1998), both of which

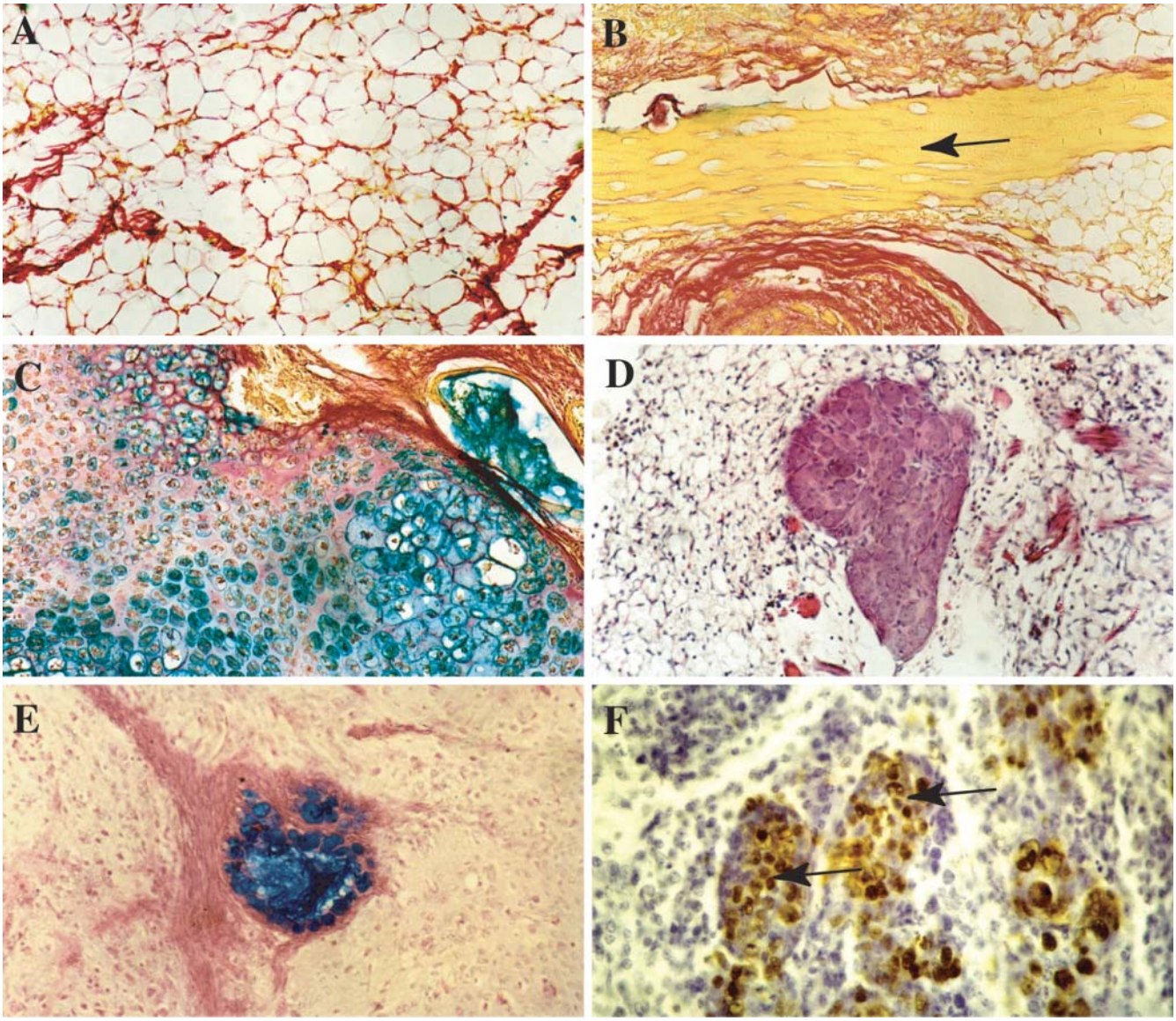

Fig. 2 EGCs formed differentiated tumors *in vivo*. EGCs derived from 12.5 dpc embryos were injected under kidney capsules of 129 SvEv males. Histological analysis of the teratomas revealed that tumors contained a broad variety of tissues. (**A**) adipose cells (**B**) striated muscle (*arrow*) (**C**) cartilage (**D**) neuron ganglia (**E**) secretory epithelia. (**F**) Immunohistochemistry of tumor section for GCNA1 showing brown staining of undifferentiated cells (*arrows*). Original magnification $\times 200$.

have been shown to be stably and ubiquitously expressed.

We have found EGCs to be similar to ESCs in that they preferentially colonize epiblast in chimeras formed with 8–16 cell embryos. The sizes of the clones generated suggest that our EGCs were at a selective advantage relative to the host epiblast cells. As EGCs are extremely efficient at forming chimeras and colonizing the germ line following aggregation with morulae, they could provide means of altering gene expression in species where ESC lines have not yet been derived. As EGCs are derived from a cell lineage that was established subsequent to the differentiation of both trophoblast and primary endoderm, we expected to observe colonization only of epiblast. However, the presence of EGC derivatives also in primary endoderm showed that the potential of these cells is not yet restricted to develop only into epiblast lineages. The morphology and positive staining for AP and SSEA1 indicate that no prior differentiation of the EGCs had occurred. In our studies, EGCs did not show any colonization of trophoblastic tissues derived from trophectoderm.

ESCs have also been occasionally found in primary endoderm tissues (Beddington and Robertson, 1989) but never in the trophectoderm-derived lineages. This is perhaps not surprising since ESCs are derived from the inner cell mass of embryos in which trophoblast differentiation has already occurred. ESCs can, however, be

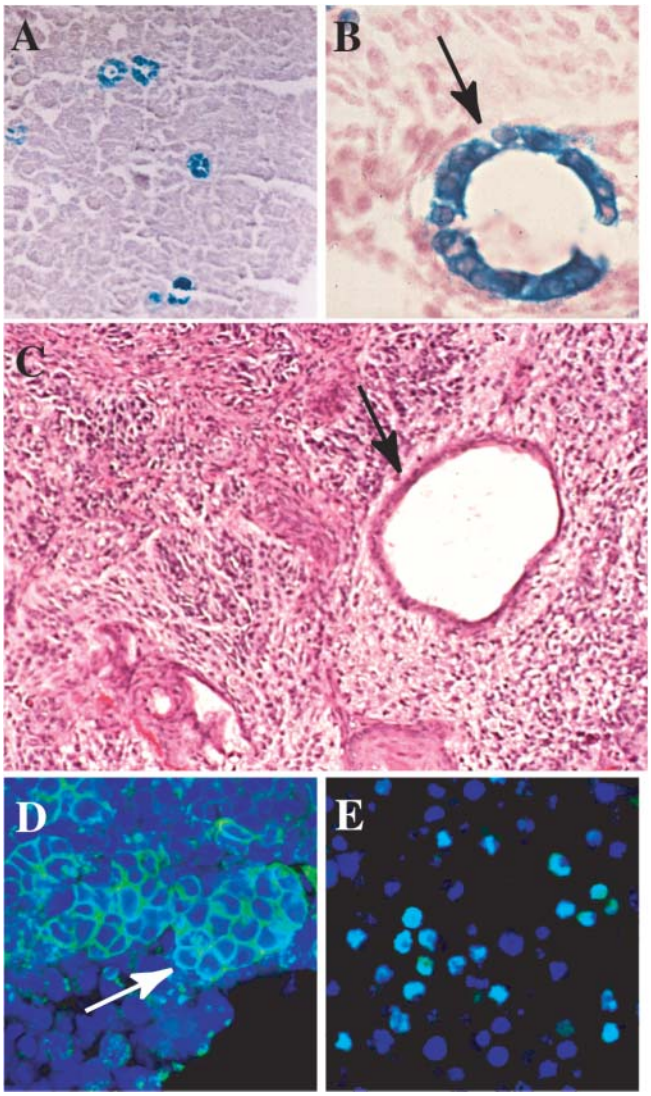

Fig. 3 Differentiation of EGCs in urogenital ridge aggregates. EGCs marked with LacZ were added to urogenital ridge aggregates and co-cultured for 4 to 6 days. (**A** and **B**, *arrow*) Some EGC derivatives formed circular epithelial structures. (**C**, from T. Tada) Section of a four-week-old tumor formed from male EGCs injected subcutaneously into the hind leg of a nude mouse. (**D**, *arrow*) EGCs formed colonies expressing SSEA1 (*green*). (**E**) Some aggregates were dissociated into single cell suspensions and stained for Oct-4, using secondary antibody coupled to FITC (*green*). Nuclei were stained with TOTO-3 (*purple*). Light blue nuclei indicate co-localization of TOTO-3 and Oct-4, i.e. EGCs. Original magnification A and C $\times 10$, B $\times 100$, D and E $\times 200$.

forced into a trophoblastic differentiation pathway by switching off the expression of the *Oct-4* gene (Niwa et al., 2000).

The pluripotency of ESCs has led to their suggested clinical use as a source of tissue. We confirmed that EGCs derived in this laboratory could form teratomas when injected under the kidney capsule. The teratomas contained a variety of differentiated tissues and cell types, as well as some patches of undifferentiated cells.

Table 2 Presence of cyst-like bodies in co-culture of EGCs marked with LacZ with genital ridge aggregates

		10^5 EGCs+ genital ridge		10^3 EGCs+ genital ridge		Total
		F	M	F	M	
Cyst-like bodies	Present	0	2	5	11	18
	Absent	3	0	8	3	14

F – female genital ridge; M – male genital ridge

To determine whether EGCs could be induced to undergo more specific patterns of differentiation *in vitro*, we tested two co-culture systems. The first used embryonic lung cells, which have been reported to be a good source of stem cell factor (Brannan et al., 1992). The aggregates provided an environment that supported the survival and proliferation of EGCs, but no differentiation occurred. Since the EGCs were derived from germ cells in the genital ridge, we hoped that aggregates formed with urogenital ridge cells would provide a more favorable situation for EGC differentiation. This hope was partially fulfilled, since some EGCs differentiated into epithelialized cyst-like structures. Thus, it appears that the ability of EGCs to differentiate depends upon the cell type with which they are co-cultured. The same EGC line is capable of differentiating into teratoma-like structures when injected subcutaneously into the hind leg (T. Tada, personal communication), so perhaps our cyst-like structures may be analogous to the cyst-like bodies seen in teratomas (Fig. 3C), thought to be neural in origin. However, the morphology of the structures formed suggests a specific pathway of differentiation, the nature of which is currently unclear.

We also have observed that direct contact of our EGCs with genital ridge somatic cells in the reaggregates did not switch on the expression of MVH in EGCs after 4–6 days culture. In contrast, Toyooka et al. (2000) reported that some EGCs derived from 8.5 dpc embryos switched on the expression of MVH after only 3 days of co-culture with somatic cells isolated from 12.5 dpc genital ridges. This suggests either that EGC lines derived from early stages are more responsive to the somatic environment of the genital ridges or that there are differences among EGC lines in their ability to differentiate.

Acknowledgements We are grateful to the Wellcome Trust for financial support (GD-H, MZG, and AM).

We are also grateful for financial support to the Cancer Research Campaign (MZG and FW) and the Lister Institute of Preventive Medicine (MZG). We thank Dr. Sheila Barton for her help in both injecting the construct to produce EF1alpha MmGFP transgenic mice and injection of EGCs under the kidney capsule and Dr Takeshi Tada for allowing us to use his LacZ-expressing EGCs.

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