

# Two paternal genomes are compatible with dopaminergic *in vitro* and *in vivo* differentiation

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**ABSTRACT** Patient derived stem cell-based therapies are considered a future treatment option for Parkinson's disease, a chronic and progressive brain neurodegenerative disorder characterized by depletion of dopaminergic neurons in the basal ganglia. While many aspects of the *in vitro* and *in vivo* differentiation potential of uniparental parthenogenetic (PG) and gynogenetic (GG) embryonic stem (ES) cells of several species have been studied, the capacity of androgenetic (AG) ES cells to develop into neuronal subtypes remains unclear. Here, we investigated the potential of murine AG ES cells to undergo dopaminergic differentiation both via directed *in vitro* differentiation, and *in vivo*, in ES cell-chimeric E12.5 and E16.5 brains. We show that similar to normal (N; developed from a zygote with maternal and paternal genomes) ES cells, AG cells generated dopaminergic neurons *in vitro* and in E12.5 and E16.5 chimeric brains following blastocyst injection. Expression of brain-specific imprinted genes was maintained in AG and normal dopaminergic cell cultures. Our results indicate that AG ES cells have dopaminergic differentiation potential *in vitro* and *in vivo*. This contrasts with previous reports of limited neural *in vivo* differentiation of AG cells in later brain development, and suggests that AG ES cells could be therapeutically relevant for future cellular replacement strategies for brain disease.

**KEY WORDS:** *embryonic stem cell, androgenetic, differentiation, dopaminergic neuron*

## Introduction

Parkinson's disease is a degenerative neurological disorder with characteristic motor symptoms that are caused by a loss of dopamine-producing cells in the *substantia nigra* (Jankovic, 2008; Olanow and Tatton, 1999). Clinical studies using striatal transplantation of human fetal ventral mesencephalic tissue grafts have already demonstrated proof-of-concept for dopaminergic cell therapy in Parkinson's disease. In these early studies, graft tissue consisting of postmitotic dopaminergic neuroblasts survived and restored the striatal dopamine release and provided clear clinical benefits for selected patients (Deierborg *et al.*, 2008; Lindvall and Kokaia, 2009). The future of this regenerative approach has been questioned after disappointing results in the follow-up trials that failed to show sufficient clinical improvements and revealed side effects potentially induced by the grafts (Deierborg *et al.*, 2008; Lindvall and Kokaia, 2009). Since graft availability and standardization will likely limit the use of human

fetal tissue for a cell replacement therapy in Parkinson's disease, several approaches to produce dopaminergic neurons from neural stem cells have been initiated. However, the generation of sufficient numbers of functional dopaminergic neurons from primary cultures of fetal, neonatal or adult neural tissues is not yet possible with the current methods. Differentiating ES cell cultures represent a potential cell source for cell replacement therapies (Parish and Arenas, 2007; Sasai, 2002).

Several protocols have succeeded to generate ES cell differentiation systems for efficient neural induction and in the production of a wide range of neural subtypes including ES cell-derived dopaminergic neurons (Barberi *et al.*, 2003; Bibel *et al.*, 2007). ES cell-derived neurons of numerous species have been successfully tested in rodent and primate models of Parkinson's disease

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*Abbreviations used in this paper:* AG, androgenetic; ES cell, embryonic stem cell; GG, gynogenetic; N, biparental; PG, parthenogenetic.

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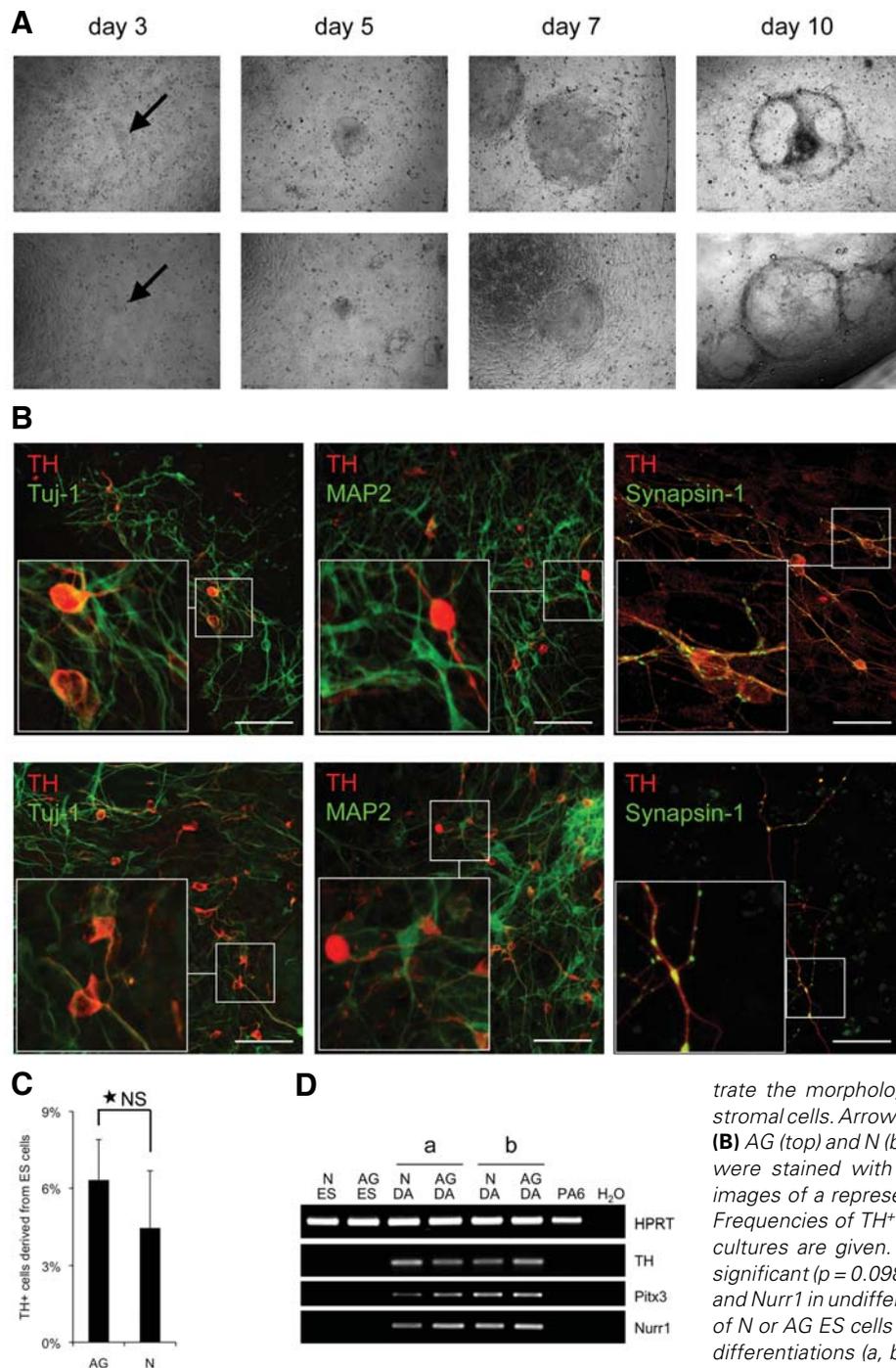
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(Lindvall and Kokaia, 2009; Takagi *et al.*, 2005). ES cell-derived dopaminergic neurons showed electrophysiological and functional properties of midbrain dopaminergic cells (Kim *et al.*, 2002). Importantly, ES cell-derived dopaminergic neurons restored behavioral function in animal models of Parkinson's disease (Bjorklund *et al.*, 2002; Cibelli *et al.*, 2002; Hedlund and Perlmann, 2009; Maxwell and Li, 2005; Rodriguez-Gomez *et al.*, 2007; Toriumi *et al.*, 2009; Yang *et al.*, 2008). An additional benefit of ES cells is that the genome of these cells can be efficiently manipulated to direct lineage specification and function (Draper and Nagy, 2007; Menendez *et al.*, 2005; Prella *et al.*, 2002).

In mammals, embryos with only maternally (PG, GG)- or paternally (AG)-derived genomes do not develop past early postimplantation stages due to the consequences of genomic imprinting. Maternal and paternal genomes are functionally non-redundant due to differential DNA methylation and transcription from paternal and maternal alleles (Solter, 2006). Abnormal levels of parent-of-origin-specific gene expression of imprinted genes during development are associated with a limited post-implantation viability of uniparental embryos (Barton *et al.*, 1984; Cattanach and Kirk, 1985; McGrath and Solter, 1984; Surani *et al.*, 1984). Despite the compromised *in vivo* developmental potential of uniparental embryos, diploid uniparental AG and PG/GG embryos develop to the blastocyst stage and can be used for the derivation of ES cell lines. Uniparental ES cell lines have been derived in mouse (Mann *et al.*, 1990; Robertson *et al.*, 1983) and primate species including humans (Cibelli *et al.*, 2002; Revazova *et al.*, 2007). Mouse uniparental ES cell lines are pluripotent and contribute to multi-lineage post-implantation development, including the germ line (Allen *et al.*, 1994; Narasimha *et al.*, 1997). While PG ES cell chimeras can survive postnatally with substantial contribution of ES cells (Sturm *et al.*, 1994), AG ES cell chimeras typically exhibit severe defects and high lethality during fetal and postnatal stages (Mann *et al.*, 1990; Narasimha *et al.*, 1997). However, similar to ES cells derived from biparental (N) embryos, uniparental ES cells generate ecto-, meso-, and endodermal cell lineages in cell cultures including neural progenitor/stem cells and engrafting hematopoietic stem cells (Dinger *et al.*, 2008; Eckardt *et al.*, 2008; Eckardt *et al.*, 2007; Lengerke *et al.*, 2007; Teramura *et al.*, 2009).

We consider the analysis of AG ES cells relevant for two reasons: 1.) basic research: so far it is unclear which types of tissue stem cells can develop in the ab-



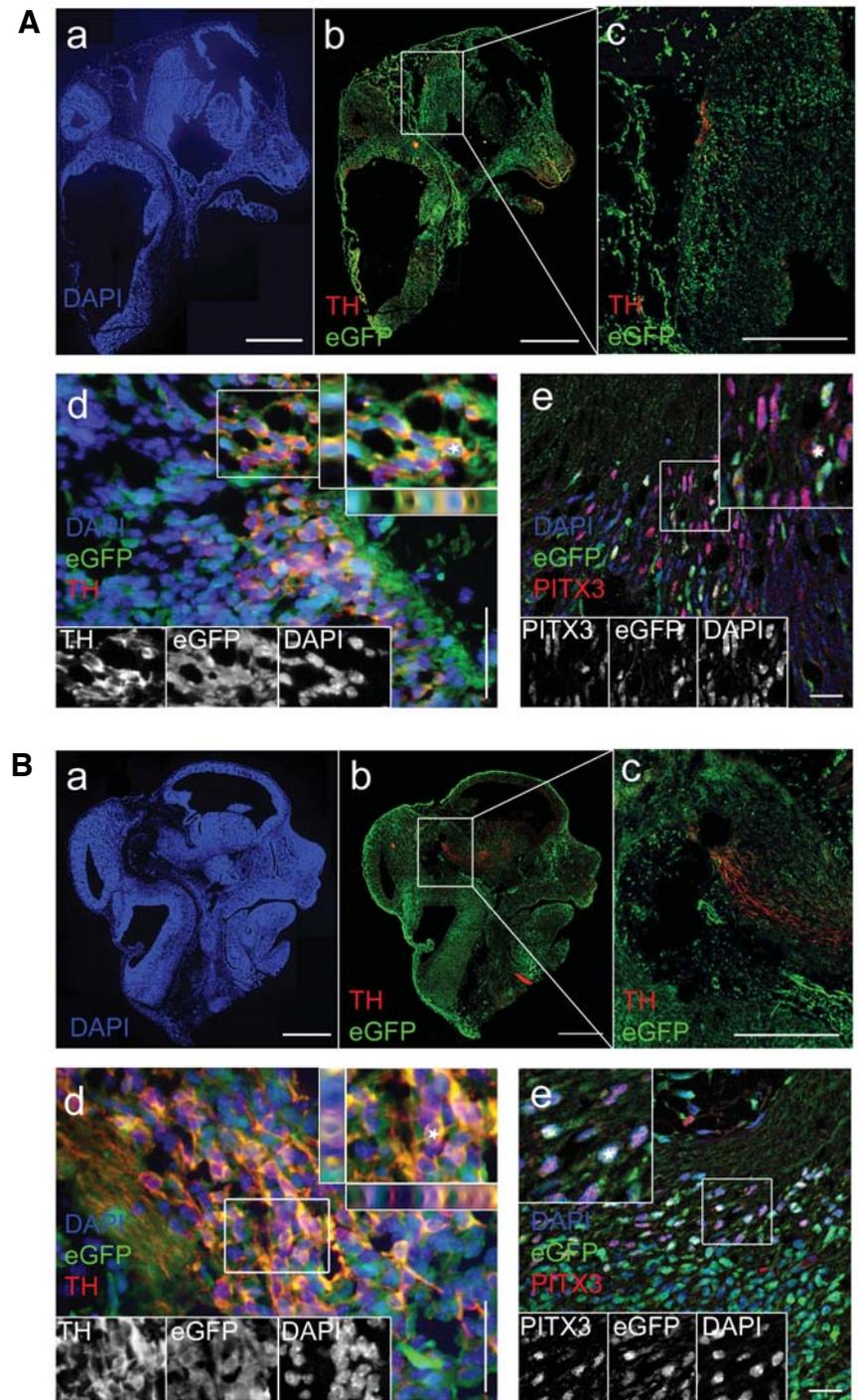
**Fig. 1. *In vitro* dopaminergic differentiation of androgenetic (AG) and normal (N) ES cells. (A) PA6 coculture-induced dopaminergic differentiation of AG (top) and N (bottom) ES cells. Time-lapse, phase-contrast images illustrate the morphological changes in ES cell colonies growing on PA6 stromal cells. Arrows indicate an ES cell-derived colony. 4x magnification. (B) AG (top) and N (bottom) ES cell-derived neurons cultured for 2 weeks were stained with TH/Tuj-1, TH/MAP2 and TH/Synapsin-1. Confocal images of a representative analysis are shown. Scale bars: 25  $\mu$ m. (C) Frequencies of TH+ cells among eGFP+ ES cell derivatives in AG and N cultures are given. Biological replica (n) = 3 for parts (A-C). \*NS: not significant (p = 0.098). (D) RT-PCR analysis for the expression of TH, Pitx3 and Nurr1 in undifferentiated AG or N ES cells, in PA6 stroma cell cultures of N or AG ES cells and in PA6 stromal cells. Analysis of 2 independent differentiations (a, b) are revealed. Shown is a representative analysis.**

sense of a maternal genome; 2.) tissue regeneration: PG- or GG-based cell replacement strategies do only cover fertile-aged females but not males. Thus uniparental strategies for the male population should be equally considered. Due to the severe phenotypes of AG ES cell chimeras AG ES cells have so far not been analyzed as extensively. Here, we show that murine AG and N ES cells do not differ in their differentiation potential *in vitro* in dopaminergic differentiation cultures and *in vivo* in fetal brain development upon blastocyst injection. Based on the analysis of brain-specific imprinted genes, differentiation into dopaminergic neurons is therefore not limited by paternal-only gene expression.

## Results

### Neural and dopaminergic *in vitro* differentiation of androgenetic ES cells

To assess the dopaminergic *in vitro* differentiation potential of AG in comparison to N ES cells, we seeded AG and N ES cells in a coculture-based differentiation system for selective generation of dopaminergic neurons using mouse stromal cells (PA6 cells) as a feeder cell layer (Barberi *et al.*, 2003). PA6 is a bone marrow-derived stromal cell line that is capable of supporting early hematopoietic and B-lineage progenitor cells (Nishikawa *et al.*, 1988). More recent reports described the capability of PA6 cells to induce dopaminergic differentiation from ES cells in the presence of specific midbrain patterning and differentiation factors (Hermann *et al.*, 2006; Kawasaki *et al.*, 2000). We plated undifferentiated AG and N ES cells on PA6 stromal cells at low density. Sequential exposure to midbrain patterning and differentiation factors FGF8, SHH, bFGF, and SHH was subsequently used to favor dopaminergic phenotypic specification during the coculture period (Fig. 1A). After a total differentiation period of 14 days, cells were immunostained for neuron-specific Tuj-1 (class III beta-tubulin), MAP2 (microtubule associated protein 2), for the dopaminergic neuron-specific marker tyrosine hydroxylase (TH), and for the presynaptic vesicle protein Synapsin-1. As shown in Fig. 1B both AG and N cells stain positive for TH/Tuj-1, TH/MAP2 and TH/Synapsin-1 indicating that AG simi-



**Fig. 2. *In vivo* differentiation of AG and N cells in E12.5 chimeric brains upon blastocyst injection.** Following blastocyst injection of AG or N ES cells donor cells were analyzed in E 12.5 chimeric brains. **(A)** Immunostaining of cryosections of an AG ES cell-chimeric brain. **(a)** A representative sample shows DAPI staining of a sagittal section of an AG chimeric head (image combined from 13 individual pictures). **(b)** An overlay of anti-eGFP (donor cells, green) and anti-TH-specific (red) immunostainings is shown. A mid-brain region is marked. **(c)** Magnification of the mid-brain region. **(d)** Shown are overlays of red (TH; dopaminergic cells), green (anti-eGFP; ES cell-derived cells) and blue signals (DAPI; nuclei). Rectangle (top center) indicates area that was further analyzed by z-stack analysis. Insert (top right) shows a 50  $\mu\text{m}$  x-y layer and orthogonal view in the z direction of 10  $\mu\text{m}$  cryosections to confirm signal colocalization. \* marks signal colocalization. Single color channels of the marked area (top center) are shown (bottom). **(e)** Overlays of red (PITX3; dopaminergic cells), green (anti-eGFP; ES cell-derived cells) and blue signals (DAPI; nuclei). Also z-stack analysis (upper right) and single color channels of a marked area are shown (bottom). Images shown in panels (a-d) were taken by a BioZero microscope, images in (e) came from a confocal microscope.  $n = 3$ . **(B)** Immunostaining of representative sagittal cryosections from a N chimeric E12.5 brain. Overview shown in (a,b) were combined from 16 individual images. Panels are as described in (A). Scale bars: (a,b) 2 mm; (c) 1 mm; (d) 50  $\mu\text{m}$  and (e) 20  $\mu\text{m}$ .

lar to N ES cells can generate cells that immunophenotypically resemble dopaminergic neurons. AG and N cocultures generated similar frequencies of TH<sup>+</sup> cells (TH<sup>+</sup> AG cells:  $6.3 \pm 1.6\%$ ; TH<sup>+</sup> N cells:  $4.5 \pm 2.2\%$ ) (Fig. 1C). Morphological presence of Synapsin-1 positive puncta was detected in both AG and N ES cultures (Fig. 1B, right panels) indicating the ability of AG and N derived dopaminergic neurons to form synaptic terminals.

To further define the cell types derived in the coculture system, we evaluated dopaminergic gene expression in AG and N ES-derived neurons. In contrast to N and AG ES cells as well as PA6 feeder cells, PA6 cultures of N and AG dopaminergic neurons from two independent experiments exhibited expression of the dopaminergic-specific transcripts TH, paired-like homeodomain transcription factor 3 (PITX3) and nuclear receptor-related 1 (Nurr1) (Fig. 1D). Together the data support the view that dopaminergic cells can be generated from AG and N ES cells *in vitro*.

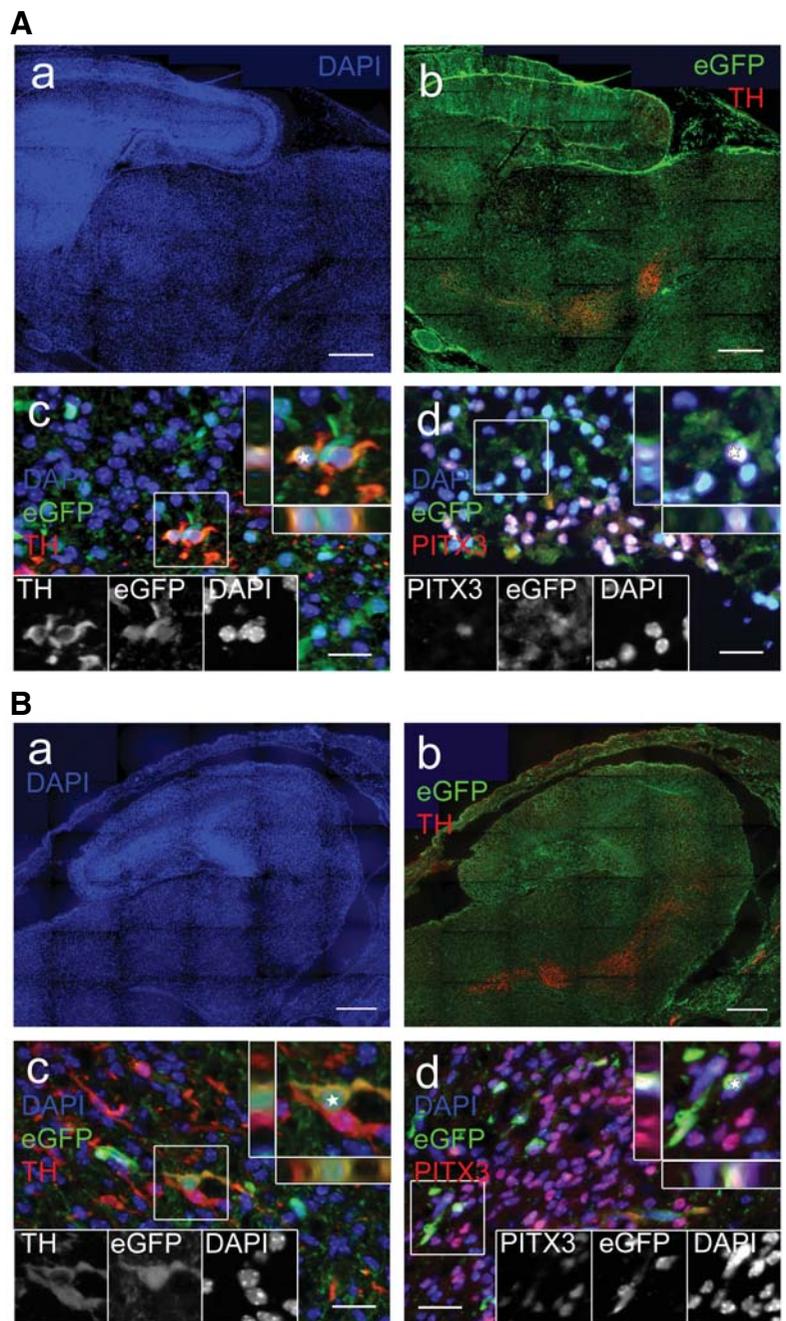
#### Neural and dopaminergic differentiation of androgenetic ES cells in chimeric animals

To study the capacity of AG ES cells to participate in neural development within a developing embryo, we generated chimeric embryos by blastocyst injection of eGFP positive AG and, as a control, N ES cells. We identified and isolated chimeric embryos both at E12.5 and E16.5, and ascertained distribution and immunophenotype of GFP positive ES cell derived cells in sagittal sections of chimeric brains. Consistent with previous observations (Mann *et al.*, 1990), the frequency of dead or absorbed embryos increased from E12.5 to E16.5 for AG ES cell-chimeric embryos ((Dinger *et al.*, 2008) and data not shown). With increasing fetal age, ES cell contribution was lower in surviving AG chimeras compared to N chimeras (Table 1).

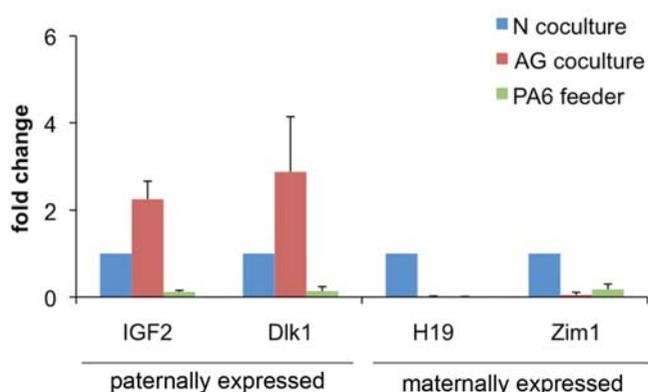
In E12.5 chimeric brains, TH- and PITX3-expressing cells of ES cell origin were present in the developing *substantia nigra* (Fig. 2). We further detected TH- and PITX3-positive ES cell derivatives in the *ventral tegmental* cell groups in E16.5 chimeric brains (Fig. 3). Z-stack analysis confirmed signal colocalization of eGFP and the dopaminergic markers TH and PITX3 (Fig. 2 and Fig. 3). Although donor chimerism varied between animals, we observed corresponding frequencies of eGFP/TH or eGFP/PITX3 double positive cells in AG and N chimeric brains, respectively (Table 1). This indicates that AG and N cells generate with similar frequencies ES cell-derived dopaminergic neurons in chimeric E12.5 and E16.5 brains.

#### Imprinted gene expression during neuronal differentiation of androgenetic ES cells

To assess imprinting in dopaminergic cultures we analyzed gene expression of imprinted genes that are expressed in the developing brain by quantitative RT-PCR. From a panel of brain-specific imprinted genes previously tested for expression in AG neurons, we restricted this analysis to genes not expressed in PA6



**Fig. 3. Androgenetic (AG) and normal (N) donor cells were analyzed in E16.5 chimeric brains. (A)** Immunostainings of a representative AG ES cell-chimeric midbrain. **(a)** A DAPI staining of a sagittal section (image combined from 28 individual photographs) is shown. **(b)** Anti-eGFP (green) and anti-TH-specific (red) immune stainings of the area as shown in **(a)** is revealed. **(c)** Overlays showing TH-, eGFP- and DAPI-specific stainings of a mid-brain region. Insert shows a z-stack analysis for TH/eGFP signal colocalization (upper right). In addition higher magnifications of a marked region displayed in single color channels are displayed (bottom). **(d)** Shown is an overlay of green (anti-eGFP), red (PITX3; dopaminergic cells), and blue signals (DAPI; nuclei). Inserts show a z-stack analysis and single color channels for signal colocalization. \* marks TH/eGFP or PITX3/eGFP signal colocalization, respectively. Images were taken by a BioZero microscope. **(B)** Immunostaining of representative sagittal cryosections from a normal chimeric E16.5 brain. Overview shown in panels **(a, b)** were combined from 35 individual images. Panels are as described in **(A)**. Scale bars: **(a, b)** 2 mm; **(c, d)** 25 μm.



**Fig. 4. Analysis for the expression of imprinted brain genes after *in vitro* dopaminergic differentiation of androgenetic (AG) and normal (N) ES cells.** Relative expression of imprinted genes was compared in N and AG ES cell-derived dopaminergic neurons in PA6 cocultures after two weeks of differentiation. Shown are quantitative RT-PCR data from representative batches of cocultures. The relative expression represents the fold change of gene expression in AG to N cells. Fold change was calculated by the 2<sup>ΔCt</sup> method. Expression levels of N cells were set to 1. In brain, IGF2 and Dik1 are preferentially paternally expressed, and Zim1, and H19 are preferentially maternally expressed. n = 3.

stromal cells (Dinger *et al.*, 2008). As shown in Fig. 4 the paternally expressed *IGF2* and *Dik1* genes exhibited higher levels of expression in AG compared to N dopaminergic neural cultures, while the maternal expressed *H19* and *Zim1* genes were not detected or are poorly expressed. This indicates that these imprinted genes maintain their parent-of-origin-specific gene expression bias in AG ES cell-derived dopaminergic neurons.

**Discussion**

Parkinson’s disease is the most common degenerative neurological movement disorder and results primarily from the loss of dopaminergic neurons in the *substantia nigra*. Although effective symptomatic therapies are available, no proven neuro-restorative therapies have been established to date. In this study we report that uniparental AG ES cells are capable of differentiating into dopaminergic neurons *in vitro* and *in vivo*, similar to N ES cells.

Uniparental ES cells are one of several pluripotent cell types that could be a source of tissue for regenerative therapies for brain disease, along with N embryo-derived ES cells, induced pluripotent stem (iPS cells), and directly reprogrammed neural cells

(Eckardt *et al.*, 2008; Vierbuchen *et al.*, 2010; Yamanaka, 2007). Despite their similarity, these cell types are distinct in their origin (maternal or paternal gamete- only versus both parental genomes for uniparental versus N ES cells, factor-reprogrammed versus germ line reprogrammed for iPS versus all ES cell types), with consequences on epigenetic status (Chin *et al.*, 2009; Deng *et al.*, 2009; Doi *et al.*, 2009; Sato *et al.*, 2010) and potentially, differentiation capacity. For example, the neural differentiation capacity of the currently available iPS cell lines is variable and far less efficient than that of ES cells (Hu *et al.*, 2010). For murine uniparental ES cells of both maternal and paternal origin, differentiation into transplantable hematopoietic derivatives has been demonstrated (Eckardt *et al.*, 2007), suggesting that at least for this tissue type, potential effects of genomic imprinting are not consequential. Here, we show that the presence of two paternal genomes in AG ES cells is compatible with dopaminergic differentiation.

It has been recognized for some time that as a result of imprinting the two parental genomes are functionally not equivalent. In about 1 % of the genes one of the two alleles is partially or completely switched off (Barton *et al.*, 1984; McGrath and Solter, 1984). The decision to silence a particular allele depends on the germ line (paternal or maternal) from which the allele was inherited. Further analyses on the cellular consequences of imprinted gene expression have revealed that maternal and paternal genomes have opposite influences on proliferation, cell-cycle length, senescence, and tumor formation (Hernandez *et al.*, 2003). There is increasing evidence that imprinted gene function affects brain development and behavior (Wilkinson *et al.*, 2007).

The literature reports that upon blastocyst injection AG donor cell contribution to chimeras decreases during development (Barton *et al.*, 1991). This phenomenon has been interpreted as a selective elimination process of AG cells (Fundele *et al.*, 1989), however, the precise reasons for the decline of AG donor cell frequencies remains unclear. It has been estimated that 10 % of AG cell contribution in chimeras leads to embryonic lethality (Narasimha *et al.*, 1997), and higher levels AG ES cell contribution to late gestation chimeras are associated with severe defects and lethality (Mann *et al.*, 1990). In our analyses we observed that AG ES cells, as previously reported for N and PG ES cells (Kim *et al.*, 2002; Sanchez-Pernaute *et al.*, 2008), generated dopaminergic neurons *in vitro* and *in vivo*. The frequencies of dopaminergic cells were similar in cultures and transplant recipients of AG and N cells. The apparent similar neural *in vitro* and *in vivo* activity of AG and N ES cells is surprising considering previous observations of substantial cell autonomous differences between the

TABLE 1

**FREQUENCIES OF TH+ AND PITX3+ DONOR CELLS IN AG AND NORMAL CHIMERIC E12.5 AND E16.5 BRAINS**

Days of gestation	12.5						16.5		
	AG-2	AG-3	AG-4	N-5	N-8	N-9	AG-b	AG-c	N-b
Donor cells* in midbrain	76.1 % 761/1000	53.5 % 535/1000	24.7 % 247/1000	86.5 % 865/1000	70 % 700/1000	58.1 % 581/1000	24.4 % 163/669	24 % 191/797	12 % 69/574
TH-positive donor cells**	60.5 % 150/248	32.5 % 108/332	23.6 % 41/174	72.0 % 126/175	65.9 % 112/170	39.3 % 103/262	18.8 % 39/207	35.3 % 72/204	3.1 % 5/162
PITX3-positive donor cells**	56.9 % 202/355	39.2 % 129/329	25.1 % 48/191	68.0 % 168/244	65.4 % 121/185	39.7 % 105/264	34.4 % 52/151	35.8 % 71/198	8.9 % 27/303

Calculation of Pearson product-moment correlation coefficients: For the eGFP/TH case we calculated the correlation coefficient of r=0.75, indicating that the combined data was well described by a linear relation between donor chimerism and frequency of eGFP/TH double positive cells. For the case of eGFP/PITX3 double positive cells the correlation coefficient was r=0.63, again indicating a linear relation. \*) eGFP+ cells / DAPI+ cells. \*\*) eGFP+ cells / TH+ or PITX3+ cells.

neural differentiation potential of AG and PG cells in developmental chimeras produced either by morula aggregation or inner cell mass injection into blastocysts (Keverne *et al.*, 1996). In these studies, AG cells contributed preferentially to hypothalamic structures and lesser to the cortex while PG/GG cells engrafted substantially to the cortex, hippocampus and striatum but not to the hypothalamic structures.

Recently, it has been reported that during dopaminergic differentiation of human ES cells a specific region of the genome located on chromosome 11p15.5 becomes transcriptionally highly activated (Freed *et al.*, 2008). As this region contains the paternally expressed *IGF2* and the maternally expressed *H19* genes, it has been proposed that the *H19-IGF2* imprinting region is involved in the differentiation of ES cells into neuronal precursors and/or to dopaminergic neurons. Our results show that during dopaminergic differentiation of AG ES cells, a parent of origin specific expression bias is maintained for the imprinted *IGF2* and *H19* genes. Despite this unbalanced gene expression, AG like N cells generate dopaminergic neurons *in vitro* and *in vivo*, suggesting that a deregulation of *IGF2* and *H19* expression due to a lack of the maternal allele does not interfere with dopaminergic development.

The apparently similar *in vitro* and *in vivo* dopaminergic potential of AG and N cells in the present study is paralleled by the comparable neuronal and glial differentiation potential of AG and N ES cells (Dinger *et al.*, 2008; Okabe *et al.*, 1996). Likewise, hematopoietic potential of the AG and N cells does not differ *in vitro* or *in vivo* (Eckardt *et al.*, 2007). This suggests that, the differentiation potential of uniparental ES cells is less restricted *in vitro* or in blastocyst injection chimeras than that of entirely AG-derived embryos. These surprising observations could either indicate that imprinting does not play a crucial role for the development of adult-type stem cells or that from the starting ES cell cultures only cells that show a relaxation of critical imprinted genes get selected. Alternatively, uniparental cells may develop other compensatory mechanisms. Indeed earlier analyses revealed that the expression of selected imprinted genes was relaxed in differentiating AG cell cultures adapting hematopoietic or neural cell fates (Dinger *et al.*, 2008; Eckardt *et al.*, 2007).

Numerous tumors are associated with the preferential loss of parental chromosomal regions. Examples include rhabdomyosarcoma (maternal chromosome 11p15.5) (Scrabble *et al.*, 1989), neuroblastoma (maternal chromosome 1p36 or paternal chromosome 2) (Caron *et al.*, 1993), and acute myeloblastic leukemia (paternal chromosome 7) (Katz *et al.*, 1992). These observations suggest an involvement of imprinted genes in tumor-initiation or progression processes. Our analyses of chimeric E12.5 and E16.5 fetuses did not show signs of tumor formation indicating that AG chimeras are not more tumor-prone than N ES chimeras. This is in line with our earlier observation of no tumor formation in uniparental HSC transplant recipients or chimeras that developed upon blastocyst injections of PG/GG or AG ES cells (Eckardt *et al.*, 2007). Our next perspective is to show whether a Parkinson's disease mouse model can be cured by transplantation of AG ES cell-derived dopaminergic neurons (Chiba *et al.*, 2008; Rodriguez-Gomez *et al.*, 2007; Toriumi *et al.*, 2009; Yang *et al.*, 2008).

In summary we have shown that AG like N ES cells can differentiate *in vitro* and *in vivo* in a blastocyst transplantation paradigm into dopaminergic neurons. AG cells are interesting cell

systems to study the involvement of imprinting on tissue stem cell generation.

## Materials and Methods

### ES cell culture

AG and N eGFP<sup>+</sup> murine ES cells (2 AG cell lines and 2 N cell lines (Eckardt *et al.*, 2007)) were cultured on inactivated primary murine embryonic fibroblasts (MEF) in DMEM high glucose (PAA Laboratories, Cölbe, Germany) supplemented with 15 % ES cell-tested Fetal Calf Serum (FCS) (PAA), leukemia inhibitory factor (LIF)-conditioned medium, 1 % Non Essential Amino Acids (PAA), Penicillin (100 U/mL) / Streptomycin (100 U/mL) (PAA), 2 mM L-glutamine (PAA), 1 mM sodium pyruvate (PAA) and 0.1 mM  $\beta$ -mercaptoethanol (Sigma-Aldrich, Schnellendorf, Germany) as previously described (Dinger *et al.*, 2008). Medium was changed every day. For passage of ES cells, medium was removed, cells were washed with PBS and incubated with trypsin/EDTA (PAA). After 5 minutes, the reaction was stopped by adding medium/10 % FCS. Cells were collected, washed, resuspended and replated on fresh MEF feeder cells.

### Neural *in vitro* differentiation

ES cells were differentiated into dopaminergic neurons using a coculture system with stromal cells as previously described (Barberi *et al.*, 2003). Briefly, ES cells were placed on gelatin-coated plates (0.1 % in PBS; Sigma-Aldrich) for 30 minutes at 37°C to allow separation of MEFs and ES cells. For differentiation, a single cell suspension of ES cells was plated at a density of 100 cells/cm<sup>2</sup> on inactivated PA6 stromal cells and cultured for up to 14 days at 37°C, 5 % CO<sub>2</sub> and 4 % O<sub>2</sub>. Culture medium was changed every second day after day 3 of culture. From seeding to day 8, cells were cultured in serum replacement medium (SRM; DMEM supplemented with 15 % Knockout™ Serum Replacement (Invitrogen, Karlsruhe, Germany), 2mM glutamine and 10  $\mu$ M  $\beta$ -mercaptoethanol). On days 5 and 7, SRM was supplemented with 200 ng/mL SHH and 100 ng/mL FGF8. From day 8 onwards, cells were cultured in N3 medium, supplemented with SHH, FGF8 and 50 ng/mL bFGF on day 8 and 10, and with 20 ng/mL BDNF and 200  $\mu$ M ascorbic acid (Sigma-Aldrich) after day 11. All growth factors were purchased from PeproTech (Hamburg, Germany). Passage numbers of ES cells used for *in vitro* differentiation and transplantation experiments ranged from passages 20 – 40.

### Immunostaining of cultured cells

For multilabel fluorescent imaging using a SP5 Confocal Microscope (Leica, Wetzlar, Germany) cells grown on coverslips were fixed in 4 % formaldehyde, permeabilized in 0.5 % Triton-X and stained with the following antibodies: rabbit anti-TH (1:500; Sigma-Aldrich), mouse/rabbit anti-Synapsin 1 (1:1000; SynapticSystems, Göttingen, Germany), mouse anti-Tuj-1 (1:1000) and mouse anti-MAP2 (1:500; Chemicon, Schwalbach, Germany), chicken anti-eGFP (1:500; Abcam, Cambridge, UK), and Cy2 (Chemicon), Cy3 and DyLight 649 (Dianova, Hamburg, Germany) labeled secondary antibodies (all in a dilution of 1:500). DAPI-Moviol was used as a mounting medium and for fluorescent counterstaining of nuclei.

### Analysis of imprinted brain gene expression using quantitative RT-PCR

Total RNA was isolated from cultured cells by using peqGOLD RNAPure™ (peqLab Biotechnologie, Göttingen, Germany). Before generation of cDNA, RNA preparations were treated with DNase I (Ambion, Applied Biosystems, Foster City, USA). RNA was reverse-transcribed into cDNA using M-MLV reverse transcriptase (Invitrogen). RT-PCR reactions were performed and quantified using a Rotor-Gene™ 3000 (Corbett Life Science, LTF Labortechnologie, Wasserburg, Germany) and ABsolute™ QPCR SYBR® Green Mix (ABgene, Hamburg, Germany). The relative gene expression levels were calculated with the 2<sup>- $\Delta$ CT</sup>

method. The  $\Delta$ Ct-values indicate a difference of Ct-values between reference gene and target gene. The housekeeping gene  $\beta$ -actin was used for the reference gene. The expression level of a target gene in N cells were set to 1 in order to determine differences of the target gene expression in AG cells. The primer sequences used were:

$\beta$ -actin f: 5'-GATATCGCTGCGCTGGTCGTC-3'  
r: 5'-ACGCAGCTCATTGTAGAAGGTGTGG-3'

Dlk1 f: 5'-TGCAATGGAGTCTGCAAGG-3'  
r: 5'-AGGGAGAACCATTGATCACG-3'

H19 f: 5'-CATGTCTGGCCTTTGAA-3'  
r: 5'-TTGGCTCCAGGATGATGT-3'

IGF2 f: 5'-CTAAGACTTGGATCCCAGAACC-3'  
r: 5'-GTTCTTCTCCTTGGGTTCTTTC-3'

Zim1 f: 5'-GAGAACCGTACTGCTGTCA-3'  
r: 5'-CTTGCACCGGTACCTGGAGT-3'.

#### Generation of chimeric embryos by blastocyst injection

Blastocyst injection was performed as described (Dinger *et al.*, 2008). Briefly, to obtain blastocysts for the injection of ES cells, 6–8 week old female NMRI mice were superovulated by intraperitoneal injection of 10 units pregnant mare's serum (PMSG), followed 48 hours later by an injection of 10 units human chorionic gonadotropin (hCG) (both Intervet, Unterschleißheim, Germany), caged with stud males and checked for vaginal plugs the next morning. The day of finding the plug was designated day 0.5 days post coitum (dpc). At 3.5 dpc, pregnant mice were sacrificed, ovaries and oviducts were removed and transferred into M16 medium (Sigma-Aldrich). Blastocysts were flushed from the oviducts and kept 1 to 2 hours in M2 medium (Sigma-Aldrich) prior to the injection of ES cells. To induce pseudopregnancy in recipient foster animals, 6–8 week old female NMRI mice were mated with vasectomized NMRI males of proven sterility. ES cells as a single cell suspension were prepared in M2 medium prior to blastocyst injection.

#### Immunohistochemistry of E12.5 and E16.5 brain cryosections

For cryosections, whole E12.5 and E16.5 embryo heads were isolated and fixed for 12 hours in PBS/ 4 % paraformaldehyde (Sigma-Aldrich). Following fixation, tissues were kept in PBS/ 16 % glucose (Applichem), embedded in TissueTek O.C.T. (Sakura Finetek, Heppenheim, Germany) and frozen at -80°C. 10  $\mu$ m sagittal brain sections were stained with primary chicken antibody against eGFP (Abcam) and with secondary Cy2-conjugated goat anti-chicken IgY (Abcam). For neuronal cell staining, primary rabbit antibody against TH (dilution 1:1000; Sigma-Aldrich) or primary rabbit antibody against PITX3 (dilution 1:500; Zymed, Carlsbad, USA) and secondary Cy3-conjugated goat anti-Rabbit IgG (Chemicon) were used. Nuclei were counterstained with DAPI (Sigma-Aldrich). For analysis of co-localization of green and red signals, Z-stacks were taken using a BioZero microscope (400x magnification) (Keyence, Neu-Isenburg, Germany), yielding XY pictures and orthogonal XZ and YZ projections of Z-stacks. In addition, a Leica SP5 Confocal Microscope was used. For intranuclear staining, tissue slices were microwaved for 6 minutes in 10 mM citrate buffer (pH 6.0) for antigen retrieval.

#### Quantification of eGFP<sup>+</sup> ES cell-derived cell-contribution in E12.5 and E16.5 chimeric brains

To quantify the contribution of eGFP<sup>+</sup> ES cell-derived cells in the midbrain, sagittal brain sections were stained using primary chicken antibody against eGFP (Abcam) and with secondary Cy2-conjugated goat anti-chicken IgY (Abcam) and DAPI. The percentage of eGFP<sup>+</sup> cells was determined by counting the numbers of DAPI-stained nuclei and the numbers of eGFP<sup>+</sup> cells in 3 representative 100  $\mu$ m x 100  $\mu$ m squares per brain. The slides were analyzed by a blinded investigator.

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