Neural Differentiation Potential of Murine Androgenetic Embryonic Stem Cells

Dissertation zur Erlangung
des naturwissenschaftlichen Doktorgrades
der Julius-Maximilians-Universität
Würzburg

vorgelegt von

Timo Christoph Dinger
geboren in Hilden

Würzburg 2008

Eingereicht am: 18. 12. 2008

Mitglieder der Promotionskommission:

Vorsitzender: Prof. Dr. Martin J. Müller

Gutachter: Prof. Dr. Albrecht M. Müller

Gutachter: Prof. Dr. Thomas Brand

Tag des Promotionskolloquiums: 20. 05. 2009

Doktorurkunde ausgehändigt am: .....

Eidesstattliche Erklärung

Hiermit erkläre ich, dass ich diese Dissertation selbstständig angefertigt und keine anderen als die von mir angegebenen Hilfsmittel und Quellen benutzt habe.

Ich erkläre außerdem, dass diese Dissertation weder in gleicher noch in anderer Form bereits in einem Prüfungsverfahren vorgelegen hat.

Ich habe früher, außer den mit dem Zulassungsgesuch urkundlichen Graden, keine weiteren akademischen Grade erworben oder zu erwerben gesucht.

Würzburg, den 16.12.2008

Timo C. Dinger

See first, think later, then test. But always see first. Otherwise you will only see what you were expecting. Most scientists forget that.

Douglas Adams (1952-2001)

für meine liebe Frau

Kristina

TABLE OF CONTENTS

1 Summary **1**

2 Zusammenfassung **3**

3 Introduction **5**

3.1 Stem Cells – Embryonic and Adult *5*

3.1.1 Properties and Potential of Stem Cells 5

3.1.2 Embryonic Stem Cells 7

3.1.3 Adult Stem Cells 9

3.1.3.1 Neurospheres and Neural Stem Cells 9

3.2 Uniparental Cells – Generation and Properties *10*

3.2.1 Androgenetic and Gynogenetic ES Cells 11

3.2.2 Parthenogenetic ES Cells 12

3.2.3 Developmental Potential of Uniparental Embryos and Uniparental ES Cells 12

3.3 Imprinting: Parent-of-Origin-Specific Gene Expression *15*

3.3.1 Imprinting in Neural Development 15

3.4 Aim of this Study and Experimental Strategy *16*

4 Material **19**

4.1 Mouse Strains *19*

4.2 Cell Lines *19*

4.3 Antibodies *20*

4.3.1 Primary Antibodies 20

4.3.2 Secondary Antibodies 20

4.4 Cell Culture-related Materials *21*

4.4.1 Media and Supplements 21

4.4.2 Growth Factors 22

4.5 Chemicals *22*

4.5.1 Buffers and Solutions 22

4.5.2 Chemicals 22

4.5.3 Oligonucleotide Primers 24

4.6 Consumables *25*

4.7 Instruments *26*

5 Methods 28

5.1 Cell Culture 28

5.1.1 Establishing Primary Murine Embryonic Fibroblasts (Feeder Cells) 28

5.1.2 Murine Embryonic Stem Cell Culture 28

5.1.3 Establishing Murine Neurosphere Cultures from Foetal Brains 29

5.1.4 Freezing of ES Cells, Neurosphere Cells and MEFs 29

5.1.5 Thawing of ES Cells, Neurosphere Cells and MEFs from Liquid Nitrogen Storage 29

5.2 Neural In Vitro Differentiation of AG ES Cells 29

5.3 Immunocytochemistry of In Vitro Differentiated ES Cells 30

5.4 Blastocyst Injection and Chimeric Embryo Generation 30

5.4.1 Blastocyst Generation 30

5.4.2 Blastocyst Injection of ES Cells 31

5.4.3 Embryo Isolation and Analysis 31

5.5 Flow Cytometry 31

5.5.1 Analysis of Chimerism in Foetal Liver and Brain 31

5.5.2 Analysis of Chimerism in Mixed Neurosphere Cultures 31

5.5.3 Fluorescence Activated Cell Sorting (FACS) of AG-derived Neurosphere Cells 32

5.6 Self-Renewal and Neural In Vitro Differentiation of Neurosphere Cells 32

5.6.1 Analysis of Neurosphere Initiating Cells 32

5.6.2 Neural In Vitro Differentiation of neurosphere cells 32

5.6.2.1 Immunocytochemistry for Neurons and Astrocytes with Cell-permeabilisation 32

5.6.2.2 Immunocytochemistry for Oligodendrocytes without Cell-permeabilisation 33

5.7 RNA Isolation and RT-PCR 34

5.7.1 RNA Isolation 34

5.7.2 DNase Treatment 34

5.7.3 First Strand Synthesis 34

5.7.4 Quantitative RT-PCR 35

5.8 Immunohistochemistry of Chimeric Brains 35

5.8.1 Sample Preparation and Cryosections 35

5.8.2 Intracellular Immunohistochemistry 36

5.8.3 Intranuclear Immunohistochemistry 36

5.8.4 Quantification of $eGFP^+$ ES Cell-derived Cell-contribution in E12.5 Chimeric Brains 37

5.9 Statistical Analysis *38*

6 Results **39**

6.1 Neural In Vitro Differentiation of AG ES Cells *39*

6.1.1 In Vitro Differentiation of AG, GG and N ES Cells into Pan-neural Progenitor Cells 39

6.1.2 In Vitro Differentiation of pnPCs in Neuronal and Astroglial Cell Types ..41

6.2 AG ES Cell Chimeric Embryos *42*

6.2.1 Chimerism in Foetal Liver and Brain of E12.5 and E14.5 Embryos..... 43

6.3 AG Cells Contribute to the Brain in Foetal AG ES Cell Chimaeras *45*

6.3.1 AG Cells Contribute to the Developing Brain and Form Neural Cell Types. 45

6.3.2 Cell Proliferation and Apoptosis in AG Chimeric Foetal Brains..... 50

6.4 AG Chimeric Foetal Brain Cells in Mixed Neurosphere Cultures..... 52

6.5 Stem Cell Properties of AG Neurospheres *54*

6.5.1 Neurosphere Initiating Number..... 54

6.5.2 Quantitative RT-PCR of Imprinted Genes..... 55

6.5.3 Neural In Vitro Differentiation of Neurosphere Cells 57

7 Discussion **59**

7.1 Differentiation Potential of AG ES Cells..... *59*

7.2 One in Many: Alternatives to Normal Embryonic Stem Cells..... *59*

7.3 Induced Pluripotent Stem Cells and Other Alternative Strategies *60*

7.4 Uniparental Cells: A Candidate for the Generation of Pluripotent Patient-Specific Stem Cells? *60*

7.5 The Full Therapeutic Circle Using Uniparental Cells: Chances and Hurdles.....*61*

7.6 Uniparental Cells in Therapies for Neural Diseases *63*

7.7 Neural Differentiation Potential of Murine AG ES Cells *63*

7.8 Neural In Vitro Differentiation of AG ES Cells *64*

7.9 In vivo Contribution of AG ES Cells to Early Foetal Brains..... *64*

7.10 Neurosphere Cells from AG Chimeric Foetal Brains *65*

7.11 Conclusion *66*

8 Literature **67**

9 Abbreviations **77**

10 Publications 80

10.1 Articles 80

10.2 Book Chapters 80

10.3 Poster Presentations / Oral Presentations 81

11 Curriculum Vitae 82

Acknowledgements 83

1 SUMMARY

Uniparental zygotes with two genomes from the same sex can be established from fertilised oocytes after pronuclear exchange. They contain two maternal (gynogenetic; GG) or paternal (androgenetic; AG) pronuclei and are not competent to develop into viable offspring but they can form blastocysts from which embryonic stem cells (ES cells) can be derived. The developmental potential of uniparental ES cells is not fully investigated. The restricted developmental potential of uniparental cells is cell-intrinsic and probably reflects the different roles maternal and paternal genomes play during development. Following blastocyst injection, both GG and AG ES cells show biased and parent-of-origin-specific chimaera formation. While the *in vitro* and *in vivo* neural differentiation potential of GG ES cells is well characterised the neural developmental potential of AG ES cells is less clear. In an earlier study the group of K. John McLaughlin reported that AG and GG ES cell-derived hematopoietic stem cells conveyed long-term, multi-lineage hematopoietic engraftment with no associated pathologies (Eckardt *et al.*, 2007).

The aim of this study was to investigate the potential of AG uniparental murine ES cells to differentiate *in vitro* and *in vivo* into neural progenitor / stem cells and further into neurons, astro- and oligodendroglia in comparison to GG and biparental (normal fertilised; N) ES cells.

Uniparental and biparental ES cells were obtained from K. John McLaughlin's group and a cell culture system was established to expand uniparental (AG, GG) and biparental N ES cells on murine embryonic fibroblasts (MEF). A multistep-protocol was used to differentiate ES cells towards pan-neural progenitor cells and neuronal and glial cell types (Brüstle *et al.*, 1997). The ability of terminal neural differentiation *in vitro* was analysed by fluorescence microscopy using neuronal and glial lineage markers. In parallel, eGFP$^+$ AG or N ES cells were injected into blastocysts prior to their transfer into foster mothers. At E12.5 and E14.5, embryos were isolated, forebrains were dissected and by means of fluorescence activated cell sorting (FACS) eGFP$^+$ donor cells were isolated from chimeric brains. Both eGFP$^+$ donor and corresponding eGFP$^-$ blastocyst-derived brain cells were expanded and analyses of differentiation potential and self-renewal capacity were performed. Also, cryosections of E12.5 chimeric brains were analysed for donor contribution to the neuronal lineage by immunofluorescence microscopy.

Here it is described that following *in vitro* differentiation, AG pan-neural progenitor cells have similar abilities to differentiate into neuronal and glial lineages as GG and N pan-neural progenitor cells. In cryosections of E12.5 chimeric brains no differences in brain engraftment and formation of immature neuronal cells between uniparental AG and N donor cells were detected. AG and N ES cell-derived cells isolated from chimeric foetal brains by FACS exhibited similar neurosphere initiating cell frequencies and neural multi-lineage differentiation potential.

Therefore, the data of this study suggest that the previously described differences in the *in vivo* engraftment pattern of uniparental inner cell mass (ICM) cells in foetal brains (Keverne *et al.*, 1996) are not primarily due to limitations in the proliferation or differentiation properties of uniparental neural progenitor cells. The results presented here indicate that AG ES cell-derived neural progenitor / stem cells did not differ from N neural progenitor / stem cells in their self-renewal and their neural multi-lineage differentiation potential. Also AG ES cell-derived cells contributed to developing brains at early foetal developmental stages showing a widespread and balanced distribution in chimeric brains. AG brain cells form neurospheres with self-renewal and neural differentiation capacity similar to N ES cell-derived brain cells.

Thus, the data of this study together indicate that the neural developmental potential *in vivo* and *in vitro* of AG and N ES cells does not differ.

2 ZUSAMMENFASSUNG

Uniparentale Zygoten mit zwei Allel-Sätzen des gleichen Geschlechtes entstehen aus befruchteten Eizellen nach dem Austauschen von einem der Pronuclei, so dass sie entweder zwei maternale Pronuclei (gynogenetisch; GG) oder zwei paternale Pronuclei (androgenetisch, AG) enthalten. Diese uniparentalen Zygoten sind nicht in der Lage, sich zu lebensfähigen Organismen zu entwickeln, aber sie erreichen das Entwicklungsstadium der Blastozyste, aus denen uniparentale embryonale Stammzellen (ES Zellen) gewonnen werden können. Das Entwicklungspotential uniparentaler ES Zellen ist bisher nicht vollständig verstanden. Das begrenzte Entwicklungspotential uniparentaler Zellen ist zell-intrinsisch und spiegelt die möglichen unterschiedlichen Rollen wieder, die maternales und paternales Genom während der Entwicklung eines Organismus spielen. Nach der Injektion in wildtypische Blastozysten zeigen sowohl AG- als auch GG-Zellen unausgewogene und spezifisch für den parentalen Ursprung der injizierten Zellen typische Entwicklungen in den chimären Embryonen. Während das neurale Entwicklungspotential von GG ES Zellen gut charakterisiert ist, ist dies für AG ES Zellen weit weniger untersucht. In einer früheren Studie zeigte die Arbeitsgruppe von K. John McLaughlin, dass AG- und GG-Zellen langzeitrepopulierende hämatopoetische Zellen mit der Fähigkeit zur Multiliniendifferenzierung sind, deren Transplantation zu keinen pathologischen Veränderungen im hämatopoetischen System führten (Eckardt *et al.*, 2007).

Das Ziel dieser Arbeit war es, das Potential muriner AG ES Zellen zur Differenzierung in neurale Stamm- und Vorläuferzellen und weiter in neurale, astro- und oligodendrogliale Zellen im Vergleich zu GG und biparentalen (normal befruchteten; N) ES Zellen *in vivo* und *in vitro* zu untersuchen.

Für uniparentale und biparentale ES Zellen, die von der Arbeitsgruppe von K. John McLaughlin zur Verfügung gestellt wurden, wurde ein Zellkultursystem etabliert, um uniparentale (AG, GG) und N ES Zellen auf murinen embryonalen Fibroblasten (MEFs) zu kultivieren. Ein mehrstufiges Differenzierungsprotokoll wurde angewandt, um aus ES Zellen pan-neurale Vorläuferzellen und neuronale und gliale Zelltypen zu generieren (Brüstle *et al.*, 1997). Die Fähigkeit zur terminalen neuronalen Differenzierung wurde mit Fluoreszenzmikroskopie unter der Verwendung von linienspezifischen neuronalen und glialen Markermolekülen analysiert. Parallel dazu wurden $eGFP^+$ uniparentale AG oder biparentale N ES Zellen in Blastozysten injiziert, die in Ammentiere transferiert wurden. An den

Tagen E12.5 und E14.5 wurden die Embryonen isoliert, die fötalen Vorderhirne wurden präpariert, und aus den daraus resultierenden Einzelzellsuspensionen wurden durch fluoreszenzaktivierte Zellsortierung aus AG ES Zellen entstandene GFP^+ Zellen isoliert. Sowohl von AG ES Zellen abstammende $eGFP^+$ Zellen als auch von den korrespondierenden, von den Blastozysten abstammende, $eGFP^-$ Zellpopulationen wurden als Kulturen expandiert. Analysen des neuronalen Differenzierungspotenzials und der Selbsterneuerungsfähigkeit wurden durchgeführt. Außerdem wurde in Kryosektionen von E12.5 chimären Gehirnen die Verteilung der von AG ES Zellen abstammenden $eGFP^+$ Zellen durch Immunfluoreszenzmikroskopie untersucht.

AG pan-neurale Vorläuferzellen zeigten in der *in vitro* Differenzierung ähnliche Fähigkeiten zur Differenzierung in neurale und gliale Zelllinien wie GG und N pan-neurale Vorläuferzellen. In Kryosektionen von E12.5 chimären Gehirnen wurden keine signifikanten Unterschiede in der Besiedlung von Gehirngeweben und der Bildung unreifer neuronaler Zellen zwischen AG und N Zellen festgestellt. Die von AG und N ES Zellen abstammenden Zellen, die durch fluoreszenzaktivierte Zellsortierung aus den chimären fötalen Gehirnen isoliert wurden, zeigten gleiche Frequenzen von Neurosphären initiierenden Zellen und ein gleiches neurales Multilinien-Differenzierungspotenzial.

Zusammenfassend deuten die Daten in der hier vorliegenden Studie darauf hin, dass die Unterschiede im *in vivo* Besiedlungsmuster fötaler chimärer Gehirne durch uniparentale Zellen (Keverne *et al.*, 1996) nicht auf Limitierungen in den Proliferations- oder Differenzierungseigenschaften von uniparentalen neuralen Vorläuferzellen zurückzuführen sind. Die Ergebnisse zeigen vielmehr, dass neurale Vorläufer- / Stammzellen, die von AG ES Zellen abstammen, sich nicht in ihrem Selbsterneuerungs- und Multilinien-Differenzierungspotenzial von N Vorläufer- / Stammzellen unterscheiden. Ebenso zeigen AG ES Zellen nach Blastocysten-Injektion in frühen fötalen Entwicklungsstufen eine ausgewogene Verteilung im gesamten chimären Gehirn. AG Gehirnzellen bilden Neurosphären mit dem gleichen Selbsterneuerungs- und neuralen Differenzierungspotenzial wie N Gehirnzellen.

Zusammen genommen zeigen die Daten dieser Studie, dass sich die Fähigkeit von AG ES Zellen zur frühen neuralen Entwicklung *in vitro* und *in vivo* nicht von N ES Zellen unterscheiden.

3 INTRODUCTION

3.1 Stem Cells – Embryonic and Adult

With the fertilisation of an oocyte a zygote is formed, the ultimate stem cell in mammalian development. Upon further development the daughter cells of the zygote gradually lose developmental potential and become further determined form functional effector cell types. Developing through the stages of morula and blastocyst, the cells of the ICM give rise to the three germ layers and the germ cells. In parallel, the cells from the throphectoderm contribute to the formation of extra-embryonic tissue. By progressive development and differentiation a complete, structured multi-cellular organism is generated, comprised of complex organs that are composed of a plethora of different specified effector cells and a small number of somatic (adult) stem cells.

3.1.1 Properties and Potential of Stem Cells

Stem cells are functionally defined and to date there are no distinct molecular or morphological markers known that can be used for their direct isolation (Ivanova *et al.*, 2002; Ramalho-Santos *et al.*, 2002). One hallmark of stem cells is the capacity to self-renew. Self-renewal means a stem cell can undergo symmetrical cell division, yielding two daughter stem cells or asymmetrical cell division, giving rise to a daughter stem cell and a more committed progenitor / effector cell. Additionally, stem cells can undergo a symmetric division yielding two daughter cells that have properties of differentiated cell types (Figure 1). The second defining property of stem cells is multi-lineage differentiation. Multi-lineage differentiation means stem cells are able regarding to their potential to form a number of different specialised progenitor and effector cells. Thus, stem cells are capable to maintain a pool of stem cells, while supplying sufficient numbers of progeny cells for the maintenance of the organism.

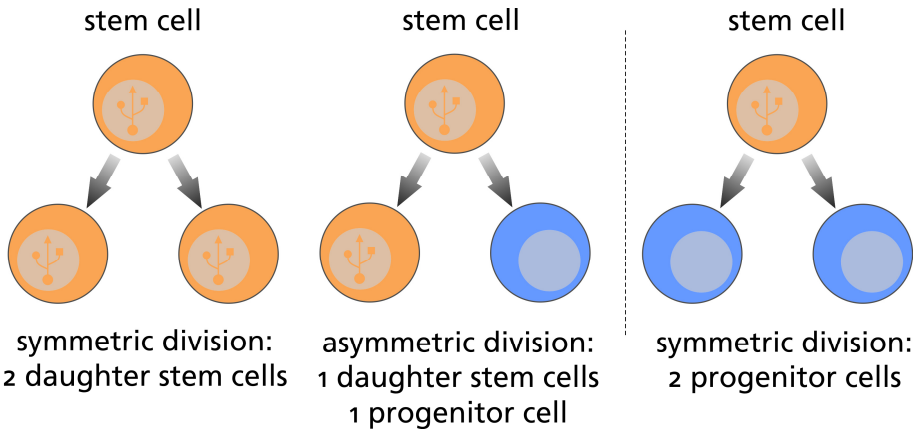


Figure 1: Symmetric and asymmetric cell division of stem cells. Symmetric division of a stem cell yields two daughter stem cells thereby expanding the stem cell pool. Asymmetric division of a stem cell maintains the stem cell pool, giving rise to one stem cell and one more differentiated progenitor cell. The symmetric division of stem cells combined with differentiation generates two daughter progenitor cells with reduced developmental potential compared to the original stem cells. This scenario finally leads to a depletion of the stem cell pool.

While totipotent cells in the stages of the zygote developing to the morula are able to form a complete organism, this ability is lost during further development. Pluripotent cells, like ES cells isolated from the ICM of the developing blastocyst, are able to give rise to all cell types from the three germ layers, endoderm, mesoderm and ectoderm, as well as germ cells. Multipotent stem cells such as somatic stem cells are restricted in their developmental potential to their originating tissue, i.e. they are able to differentiate into all cell types of this tissue (Figure 2).

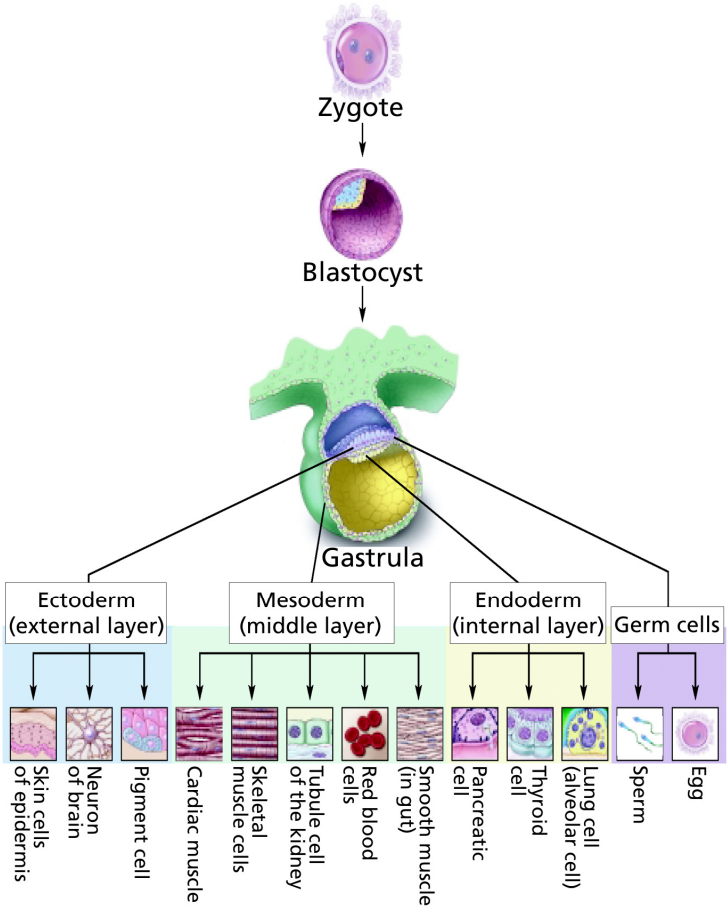


Figure 2: Stem cell hierarchy and potential during development. During embryogenesis the zygote progresses via the morula stage to the blastocyst. The ICM of the blastocyst forms the three germ layers, the endoderm, mesoderm and ectoderm, and the germ cells. As the embryo develops further through organogenesis, the developmental potential of the cells decreases as they become more specialised and determined. Only a small number of somatic stem cells retain proliferation and differentiation potential in the adult organism. (Picture: © 2001 Terese Winslow, Caitlin Duckwall, http://stemcells.nih.gov/info/scireport/chapter1.asp)

3.1.2 Embryonic Stem Cells

The isolation and stable cultivation of pluripotent embryonic cells from the ICM of pre-implantation blastocyst has first been described for murine cells in 1981 (Evans and Kaufman, 1981; Martin, 1981). In 1998 the establishment of pluripotent ES cells from human blastocysts was reported (Thomson *et al.*, 1998).

Murine ES cells remain undifferentiated when co-cultured on a feeder layer of MEFs in the presence of leukaemia inhibitory factor (LIF) (Williams *et al.*, 1988; Gough *et al.*, 1989). While maintaining an undifferentiated state over an extensive period of cell culture time, murine ES cells can contribute to the developing embryo when injected in blastocyst. The arising embryo is chimeric, formed from cells that originate from the blastocysts ICM as well as from cells derived from the

injected ES cells. When these blastocysts are transferred into pseudo-pregnant fosters, chimeric embryos develop. Injected ES cells contribute to all three germ layers and to the germ line (Bradley *et al.*, 1984; Beddington and Robertson, 1989).

ES cells have a second fascinating property. ES cells are capable to differentiate *in vitro* into cells of all three germ layers (Doetschman *et al.*, 1985; Wobus *et al.*, 1988; Reubinoff *et al.*, 2000; Schuldiner *et al.*, 2000) and into germ cells (Hubner *et al.*, 2003; Toyooka *et al.*, 2003). The withdrawal of LIF and feeder cells leads to the spontaneous differentiation of ES cells and the formation of embryoid bodies consisting of a plethora of progenitor and effector cell types (Doetschman *et al.*, 1985; Wobus *et al.*, 1988). The enrichment and selection of specific cell population for further utilisation is possible by adjusting differentiation conditions and by the addition of cell lineage-specific growth factors.

Modifying the ES cell genome prior to *in vitro* differentiation yields genetically engineered progenitor / effector cells. This technique is the basis for the potential use of "genetically repaired" patient specific ES cells in therapy. Furthermore, the genetic modification of ES cells enables the generation of transgenic animals (Evans and Kaufman, 1981; Robertson *et al.*, 1986; Doetschman *et al.*, 1987; Thomas and Capecchi, 1987; Rideout *et al.*, 2002; Christoforou and Gearhart, 2007).

A wide range of *in vitro* differentiation procedures that generate ES cell-derived neural progeny is available (Okabe *et al.*, 1996; Brüstle *et al.*, 1997; Lee *et al.*, 2000; Barberi *et al.*, 2003; Ying *et al.*, 2003). With these procedures mature neural cell types have been generated, utilizing an intermediate progenitor cell stage, where cells can be expanded *in vitro* for several passages. These progenitors can give rise to a variety of differentiated neural cell types such as GABAergic, glutaminergic and dopaminergic neurons, astrocytes and oligodendroglia (Brüstle *et al.*, 1999; Kim *et al.*, 2002). Therefore ES cells are capable of unlimited self-renewal, *in vitro* multi-lineage differentiation and *in vivo* contribution to all tissues of a developing embryo. Since ES cells are not capable to form extra-embryonic tissues and to give rise to a complete organism, they are termed pluri- but not totipotent (Nagy *et al.*, 1990). Pluripotent ES cells are promising cell types for basic research and regenerative medicine.

3.1.3 Adult Stem Cells

Following gastrulation and the formation of endo-, meso- and ectoderm, somatic stem cell systems are established. The different stem cell types arise in different tissues at different time points during embryogenesis (Müller *et al.*, 1994; Qian *et al.*, 2000). In the developing embryo, these stem cells enable the formation of specialised cells and tissues during organogenesis. In the adult organism, stem cell systems are responsible for maintaining tissue homoeostasis and for regeneration and repair of damaged tissues. To fulfil this function, somatic stem cell systems are hierarchically organised, with a small population of undifferentiated stem cells at the top of the hierarchy. These rare cells give rise to more committed progenitor cells or transient amplifying cells, which generate the terminally differentiated effector cells. Somatic stem cells have so far been described for a number of adult tissues in mammals, e.g. epidermal stem cells in the skin (Watt, 1998), stem cells of the hematopoietic system (Morrison *et al.*, 1995), mesenchymal stem cells (Bianco *et al.*, 2008), intestinal crypt cells in the gut (Moore and Lemischka, 2006) and neural stem cells of the nervous system (Weiss *et al.*, 1996a; Weiss *et al.*, 1996b).

3.1.3.1 Neurospheres and Neural Stem Cells

In contrast to tissues with high cell turnover, e.g. the hematopoietic system, intestine or skin, the mammalian central nervous system has been considered as incapable to compensate damage to its cellular structure by the regeneration of lost cells. Newer studies rebut this long-held assumption and show formation of newborn neurons also in the adult brain albeit restricted to two brain areas: the subependymal layer and the dentate gyrus (Mayer-Proschel *et al.*, 1997; Mujtaba *et al.*, 1998; Vescovi *et al.*, 1999; Vescovi and Snyder, 1999; Colombo *et al.*, 2005). Neural stem cells (NSC) can be isolated from adult brain tissue of these regions and from foetal forebrains of mice, rats and humans (Doetsch, 2003; Morshead and van der Kooy, 2004). Supplemented with the growth factors epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF), NSCs can be expanded in culture. Depending on culture conditions, NSCs grow either as an adherent cell layer (Conti *et al.*, 2005; Pollard *et al.*, 2006) or they form free-floating and tightly packed cellular aggregates, so called neurospheres. Neurospheres contain a variety of neural cell types at diverse differentiation stages. In optimal culture conditions, a population of NSCs is maintained within the neurospheres over an extended number of passages, with full multi-lineage differentiation potential (Figure 3). Upon withdrawal of growth factors and the induction of differentiation either by

adding serum or neural differentiation supplement to the culture medium, NSCs differentiate into neurons, astro- and oligodendroglia (Mayer-Proschel *et al.*, 1997; Rao and Mayer-Proschel, 1997; Doetsch *et al.*, 1999; Gritti *et al.*, 1999).

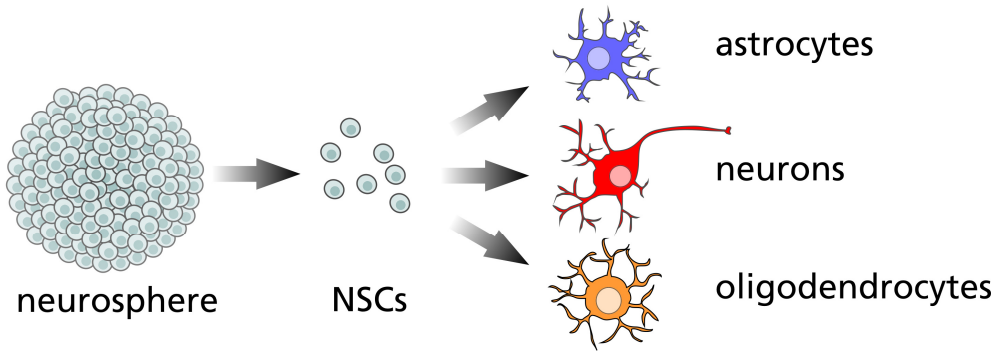


Figure 3: Differentiation of neurosphere cells. Neurospheres are free-floating cell aggregates that contain neural stem cells (NSC). NSCs are capable to clonally differentiate into the three neural lineages, neuronal cells, astrocytes and oligodendrocytes.

3.2 Uniparental Cells – Generation and Properties

Uniparental cells are cells which inherited genetic material from only one parental side, so that they contain either only maternal or paternal DNA, respectively. While parthenogenesis, the development of viable offspring from an unfertilised oocyte, is described as an obligate route of reproduction in several vertebrates e.g. snakes, lizards, sharks, in which it occurs spontaneously and occasionally, it is no option in mammalians. Similar to parthenogenetic (PG) cells that are derived from unfertilised oocytes, upon activation by chemical or other means, uniparental cells can also be established from zygotes by pronuclear exchange (see 3.2.1). Maternal-only, gynogenetic (GG) cells originate from zygotes with two maternal pronuclei. Paternal-only, androgenetic (AG) cells derive from zygotes with two paternal pronuclei.

Uniparental PG embryos have been experimentally generated in several mammalian species including mice (Graham, 1970), cattle (Lagutina *et al.*, 2004)), primates (Macaca fascicularis (Cibelli *et al.*, 2002)) and humans (Revazova *et al.*, 2007). Similarly, AG and / or GG embryos have been produced from a variety of mammals, e.g. of mice (AG/GG embryos (Surani and Barton, 1983; McGrath and Solter, 1984; Surani *et al.*, 1984)) and cattle (AG embryos (Lagutina *et al.*, 2004)), but not from humans so far.

3.2.1 Androgenetic and Gynogenetic ES Cells

For the generation of AG or GG embryos by pronuclear transfer, oocytes are either fertilised by *in vitro* fertilisation (IVF) or by intracytoplasmatic sperm injection (ICSI). To generate a uniparental AG embryo the maternal pronucleus is removed from the fertilised oocyte before the fusion of the pronuclei. From a second fertilised oocyte in the state before pronuclear fusion the paternal pronucleus is extracted and injected into the fertilised oocyte lacking the maternal pronucleus. The pronuclei fuse and the uniparental zygote develops further till the blastocyst stage. Zona prelucida-free blastocysts are placed on MEF layers and ES cells are established from the outgrowth of the ICM (McGrath and Solter, 1983; Eckardt *et al.*, 2007) (Figure 4). Uniparental GG ES cells can be produced in the same way, by removing the paternal pronucleus from the fertilised oocyte, replacing it by a second maternal pronucleus.

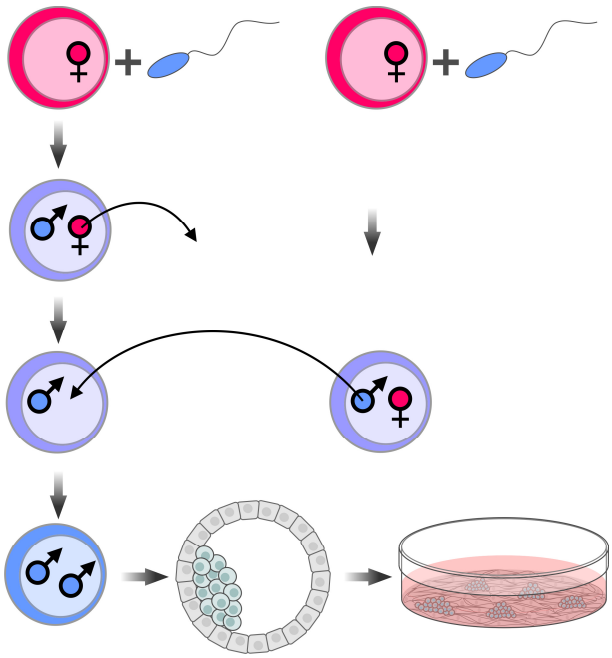


Figure 4: Generation of uniparental AG ES cells. By removing the maternal pronucleus from a fertilised oocyte prior to pronuclear fusion and replacing it by a second paternal pronucleus, a uniparental zygote is produced with only paternal genetic information. This uniparental zygote is not capable of developing into a viable offspring, but it can develop to the blastocyst stage. AG ES cells are generated from the ICM of uniparental blastocysts.

3.2.2 Parthenogenetic ES Cells

Uniparental PG embryos are generated by arresting oocytes in the second meiotic metaphase with cytochalasin, which prevents the extrusion of the second polar body (Balakier and Tarkowski, 1976). The arrested oocytes are chemically activated to continue development, using e. g. strontium chloride (Eckardt *et al.*, 2007). Diploidy is maintained and the resulting pseudo-zygotes can develop further into blastocysts from which uniparental PG ES cells can be established (Graham, 1974; Mann, 2001; Kim *et al.*, 2007).

3.2.3 Developmental Potential of Uniparental Embryos and Uniparental ES Cells

Mammalian, including human, uniparental embryos are not viable. Still, they can advance in embryonic development up to the blastocyst stage. This developmental state is sufficient for the derivation of ES cells (Robertson *et al.*, 1983; Mann *et al.*, 1990).

Uniparental cleavage stage blastomeres, ICM cells and ES cells can contribute to foetal and postnatal development, when combined at preimplantation stages with normal mouse embryos (Stevens *et al.*, 1977; Surani *et al.*, 1977; Mann *et al.*, 1990; Barton *et al.*, 1991; Mann and Stewart, 1991). Both AG and PG/GG cells have been found to contribute in chimaeras to all cell lineages including the germ line (Stevens, 1978; Narasimha *et al.*, 1997). Studies on murine chimeric embryos that develop following blastocyst injection of uniparental ICM cells or uniparental ES cells have provided insights into the parent-specific contribution to foetal development. Both AG and PG/GG cells contribute with a high percentage to chimaeras in the first half of gestation. In the second half of gestation an overall reduction of uniparental cells in chimeric embryos occurs (Barton *et al.*, 1991). This has been interpreted as a selective elimination process, while the reason and mechanism of this reduction remain open (Fundele *et al.*, 1989). PG and GG cells preferentially generate brain and less mesodermal tissues and are rarely found in skeletal muscle. AG cells, on the other hand, are preferentially detected in mesodermal derivatives and lesser in brain. In general, AG chimaeras are affected stronger than PG chimaeras. AG cells cause severe defects in foetal growth, causing organomegaly, skeletal deformations and frequent lethality when combined with N cells in chimaeras (Mann *et al.*, 1990; Barton *et al.*, 1991; Mann and Stewart, 1991). Typically, an overall contribution of more than 10% of AG cells results in increased embryonic and perinatal lethality (Narasimha *et al.*, 1997). For PG cells on the contrary, proliferative defects have been observed (Nagy *et al.*,

1989; Jagerbauer *et al.*, 1992; Newman-Smith and Werb, 1995; Hernandez *et al.*, 2003). The bias in their contribution to developing embryos could indicate differences in the developmental competence of uniparental maternal and paternal cells (Fundele *et al.*, 1989; Nagy *et al.*, 1989; Paldi *et al.*, 1989; Fundele *et al.*, 1990; Mann *et al.*, 1990; Barton *et al.*, 1991).

Analysis of foetal brain development in AG and PG/GG chimaeras developing either from aggregating N morulas and PG or AG embryos at an early stage of development or following injection of uniparental ICM cells into N blastocysts, which produced PG–N and AG–N chimaeras, respectively, revealed that low contribution mice survived to term, but they had different brain phenotypes (Allen *et al.*, 1995; Keverne *et al.*, 1996). The brains of PG–N chimaeras, especially the forebrains, were relatively large in comparison to non-chimeric controls, contrasting the animals' smaller overall body size. In contrast, AG–N chimaeras had relatively small brains but larger bodies. Using PG and AG cells expressing the reporter gene *lacZ* enabled the localisation of AG- or PG-derived cells in the brain in more detail. This analysis revealed that the distribution of PG and AG cells was not random — rather, distinct patterns were detected. Although both AG and PG cells were distributed throughout the brain early in development, AG and PG cells established at later stages of embryogenesis defined and persistent localisation-patterns in distinct brain regions. AG cells substantially contribute to hypothalamic structures but less to the cortex and striatum. Conversely, PG cells contributed substantially to neocortical areas, to the striatum and to the hippocampus, but were not present in the brain regions that were colonised by the AG cells. The chimaera data were important because they pointed towards imprinted genes in the processes of neural development and indicated that the maternal and paternal genomes have dissociable effects on brain development (Keverne *et al.*, 1996).

Also, AG ES cells contributed lesser to brain upon blastocyst injection although the regional distribution of AG cells in brain was not further analysed (Mann *et al.*, 1990). Together, these observations argue, that paternal and maternal genomes have differential influences on brain development.

While various properties, in particular the phenotype of ES cell chimaeras generated with AG ES cells (Mann *et al.*, 1990; Allen *et al.*, 1994) indicate that genomic imprinting is similar in uniparental ES cells compared to primary embryonic cells such as those derived from cleavage stage embryos or the ICM of uniparental blastocysts, some phenotypic differences do exist between PG ES cell

and PG aggregation chimaeras (Allen *et al.*, 1994; Hernandez *et al.*, 2003). PG ES cell chimaeras do not exhibit the growth deficits that are observed in PG aggregation chimaeras (Allen *et al.*, 1994). Also the exclusion of PG cells from liver or muscle (Fundele *et al.*, 1989; Nagy *et al.*, 1989) has not been similarly observed with PG ES cells, indicating differences in the developmental potential of these cells that could be associated with epigenetic changes resulting from ES cell derivation and culture (Dean *et al.*, 1998; Humpherys *et al.*, 2001; Jiang *et al.*, 2007).

In a recent study, the functionality of GG and AG ES cell-derived transplants has been demonstrated in a mammalian species (Eckardt *et al.*, 2007). Both, murine AG and GG ES cell-derived foetal liver hematopoietic stem cells (HSCs) conveyed long-term and multi-lineage reconstitution of the entire hematopoietic system in transplant recipients. In contrast to the defects observed in AG chimaeras, AG ES cell-derived hematopoietic cells in adult recipients did not exhibit any abnormal phenotypes. The expression levels of imprinted genes in lymphocytes of either AG or GG origin were typically low, without parent-of-origin-specific bias. It seems therefore that correct expression of imprinted genes is not required for normal function or that normal expression is re-established by resetting the imprinting status, at least in hematopoietic cells.

The derivation of pluripotent monkey PG ES cells capable of differentiation into ecto-, endo- and mesodermal derivatives opened the discussion of using PG ES cells as a source of autologous material for transplantation (Cibelli *et al.*, 2002). PG ES cells isolated from monkey demonstrate the ability to differentiate into neural precursors and into cells with neuronal morphologies and characteristics of functional neurons. Further *in vivo* analyses show the long-term survival of dopaminergic neurons derived from PG monkey ES cells after transplantation into immunosuppressed rats (Sanchez-Pernaute *et al.*, 2005).

Looking into the problem of graft rejection, Kim *et al.* (Kim *et al.*, 2007) demonstrated that murine PG ES cells can be selected for major histocompatibility complex (MHC) compatibility and that injection of differentiated PG ES cells expressing matched MHC antigens leads to teratoma formation in immune competent hosts. In addition, the isolation and characterisation of six human PG ES cell lines was described (Revazova *et al.*, 2007). Human PG ES cells are able to differentiate into cell types from all three germ layers and they are MHC-matched with the oocyte donors (Revazova *et al.*, 2007). In contrast to PG ES cells, AG ES cells have not been similarly examined, in part because of the abnormal

phenotype of murine AG ES cell chimaeras (Mann *et al.*, 1990; McLaughlin *et al.*, 1997) and the lack of derivation of non human primate and human AG ES cells.

3.3 Imprinting: Parent-of-Origin-Specific Gene Expression

Insights into genomic imprinting and its role in adult tissue formation are important prerequisites for the therapeutic applicability of uniparental ES cell-derived cells. Imprinting is the epigenetic mechanism by which gene expression is modulated so that maternal and paternal alleles are differentially expressed (Efstratiadis, 1994; Solter, 1998). Imprinted gene expression is a spatiotemporally dynamic process, i.e. differential gene expression from the parental alleles can be limited to specific tissues or even specific cells at certain times in development. In contrast to non-imprinted genes, whose gene expression is either "on" (activated) or "off" (silenced), the expression of imprinted genes has three states, silenced, bi-allelic or mono-allelic activated (Solter, 1998; Wilkinson *et al.*, 2007). The allele-specific expression is achieved on the molecular level by epigenetic modification of the DNA, primarily by cytosine-methylation (Jaenisch, 1997; Reik and Walter, 2001). Most imprinted genes are clustered around imprinting centres, which are differentially methylated (Delaval and Feil, 2004). During gametogenesis, these epigenetic marks are erased and subsequently re-established in a parent-of-origin-specific pattern (Walter and Paulsen, 2003).

To date, more than 75 imprinted genes have been identified in the genomes of mice and humans (Morison and Reeve, 1998; Wilkinson *et al.*, 2007) (www.geneimprint.com). A large number of imprinted genes is expressed in the placenta and in the brain (Constancia *et al.*, 2005; Davies *et al.*, 2005b). The deregulation of the imprinting-driven gene expression of several genes has been observed in a number of human diseases (Falls *et al.*, 1999; Walter and Paulsen, 2003).

3.3.1 Imprinting in Neural Development

A large number of imprinted genes that have been discovered so far are expressed in the brain (although not for all of these genes the expression is exclusively restricted to brain tissue). These genes show complex spatial and temporal expression patterns. Expression strongly varies in terms of where (i.e. what brain region or even which cell type) and when during development they are expressed. Their imprinting status is modified in some cases as well. For example, imprinting of *Ube3a*, which encodes the ubiquitin ligase protein Ube3A, is maternally expressed. Imprinted *Ube3A* expression is confined to distinct

neuronal populations of the olfactory bulb, the hippocampus and the cerebellum. Elsewhere in the brain and in other body tissues, *Ube3A* is biallelically expressed (Albrecht *et al.*, 1997; Rougeulle *et al.*, 1997). The imprinted gene *SnrpN* (small nuclear ribonucleoprotein polypeptide N) is expressed paternally and imprinting is maintained into adulthood (Runte *et al.*, 2004). The paternally imprinted gene *Zim1* (Zink finger, imprinted 1) is only maternally expressed in all tissues apart from the neonatal and adult brain, where it is biallelically expressed (Kim *et al.*, 1999). The maternally expressed gene *H19* (non-protein coding imprinted maternally expressed transcript) is highly expressed during embryogenesis and in the neonatal period. It is only low and spatially limited expressed in the adult brain (Pham *et al.*, 1998). The paternally expressed, maternally imprinted gene *Igf2* (Insulin-like growth factor 2) is biallelically expressed in the foetal brain, with monoallelic expression in other foetal tissues. In the adult brain, there is biallelic expression in the pons and monoallelic expression in the globus pallidus, the Raphe nucleus and the hypothalamus (Pham *et al.*, 1998).

These examples emphasise the dynamic nature of imprinted-gene expression in the brain, which adds a further level of control to imprinted-gene function. A full developmental profile of brain-expressed imprinted genes has not yet been established so far. Furthermore, no organisational patterns have yet emerged with regard to the direction of imprinting (i.e. whether a gene is maternally or paternally expressed). Nonetheless, it is clear that imprinted genes are active at key developmental time points and that the expression of these genes is not confined to the prenatal period. Expression and imprinting can persist into the postnatal period and beyond, sometimes into the adult brain (Albrecht *et al.*, 1997; Davies *et al.*, 2005a). This suggests that many imprinted genes being in a position to influence early brain development and differentiation *in utero* might also contribute to processes of brain development and sculpting that continue after birth. The persistence of imprinting in the adult brain might also, indicate that imprinted genes have functions that are independent of neural development (Wilkinson *et al.*, 2007).

3.4 Aim of this Study and Experimental Strategy

While the *in vitro* and *in vivo* neural differentiation potential of GG/PG ES cells is well characterised, the neural developmental potential of AG ES cells is less clear. Since AG ICM cells showed a biased neural development (Keverne *et al.*, 1996), AG ES cells were not analysed equally. In an earlier study the group of K.

John McLaughlin reported that AG and GG cells conveyed long-term, multi-lineage, pathology-free hematopoietic engraftment (Eckardt *et al.*, 2007).

These findings led to this study that aimed to investigate the potential of AG uniparental murine ES cells to differentiate *in vitro* and *in vivo* into neural progenitor / stem cells and further into neuronal and glial cell types in comparison to GG and N ES cells (Figure 5).

A cell culture system was established to expand uniparental (AG, GG) and biparental N ES cells on MEFs. To assess the capability of *in vitro* neural differentiation of uniparental ES cells, a multi step-protocol was used to differentiate ES cells towards neuronal and glial cell types (Okabe *et al.*, 1996; Brüstle *et al.*, 1997). The ability of terminal neural differentiation was analysed by fluorescence microscopy using neuronal and glial lineage markers.

To analyse the *in vivo* contribution of AG ES cells to neural tissue of developing embryos, $eGFP^+$ AG uniparental or $eGFP^+$ N ES cells were injected into blastocysts prior to transfer into foster mothers. At E12.5 and E14.5, embryos were isolated, the overall grade of chimerism was determined by flow cytometry, forebrains were dissected and $eGFP^+$ donor cells were isolated from chimeric brains by FACS. To further investigate the neural developmental capability of AG cells from chimeric brains, $eGFP^+$ donor and corresponding $eGFP^-$ host neurosphere cell populations were expanded and analyses of the neural differentiation potential and self-renewal capacity were performed. In parallel, cryosections of E12.5 chimeric brains were analysed for donor contribution to the neuronal lineage to detect the contribution and localisation of AG ES cell-derived cells in the chimeric brains by immunofluorescence microscopy.

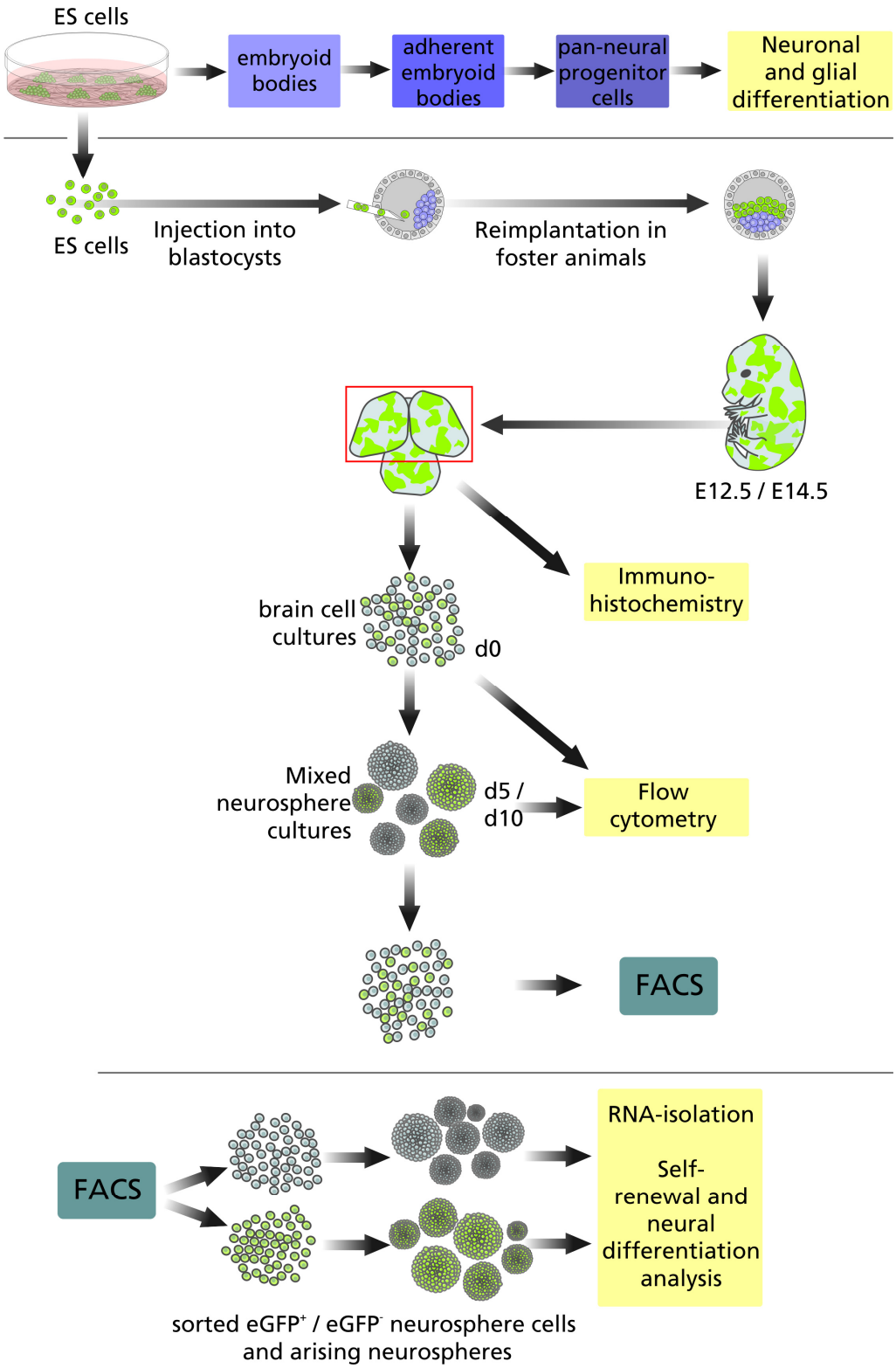


Figure 5: Experimental strategy. ES cells with ubiquitinous eGFP-transgene expression were expanded on murine MEFs and harvested afterwards. ES cells were differentiated towards neural phenotype. The ability of terminal neural differentiation was analysed by fluorescence microscopy using neuronal and glial lineage markers. In parallel AG chimeric embryos were generated by blastocyst injection. Chimeric brains were either immunohistochemically analysed or dissected and neurospheres were cultured. Single cell suspensions of neurospheres were sorted. Both eGFP$^+$ and eGFP$^-$ cell populations were analysed to study the neural differentiation potential and the self-renewal capacity.

4 MATERIAL

4.1 Mouse Strains

Mouse Strain	Utilisation	Source
NMRI	Blastocyst generation, foster animals, murine embryonic fibroblast generation	Harlan Winkelmann, Borchen, Germany (www.harlan-winkelmann.de)
C57Bl/6J	Wild-type embryos and embryonic cells for controls (flow cytometry, immunohistochemistry)	Harlan Winkelmann
OSB (eGFP-transgenic)	eGFP-transgenic embryos and embryonic cells for controls (flow cytometry, immunohistochemistry)	Masaru Okabe (Okabe *et al.*, 1997) Animal Facility (MSZ)

All animals were bred and maintained in the animal facility at the Institute for Medical Radiation and Cell Research (MSZ), University of Würzburg or obtained from Harlan Winkelmann (Borchen, Germany), and used for experimentation in accordance to the animal protection guidelines of the Government of Unterfranken (Würzburg, Germany).

4.2 Cell Lines

Cell Line	Cell Type	Source
A1 (AGA2) (B6Osb x 129)	Uniparental murine ES cells, **androgenetic**, eGFP-transgenic	S. Eckardt and K. J. McLaughlin (Eckardt *et al.*, 2007) University of Pennsylvania
A2 (AGB6) (B6Osb x 129)	Uniparental murine ES cells, **androgenetic**, eGFP-transgenic	S. Eckardt and K. J. McLaughlin
GG (GGA5) (B6Osb x 129)	Uniparental murine ES cells, **gynogenetic**, eGFP-transgenic	S. Eckardt and K. J. McLaughlin
N (NB3+) (129Sv GFPX)	Biparental (normal fertilised; N) murine ES cells, eGFP-transgenic	S. Eckardt and K. J. McLaughlin

4.3 Antibodies

4.3.1 Primary Antibodies

Specificity	Clone	Isotyp	Supplier
eGFP	Polyclonal	Chicken IgY	Abcam, Cambridge, UK (www.abcam.com)
Tubulin-ß-III	Tuj-1	Mouse IgG	R&D Systems, Minneapolis, USA (www.rndsystems.com)
NeuN	A60	Mouse IgG	Chemicon, Schwalbach, Germany (www.chemicon.com)
PCNA	PC10	Mouse IgG	BD Biosciences, Heidelberg, Germany (www.bdbiosciences.com)
Cleaved Caspase 3	Asp175 polyclonal	Rabbit Ig	Cell Signaling Technology, NEB, Frankfurt, Germany (www.cellsignal.com)
GFAP	polyclonal	Rabbit Ig	Dako, Hamburg Germany (www.dakogmbh.de)
O4	O4	Mouse IgM	R&D Systems, Minneapolis, USA

4.3.2 Secondary Antibodies

Specificity	Host	Conjugate	Supplier
Chicken IgY	Goat	Cy2	Abcam, Cambridge, UK
Mouse IgG	Goat	Cy3	Chemicon, Schwalbach, Germany
Rabbit IgG	Goat	Cy3	Chemicon, Schwalbach, Germany

4.4 Cell Culture-related Materials

4.4.1 Media and Supplements

AccuMax	PAA Laboratories, Cölbe, Germany (www.paa.at)
B27 supplement	Gibco Invitrogen, Karlsruhe, Germany (www.invitrogen.com)
DMEM / Ham's F12	PAA Laboratories, Cölbe, Germany
DMEM high glucose	PAA Laboratories, Cölbe, Germany
FCS cell-culture grade	Biochrom, Berlin, Germany (www.biochrom.de)
FCS ES cell-culture grade	HyClone, Thermo Fisher Scientific, Schwerte, Germany (www.hyclone.com)
HBSS with Mg^{2+}/Ca^{2+}	Gibco Invitrogen, Karlsruhe, Germany
HEPES buffer (100 mM)	PAA Laboratories, Cölbe, Germany
L-glutamine (200 mM)	PAA Laboratories, Cölbe, Germany
M2 medium	Sigma-Aldrich, Schnelldorf, Germany (www.sigmaaldrich.com)
M16 medium	Sigma-Aldrich, Schnelldorf, Germany
ß-Mercaptoethanol	Sigma-Aldrich, Schnelldorf, Germany
Neurobasal Medium	Gibco Invitrogen, Karlsruhe, Germany
NeuroCult Differentiation Supplement	StemCell Technologies, CellSystems Biotechnologie, St. Katharinen, Germany (www.stemcell.com) (www.cellsystems.de)
Non Essential Amino Acids	PAA Laboratories, Cölbe, Germany
PBS without Mg^{2+}/Ca^{2+}	PAA Laboratories, Cölbe, Germany
Penicillin / Streptomycin (10000 U/mL)	PAA Laboratories, Cölbe, Germany
Sodium pyruvate (100 mM)	PAA Laboratories, Cölbe, Germany
Trypsin / EDTA	PAA Laboratories, Cölbe, Germany

4.4.2 Growth Factors

Fibronectin	Sigma-Aldrich, Schnelldorf, Germany
Human EGF, recombinant	PeproTech, Hamburg, Germany (www.peprotech.com)
Human FGF-basic, recombinant	PeproTech, Hamburg, Germany
Human transferrin	Sigma-Aldrich, Schnelldorf, Germany
Insulin	Sigma-Aldrich, Schnelldorf, Germany
LIF-Conditioned Medium	AG Müller, MSZ, Würzburg, Germany (Williams *et al.*, 1988; Wenger *et al.*, 1995)
Progesterone	Sigma-Aldrich, Schnelldorf, Germany
Putrescine	Sigma-Aldrich, Schnelldorf, Germany

4.5 Chemicals

4.5.1 Buffers and Solutions

Phosphate buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.3 in H_2O.

MPS buffer: 80 mM PIPES, 1 mM $MgCl_2$, 5 mM EGTA, 0.5 % Triton X-100; pH 6.8 in H_2O.

10 mM Citrate buffer pH6: 9 mL 0.1 M citric acid in H_2O + 41 mL 0.1 M Trisodium citrate dehydrate in H_2O + 450 mL H_2O.

Endogenous blocking solution: 3 % H_2O_2 in Methanol.

4.5.2 Chemicals

Acetic acid	Applichem, Darmstadt, Germany (www.applichem.de)
Boric acid	Applichem, Darmstadt, Germany
BSA (30 % sterile solution)	PAA Laboratories, Cölbe, Germany
DAPI	Sigma-Aldrich, Schnelldorf, Germany
DEPC	Applichem, Darmstadt, Germany
DMSO	Sigma-Aldrich, Schnelldorf, Germany

DNA size marker	MBI Fermentas (www.fermentas.de)
DNase I	Ambion, Austin, USA (www.ambion.com)
DNase 10X buffer	Ambion, Austin, USA
dNTPs	Gibco Invitrogen, Karlsruhe, Germany
DTT	Sigma-Aldrich, Schnelldorf, Germany
EDTA	Applichem, Darmstadt, Germany
Ethanol	Applichem, Darmstadt, Germany
Ethidium bromide	Merck, Darmstadt, Germany (www.merck.de)
First Strand Buffer 5X	Gibco Invitrogen, Karlsruhe, Germany
Fluorescence mounting medium	Dako, Hamburg, Germany
Gelatine	Sigma-Aldrich, Schnelldorf, Germany
Glycerol	Applichem, Darmstadt, Germany
Goat serum	Sigma-Aldrich, Schnelldorf, Germany
H_2O_2 (30 %)	Applichem, Darmstadt, Germany
hCG	Intervet, Unterschleißheim, Germany (www.intervet.de)
HCl, 37%	Applichem, Darmstadt, Germany
Isopropanol	Applichem, Darmstadt, Germany
L-glycine	Sigma-Aldrich, Schnelldorf, Germany
Laminin	Sigma-Aldrich, Schnelldorf, Germany
Methanol	Applichem, Darmstadt, Germany
M-MLV reverse transciptase	Gibco Invitrogen, Karlsruhe, Germany
Oligo-dT	Gibco Invitrogen, Karlsruhe, Germany
Paraformaldehyde	Applichem, Darmstadt, Germany
peq Gold RNA pure	peq Labs, Göttingen, Germany (www.peqlab.de)
Phenol-chloroform-isoamyl alcohol	Applichem, Darmstadt, Germany
PMSG	Intervet, Unterschleißheim, Germany

poly-L-ornithine	Sigma-Aldrich, Schnelldorf, Germany
Potassium chloride	Applichem, Darmstadt, Germany
QPCR SYBR green mix	ABgene, Hamburg, Germany (www.abgene.com)
SDS	Sigma-Aldrich, Schnelldorf, Germany
Sodium azide	Applichem, Darmstadt, Germany
Sodium borate	Applichem, Darmstadt, Germany
Sodium chloride	Applichem, Darmstadt, Germany
Sodium citrate	Applichem, Darmstadt, Germany
Sodium hydroxide	Applichem, Darmstadt, Germany
Sodium selenite	Sigma-Aldrich, Schnelldorf, Germany
Tris	Applichem, Darmstadt, Germany
Triton X-100	Fluka BioChemika (Sigma-Aldrich), Schnellendorf, Germany
Trypan blue	Sigma-Aldrich, Schnelldorf, Germany
Tween 20	Gibco Invitrogen, Karlsruhe, Germany
Water Ultra pure	Merck, Darmstadt, Germany

4.5.3 Oligonucleotide Primers

ß-*actin* (Eckardt *et al.*, 2007)

f: 5'-GATATCGCTGCGCTGGTCGTC-3'/ r: 5'-CGCAGCTCATTGTAGAAGGTGTGG-3';

Igf2 (Eckardt *et al.*, 2007)

f: 5'-CTAAGACTTGGATCCCAGAACC-3'/ r: 5'-GTTCTTCTCCTTGGGTTCTTTC-3';

Impact (Eckardt *et al.*, 2007)

f: 5'-ACGTTTCCCCATTTTACAAG-3'/ r: 5'-CTCTACATATGATTTTCTCTAC-3';

Igf2r (Eckardt *et al.*, 2007)

f: 5'-TAGTTGCAGCTCTTTGCACG-3'/ r: 5'-ACAGCTCAAACCTGAAGCG-3';

Ube3a (Eckardt *et al.*, 2007)

f: 5'-CACATATGATGAAGCTACGA-3'/ r: 5'-CACACTCCCTTCATATTCC-3';

Zim1 (Kim *et al.*, 1999)

f: 5'-GAGAAGCCGTACTGCTGTCA-3'/ r: 5'-CTTGCACCGGTACCTGGAGT-3'

H19 (Wu *et al.*, 2006)

f: 5'-CATGTCTGGGCCTTTGAA-3'/ r: 5'-TTGGCTCCAGGATGATGT-3'

All Oligonucleotide Primers were ordered at and produced by eurofins MWG opreon, Ebersberg, Germany (http://www.eurofinsdna.com/de/).

4.6 Consumables

Bacteriological Petri dishes	Hartenstein Laborbedarf, Würzburg, Germany (www.laborbedarf.de)
0,5 mL and 1,5 mL caps	Eppendorf, Wesseling-Berzdorf, Germany, (www.eppendorf.de)
Cell culture flasks	Greiner Bio One, Essen, Germany (www.gbo.com/bioscience) Sarstedt, Nümbrecht, Germany (www.sarstedt.com) Nunc (Thermo Fisher Scientific), Wiesbaden, Germany (www.nunc.de)
Cell Strainer (70 µm)	BD Biosciences, Heidelberg, Germany
Centrifuge tubes (15 mL & 50 mL)	Greiner Bio One, Essen, Germany Nunc (Thermo Fisher Scientific), Wiesbaden, Germany
Cover slips	Hartenstein Laborbedarf, Würzburg, Germany
Disposable gloves	Kimberly-Clark, Koblenz, Germany (www.kimberly-clark.com)
FACS tubes	Greiner Bio One, Essen, Germany
Glass beads 5 mm	Hartenstein Laborbedarf, Würzburg, Germany
Multi-well cell culture plates	Greiner Bio One, Essen, Germany Nunc (Thermo Fisher Scientific), Wiesbaden, Germany
Parafilm M	Brand, Wertheim, Germany (www.brand.de)

Pipette tips	Hartenstein Laborbedarf, Würzburg, Germany
Scalpels	Ratiomed, Schloß Holte-Stukenbrock, Germany (Website not available)
Sterile filter	Schleicher & Schuell, Dassel, Germany (www.schleicher-schuell.de)
Super-Frost® Plus slide	Menzel-Gläser, Braunschweig, Germany (www.menzel.de)
Syringes	B. Braun, Melsungen, Germany (www.bbraun.de) Dispomed, Gelnhausen, Germany (www.dispomed.de)
Tissue culture plates	Greiner Bio One, Essen, Germany

4.7 Instruments

BioZero microscope	Keyence, Neu-Isenburg, Germany (www.keyence.de)
Cell freezing container	Nalgene, Hereford, UK (www.nalgenelabware.com)
Centrifuges	Hettich Zentrifugen, Tuttlingen, Germany (www.hettich-zentrifugen.de/) Eppendorf, Wesseling-Berzdorf, Germany, Thermo Scientific (Sorvall), Dreieich, Germany (www.thermo.com)
Digital camera	Olympus, Hamburg, Germany (www.olympus.de)
Digital weighting balance	Sartorius, Göttingen, Germany (www.sartorius.de)
FACS Diva Cell Sorter	BD Biosciences, Heidelberg, Germany
FACSCalibur	BD Biosciences, Heidelberg, Germany
Gene Rotor Real Time PCR	Corbett Life Sciences (LTF Labortechnologie), Germany (www.corbettlifesciences.com)

Incubator	Thermo Scientific (Forma Scientific), Dreieich, Germany
Inverted microscope	Carl Zeiss, Jena, Germany (www.zeiss.de)
Neubauer chamber	Marienfeld, Lauda-Königshofen, Germany (www.marienfeld-superior.com)
pH-Meter	Knick, Berlin, Germany (www.knick.de)
Pipettes	Gilson, Middleton, WI, USA (www.gilson.com)
	Abimed, Hilden, Germany (www.abimed.de)
BioPhotometer	Eppendorf, Wesseling-Berzdorf, Germany
Sterile bench	Heraeus, Hanau, Germany (www.heraeus.de)
Vortexer	Scientific Industries, New York, USA (www.scientificindustries.com)
Water bath	GFL Gesellschaft für Labortechnik, Burgwedel, Germany (www.gfl.de)

5 METHODS

5.1 Cell Culture

5.1.1 Establishing Primary Murine Embryonic Fibroblasts (Feeder Cells)

For the generation of primary MEFs pregnant female mice were sacrificed by cervical dislocation at 13 days post coitum (dpc). The uterine horns, were dissected out, briefly rinsed in 70 % (v/v) ethanol and placed into a Petri dish containing PBS without bivalent cations. Each embryo was separated from its placenta and surrounding membranes. Embryonic head and dark red organs were removed; the remaining embryo was washed with fresh PBS to remove as much blood as possible. Using a minimal amount of PBS and a scalpel, the embryos were finely minced until they were "pipettable". Triturated tissue was suspended in 1 mL Trypsin / EDTA per embryo and incubated with gentle shaking at 37°C for 15 minutes together with a 15-20 5 mm glass beads. To digest genomic DNA from ruptured or lysed cells 100 kU DNase I per mL Trypsin / EDTA were added before the incubation. To remove remaining pieces of tissue, the suspension was transferred to a 50 mL tube and two volumes of Dulbecco's modified Eagle's medium (DMEM) high glucose supplemented with 10 % cell culture-grade FCS Penicillin (100 U/mL) / Streptomycin (100 U/mL), L-glutamine (2 mM) was added. Tissue pieces were let settle down to the bottom of the tube for a few minutes and the supernatant was carefully transferred in a fresh 50 mL tube to be subjected to low speed centrifugation for five minutes at 4°C. The resulting cell pellet was resuspended in warm DMEM high glucose supplemented with 10 % cell culture-grade FCS, Penicillin (100 U/mL) / Streptomycin (100 U/mL), L-glutamine (2 mM) and plated out at one embryo equivalent per 10 cm cell culture dish for adherent cells. The fibroblasts were cultured 24 hours at 37°C, 5 % CO_2. Medium was changed on the following day to remove all non-adherent cells. Remaining adherent fibroblasts were designated passage 0 and were either expanded for usage or frozen.

5.1.2 Murine Embryonic Stem Cell Culture

The AG and N eGFP$^+$ murine ES cells used in this study have been previously described (Eckardt *et al.*, 2007). ES cells were cultured in DMEM high glucose supplemented with 15 % Foetal Calf Serum (FCS), ES cell-culture grade, leukaemia inhibitory factor (LIF)-conditioned medium (Williams *et al.*, 1988; Wenger *et al.*, 1995), 1 % Non Essential Amino Acids (NEAA), Penicillin (100 U/mL) / Streptomycin

(100 U/mL), L-glutamine (2 mM), sodium pyruvate (1 mM) and ß-mercaptoethanol (0.1 mM) on confluent layers of primary MEFs. Medium was changed daily. For passage of ES cells, medium was removed, cells were washed with twice PBS and Trypsin / EDTA was added. After ten minutes at 37°C trypsinisation was stopped by adding 10 mL DMEM containing 15 % FCS. Cell suspensions were put on gelatine-coated plates (0.1 % in PBS) for 45 minutes at 37°C to allow separation of MEFs and ES cells. 5 x 10^5 ES cells were plated on new MEF layers. Passage numbers of ES cells used for *in vitro* differentiation and transplantation experiments ranged from passages 15-25 (*in vitro* analyses) to 15-36 (*in vivo* analyses).

5.1.3 Establishing Murine Neurosphere Cultures from Foetal Brains

To establish neurosphere cultures, E12.5 foetal brains were isolated from chimeric embryos. Whole brains were triturated and single-cell suspensions were obtained by pipetting cells through a cell strainer (70 µm). Cell suspensions were cultured under neurosphere growth conditions (Neurobasal medium with 2 % B27 supplement, Penicillin (100 U/mL) / Streptomycin (100 U/mL) and L-glutamine (2 mM), supplemented with human growth factors EGF (20 ng/mL) and bFGF (20 g/mL) (Schmittwolf *et al.*, 2005). After two days of culture, free-floating neurospheres started to grow.

5.1.4 Freezing of ES Cells, Neurosphere Cells and MEFs

Cells were spun down at 200 x *g* (RCF) for ten minutes at 4°C and resuspended in 90 % cell culture-grade FCS and 10% DMSO. Cells were frozen at densities ranging from 1 x 10^6 / mL (min) to 5 x 10^6 / mL (max), approximately 1.5 mL per vial. Freezing took place in a freezing box with isopropanol at -70°C for two days; afterwards vials were transferred in liquid nitrogen storage.

5.1.5 Thawing of ES Cells, Neurosphere Cells and MEFs from Liquid Nitrogen Storage

Vials with frozen cells were thawed rapidly in a water bath at 37°C, cell suspensions were diluted with 5 mL appropriate cell culture medium, spun down at 200 x *g* (RCF) for ten minutes at 4°C, supernatant was removed and the cells were resuspended in appropriate cell culture medium, seeded and cultured.

5.2 Neural *In Vitro* Differentiation of AG ES Cells

ES cells were differentiated toward neural phenotypes as previously described (Okabe *et al.*, 1996; Brüstle *et al.*, 1997; Haupt *et al.*, 2007).

To obtain embryoid bodies (EBs), undifferentiated ES cells were cultured in DMEM high glucose supplemented with 10 % cell culture-grade FCS, HEPES (10 mM), NEAA, Penicillin (100 U/mL) / Streptomycin (100 U/mL), L-glutamine (2 mM), sodium pyruvate (1 mM) and ß-mercaptoethanol (0.1 mM) for four days in bacteriological Petri dishes. On day four, free-floating EBs were transferred to tissue culture plates for adherent cells with DMEM/Ham's F12 containing Penicillin (100 U/mL) / Streptomycin (100 U/mL), L-glutamine (2 mM), human transferrin (50 g/mL), sodium selenite (30 nM), insulin (5 µg/mL) and fibronectin (2.5 µg/mL) in order to obtain attached EBs. Fresh medium was added on day two. After four days of cultivation, attached EB were trypsinised (10 minutes, 37°C), cells were transferred to poly-L-ornithine- and laminin-coated plates and cultured in DMEM/Ham's F12 containing Penicillin (100 U/mL) / Streptomycin (100 U/mL), L-glutamine (2 mM), insulin (25 µg/mL), progesterone (20 nM), putrescine (0.1 µM), sodium selenite (30 nM), human transferrin (50 µg/mL), bFGF (50 ng/mL) and laminin (0.7 µg/mL) for generation of pan-neural progenitor cells (pnPCs). On day one, two and three fresh medium supplemented with bFGF (50 ng/mL) was added. After four days of cultivation, pnPCs were trypsinised and replated under neural differentiation conditions (Neurobasal medium with 2 % B27 supplement, Penicillin (100 U/mL) / Streptomycin (100 U/mL), L-glutamine (2 mM), supplemented with 10 % NeuroCult Differentiation Supplement) for terminal differentiation into neural cell types on cover slips coated with poly-L-ornithine and laminin. Cells were cultured at 37°C, 5 % CO_2 for up to 13 days. Medium was changed every other day.

5.3 Immunocytochemistry of *In Vitro* Differentiated ES Cells

Immunocytochemical staining for neuronal and astroglial differentiation markers was performed as described for *in vitro* differentiated neurosphere cells (see below, 5.6.2.1). Percentages of tubulin-ß-III$^+$ cells and GFAP$^+$ cells were compared between differentiated AG and N pnPCs by two-sided student's t-test. Differences were considered statistically significant if $p < 0.05$ (two-sided student's t-test).

5.4 Blastocyst Injection and Chimeric Embryo Generation

5.4.1 Blastocyst Generation

To obtain blastocysts for the injection of ES cells, six – eight week old female NMRI mice were superovulated by intraperitoneal injection of ten units pregnant

mare's serum (PMSG), followed 48 hours later by an injection of ten units human chorionic gonadotropin (hCG), caged with stud males and checked for vaginal plugs the next morning. The day of finding the plug was designated day 0.5 dpc. At 3.5 dpc, pregnant mice were sacrificed, ovaries and oviducts were removed and transferred into M16 medium. Blastocysts were flushed from the oviducts and kept one to two hours in M2 medium prior to the injection of ES cells. To induce pseudopregnancy in recipient foster animals, six - eight week old female NMRI mice were mated with vasectomised NMRI males of proven sterility.

5.4.2 Blastocyst Injection of ES Cells

ES cells were trypsinised, MEFs were removed by incubation on gelatine-coated plates for 45 minutes. ES cell single-cell suspensions were prepared in M2 medium prior to blastocyst injection. 10-15 ES cells were injected per blastocyst. Afterwards, blastocysts were transferred into pseudopregnant foster animals.

5.4.3 Embryo Isolation and Analysis

At 12.5 and 14.5 dpc, pregnant mice were sacrificed and embryos were isolated from the uteri. Pictures of embryos were taken, embryo sizes were measured and foetal livers and brains were isolated for further analyses.

5.5 Flow Cytometry

5.5.1 Analysis of Chimerism in Foetal Liver and Brain

Foetal livers of E12.5 and E14.5 embryos were isolated, triturated and liver single-cell suspensions were analysed by flow cytometry for grade of chimerism by measuring the percentage of $eGFP^+$ cells. Gates were defined by using foetal liver cells from wild-type and eGFP-transgenic embryos (Okabe *et al.*, 1997). Embryos were considered chimeric if the $eGFP^+$ cell frequency was ≥ 1 %.

Heads of chimeric embryos were either fixed for cryosections and immunohistochemistry or used to establish neurosphere cultures.

5.5.2 Analysis of Chimerism in Mixed Neurosphere Cultures

At the day of foetal brain isolation (d0), at day five (d5) and day ten (d10), the grade of $eGFP^+$ ES cell-derived contribution was assessed by flow cytometry. For flow cytometric analysis at d5 and d10, single-cell suspensions of neurospheres were prepared by incubating neurospheres with AccuMax for seven minutes at 37°C and pipetting cells through cell strainers.

5.5.3 Fluorescence Activated Cell Sorting (FACS) of AG-derived Neurosphere Cells

To isolate AG brain cells, single-cell suspensions were prepared from chimeric brains and cells were expanded for two passages under neurosphere growth conditions, as primary brain cells were found to be sensitive to FACS. Single-cell suspensions were prepared from neurospheres and cells were subjected to FACS according eGFP fluorescence. The purity of $eGFP^+$ and $eGFP^-$ populations after sorting was > 98 %. Sorted $eGFP^+$ and $eGFP^-$ neurosphere cells were expanded for two - four passages under neurosphere growth conditions to obtain sufficient cell numbers for analysis.

5.6 Self-Renewal and Neural *In Vitro* Differentiation of Neurosphere Cells

5.6.1 Analysis of Neurosphere Initiating Cells

To determine the neurosphere initiating cell number, single-cell suspensions from neurosphere cultures were produced by AccuMax treatment. Cells were counted und viability was assessed by trypan blue exclusion. 5000 single neurosphere cells were seeded per well, cells were cultured under neurosphere growth conditions. After five days, newly formed neurospheres were counted. Percentages of neurosphere initiating cells were calculated and considered significantly different if $p < 0.05$ (two-sided student's t-test).

5.6.2 Neural In Vitro Differentiation of neurosphere cells

For neural differentiation, single-cell suspensions from neurospheres were cultured under differentiation conditions. Single-cell suspensions from neurospheres were generated by AccuMax treatment. Thereafter, cells were seeded onto poly-L-ornithine / laminin coated cover slips at 5×10^5 cells per cover slip and cultured for up to 13 days under neural differentiation conditions (see above, 5.2). Subsequent to differentiation, cells were stained using the following protocol:

5.6.2.1 Immunocytochemistry for Neurons and Astrocytes with Cell-permeabilisation

After up to 13 days of culture, cover slips with differentiated cells were transferred into the wells of 24-well-plates for suspension culture filled with PBS. After removal of PBS, cells were permeabilised with 1 mL MSP buffer each well for 30 seconds. Afterwards, cells were fixed with 1 mL Methanol for three minutes at –20°C and washed three times with 1 mL PBS + 0.1 % Triton X-100. For blocking

unspecific binding, cells were treated with antibody-dilution-solution with 0.1 % Triton X-100, 5 % goat serum and 0.1 % sodium acid (NaN_3) in PBS. Then cells were incubated for 30 minutes with 250 µL of different antibodies: with α-glial fibrillary acidic protein (GFAP) (diluted 1:600 in antibody-dilution-solution) for visualisation of astrocytes, with α-tubulin-ß-III (clone Tuj-1) for neurons (mouse-IgG; 1:600 in antibody-dilution-solution), respectively. After washing four times with 1 mL 0.1 % Triton X-100 in PBS for five minutes, 250 µL of secondary antibodies labelled with the fluorochrome Cy3 were added to the cover slips and cells were incubated for 30 minutes in the dark at room temperature: α-rabbit-IgG for GFAP-staining, α-mouse-IgG for tubulin-ß-III-staining (both diluted 1:200 in antibody-dilution-solution). In the following, cells were washed 4 times with 0.1 % Triton X-100 in PBS and after that, nuclei were stained with 1 mL DAPI-solution (5 mg/mL DAPI stock solution was diluted 1:500 in PBS with 0.1 % Triton X-100, yielding a DAPI concentration of 10 µg/mL) for 1 min. Then cells were washed with PBS and cover slips were put upside down on a drop of Fluorescent Mounting Medium on slides.

5.6.2.2 Immunocytochemistry for Oligodendrocytes without Cell-permeabilisation

After 13 days of culture, cover slips with differentiated cells were removed from the plates and washed with PBS in another 24-well-plate. After disposal of PBS, 250 µL of primary O4-antibody diluted 1:100 in 5 % goat serum in PBS was added to each well and cells were incubated for 20 minutes at 37°C, 5 % CO_2. Next, cells were washed twice with 1 mL warm HBSS containing Ca^{2+}/Mg^{2+}. For Fixation, cells were treated with freshly prepared 4 % PFA in PBS for 20 min. After three times of washing with PBS containing 10 mM L-glycine, unspecific binding was blocked with 1 mL 1 x PBS + 5 % goat serum each well for ten minutes. In the following, 250 µL of Cy3-labelled secondary α-mouse-IgG diluted 1:200 in PBS containing 5 % goat serum was given to each well and cells were incubated for 90 minutes in the dark at room temperature. Then cells were washed thrice with PBS and afterwards, nuclei were stained with DAPI-solution (see 5.6.2.1) for one minute. After the last washing-step with 1 mL PBS for each well, cover slips were put upside down on a drop of Fluorescent Mounting Medium on slides. Slides were stored in the dark at 4°C.

Percentages of neurons, astroglia and oligodendroglia were assessed by differential counting total cell numbers (DAPI signals) and numbers of tubulin-ß-III^+, $GFAP^+$ and $O4^+$ cells. Mean values for AG and N neurosphere cells as well as

values for AG and blastocyst-derived cells were analysed by two-sided student's t-test. Values were considered statistically different if $p < 0.05$ (two-sided student's t-test).

5.7 RNA Isolation and RT-PCR

5.7.1 RNA Isolation

RNAs were isolated from AG and N pnPCs by using peqGOLD RNAPure™. One million cells were washed once in PBS and pelleted by centrifuging for ten minutes at 5000 x *g* (RCF). The cell pellet was resuspended in 700 µL of RNA peqGOLD, mixed by gentle pipetting, then incubated for three minutes. 100 µL of chloroform were added and mixed by gentle vortexing. After that, samples were centrifuged for ten minutes at 20000 x *g* (RCF). The aqueous supernatants were transferred to fresh tubes. Equal volumes of isopropanol were added to the aqueous phase and mixed gently by inverting. The nucleic acid was left to precipitate at -20°C for 20 minutes. Following precipitation samples were centrifuged for ten minutes at 20000 x *g* (RCF). Following centrifugation, the supernatant was discarded and the pellet was resuspended in 1 mL of 70 % Ethanol. Samples were centrifuged for 10 minutes at 20000 x *g* (RCF). The supernatant was discarded, the pellet resuspended again with 1 mL of ethanol for a second wash and centrifuged for ten minutes at 20000 x *g* (RCF). Finally, the supernatant was decanted and the pellet was resuspended in 20 µL DEPC-treated water. The amount of RNA in the samples was quantified photometrically using an Eppendorf BioPhotometer.

5.7.2 DNase Treatment

To remove the contaminating genomic DNA from the total RNA, the RNA samples were treated with RNase-free recombinant DNase I. 1 µg of RNA was mixed with 1.3 µL of 10X DNase buffer, DEPC treated water was added to 12 µL and 1 µL (2 U) of DNase I were added. Samples were incubated for 30 minutes at 37°C. DNase I was inactivated by adding 1 µL of 25 mM EDTA and subsequent incubation at 65°C for ten minutes. Half of the reaction mixture containing 0.5 µg RNA was used for cDNA synthesis

5.7.3 First Strand Synthesis

Reverse transcription reaction to synthesise first cDNA strand from DNase I-treated total RNA included 0.5 µg of RNA in 6.5 µL DEPC-water, 1µL (200 ng) of oligo-dT (16-mer) and 5 µL of DEPC treated water, the reaction-mix was heated at

65°C for five minutes to resolve all secondary RNA structures. Then 1 µL of dNTPs (10 mM), 4 µL of 5X first strand synthesis buffer, 2 µL of 100 mM DTT and 0.5 µL of M-MLV reverse transcriptase were added, yielding final 20 µL reaction-mix per sample. Samples were incubated for one hour at 37°C and cDNAs were subsequently stored at -20°C.

5.7.4 Quantitative RT-PCR

RT-PCR reactions were performed and quantified using a Rotor-Gene™ 3000 and ABsolute™ QPCR SYBR® Green Mix. Differences in gene expression were calculated with the $2^{-\Delta\Delta Ct}$ method. The housekeeping gene ß-*actin* was used for normalisation. $2^{-\Delta\Delta Ct}$ values of N pnPCs were set 1 for tested genes to determine expression differences between AG and N pnPCs. Primers were used for the housekeeping gene ß-*actin* and imprinted genes *Impact*; *Igf2r*; *Ube3a* (Eckardt *et al.*, 2007); *Zim1* (Kim *et al.*, 1999); *H19* (Wu *et al.*, 2006).

5.8 Immunohistochemistry of Chimeric Brains

5.8.1 Sample Preparation and Cryosections

For cryosections, whole E12.5 embryo heads were fixed for 12 hours in PBS containing 4 % paraformaldehyde. Following fixation, tissues were dehydrated in PBS containing 16 % glucose, embedded in TissueTek O.C.T. and frozen at -80°C. 10 µm sagittal sections were stained with primary chicken antibody against eGFP and with secondary goat anti-chicken IgY Cy2-conjugated antibody. For neuronal cell staining, primary mouse antibody against tubulin-ß-III or primary mouse antibody against neuronal nuclei (NeuN) and secondary mouse IgG Cy3-conjugated antibody were used. Nuclei were counterstained with DAPI. For analysis of co-localisation of green and red signals, 10 µm Z-stacks with 1 µm picture distance were taken using a BioZero microscope (600x magnification), yielding XY pictures and orthogonal XZ and YZ projections of Z-stacks. For intranuclear staining, tissue slices were incubated for 20 minutes at 96°C in 10 mM citrate buffer (pH 6.0) for antigen retrieval. To visualise proliferating cells, 5 µm transversal sections were stained with primary rabbit antibody against proliferating cell nuclear antigen (PCNA) and secondary goat anti-rabbit IgG Cy3-conjugated antibody. Apoptotic cells were detected using primary mouse antibody against cleaved-caspase-3 and secondary goat anti-mouse IgG Cy3-conjugated antibody (Chemicon). Nuclei were counterstained with DAPI (Sigma-Aldrich).

5.8.2 Intracellular Immunohistochemistry

For immunohistochemical detection of intracellular neuronal marker tubulin-ß-III cryosections were stained according to the following protocol:

Slides with fixed cryosections were thawed for 20 minutes at room temperature, washed for five minutes in PBS and afterwards incubated for 15 minutes with 300 µL endogenous blocking solution (3 % H_2O_2 in methanol) per slide in a moist chamber. Slides were washed twice for ten minutes in PBS while gently shaken and subsequently incubated for 30 minutes with 300 µL blocking solution (2 % BSA, 5 % goat serum, 0.1 % Triton X-100 in PBS) per slide in a moist chamber. Following this second blocking step, blocking solution was discarded and the slides were incubated overnight with 100 µL blocking solution with primary antibody mix: α-eGFP 1:1000 and α-tubulin-ß-III 1:250 (Tuj-1) at 4°C in a moist chamber. To avoid evaporation slides were covered with Parafilm.

The next day, slides were dipped in PBS to float away the Parafilm, then were washed twice for ten minutes in PBS with 0.1 % Triton X-100 while gently shaken. Subsequent to the wash steps slides were incubated 1.5 hours with 200 µL blocking solution containing secondary antibody 1:200 for detection of the α-eGFP primary antibody (goat α-chicken IgY Cy2-conjugated). Incubation was performed at room temperature in a moist chamber in the dark. All following steps were conducted in the dark to protect the fluorochromes from bleaching. Following two washing steps for ten minutes each in PBS containing 0.1 % Triton X-100 on a shaker, slides were incubated for 1.5 hours with 200 µL PBS with 0.1 % Triton X-100 with secondary antibody against the primary antibody labelling tubulin-ß-III 1:200 (goat α-mouse IgG Cy3-conjugated). Slides were washed twice for ten minutes with PBS containing 0.1 % Triton X-100 and subsequently incubated for 15 minutes in DAPI-solution (see 5.6.2.1) at 37°C. Slides were washed for five minutes in PBS at room temperature, 20 seconds in H_2O and embedded in Fluorescence Mounting Medium. Prior to microscopy slides were stored at 4°C in the dark.

5.8.3 Intranuclear Immunohistochemistry

For immunohistochemical detection of nuclear proteins as proliferating cell nuclear antigen (PCNA), Neuronal Nuclei (NeuN) and active form (cleaved) Caspase 3 (Casp3) cryosection were stained according to the following protocol:

Slides with fixed cryosections were thawed for 20 minutes at room temperature, washed for one minute in PBS and for one minute in H_2O. Afterwards slides were incubated for antigen retrieval in preheated 10 mM citrate buffer (pH 6) for 20 minutes at 96°C and cooled down to room temperature in 45 minutes in the citrate buffer, followed by five minutes incubation in H_2O at room temperature. Slides were washed twice for ten minutes with PBS and incubated for 15 minutes with 300 µL endogenous blocking solution (3 % H_2O_2 in methanol) per slide in a moist chamber. Slides were washed twice for ten minutes in PBS while gently shaken and subsequently incubated for 30 minutes with 300 µL blocking solution (2 % BSA, 5 % goat serum, 0.1 % Triton X-100 in PBS) per slide in a moist chamber. Following this second blocking step, blocking solution was discarded and the slides were incubated overnight with 100 µL blocking solution with primary antibody mix: α-eGFP 1:1000 and α-NeuN 1:250 (or α-PCNA 1:250 or α-Casp3 1:250, respectively) at 4°C in a moist chamber. To avoid evaporation slides were covered with Parafilm.

The next day, the Parafilm was carefully removed and slides were twice washed for ten minutes in PBS with 0.1 % Triton X-100 while gently shaken. Subsequent to the washing steps slides were incubated 1.5 hours with 200 µL blocking solution containing secondary antibody 1:200 for detection of the α-eGFP primary antibody (goat α-chicken IgY Cy2-conjugated). Incubation was performed at room temperature in a moist chamber in the dark. All following steps were conducted in the dark to protect the fluorochromes from bleaching. Following two wash steps for ten minutes each in PBS containing 0.1 % Triton X-100 on a shaker, slides were incubated for 1.5 hours with 200 µL PBS with 0.1 % Triton X-100 with secondary antibody specifically labelling the antibody against the antigen of interest other than eGFP 1:200 (goat α-rabbit Ig Cy3-conjugated / goat α-mouse IgG Cy3-conjugated). Slides were washed twice for ten minutes with PBS containing 0.1 % Triton X-100 and subsequently incubated for 15 minutes at 37°C in with DAPI-solution (see 5.6.2.1). Slides were washed for five minutes in PBS at room temperature, 20 seconds in H_2O and embedded in Fluorescence Mounting Medium. Prior to microscopy slides were stored at 4°C in the dark.

5.8.4 Quantification of eGFP$^+$ ES Cell-derived Cell-contribution in E12.5 Chimeric Brains

To quantify the contribution of eGFP$^+$ ES cell-derived cells, the percentage of eGFP$^+$ cells was determined for distinct brain regions by counting the numbers of

DAPI-stained nuclei and the numbers of $eGFP^+$ cells in three representative 100 µm x 100 µm squares per brain region. Regions analysed were striatum, hypothalamus, cortex and brain stem. Percentages of $eGFP^+$ cells in striatum, hypothalamus and cortex were calculated relative to brain stem. Means of $eGFP^+$ cell percentages relative to brain stem for striatum, hypothalamus and cortex from AG and N chimeric brains were considered significantly different if $p < 0.05$ (two-sided student's t-test).

5.9 Statistical Analysis

Two-sided student's t-test was performed using Microsoft Excel 2003, Microsoft, Seattle, USA (www.microsoft.com).

6 RESULTS

6.1 Neural *In Vitro* Differentiation of AG ES Cells

As the neural *in vitro* differentiation potential of AG in comparison to GG and N ES cells was unknown, AG and as control GG and N ES cells were subjected to a multi-step differentiation protocol that induced differentiation of ES cells into pnPCs and later into neuronal and glial cell types (Okabe *et al.*, 1996; Brüstle *et al.*, 1997).

6.1.1 In Vitro Differentiation of AG, GG and N ES Cells into Pan-neural Progenitor Cells

AG, GG and N ES cells growing as tightly packed colonies on MEF layers were harvested and separated from MEFs. Under differentiation conditions, AG, GG and N ES cells formed free-floating embryoid bodies in the absence of LIF, feeder layers and under reduced FCS concentrations (Figure 6, d - f). In a second and a third differentiation step, the cells adhered to the cell culture dish (Figure 6, g – i) and cells acquired a phenotype reminiscent of neural epithelia precursor cells (Brüstle *et al.*, 1997) (Figure 6, j -l).

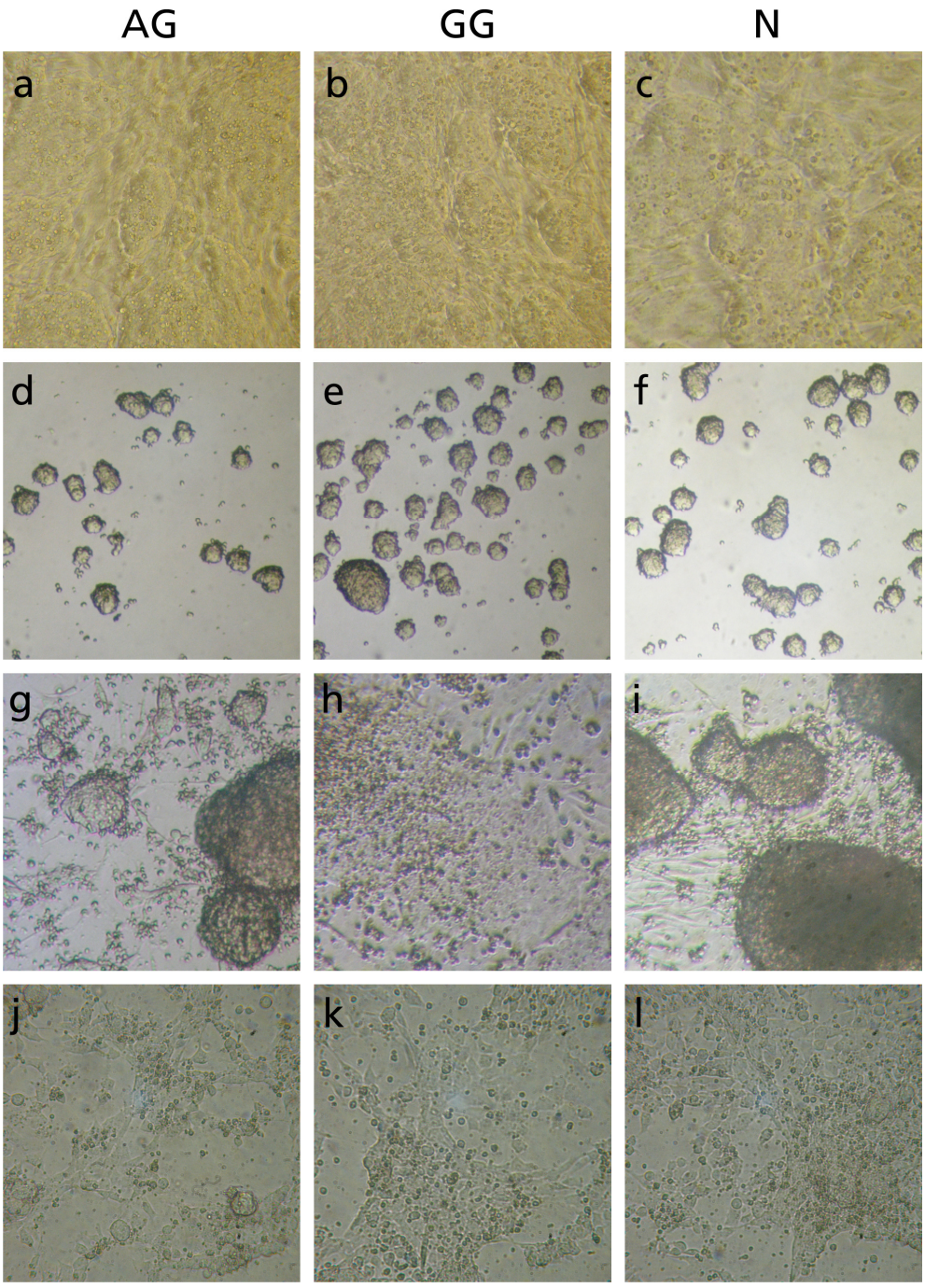


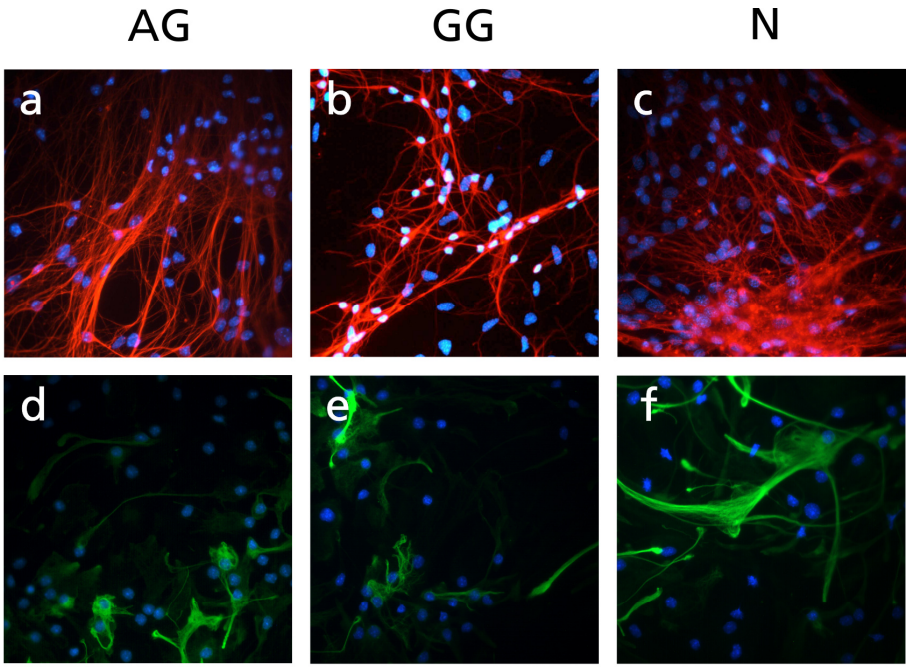
Figure 6: Multi-step neural *in vitro* differentiation of AG, GG and N ES cells. Phase contrast images of AG, GG and N ES cells differentiating *in vitro* into pan-neural progenitor cells. (**a**)(**b**)(**c**) Tightly packed ES cell colonies growing on MEF layers. (**d**)(**e**)(**f**) Free floating embryoid bodies developing from AG, GG and N ES cells (4 days under differentiation). (**g**)(**h**)(**i**) Attached embryoid bodies (8 days subject to differentiation) (**j**)(**k**)(**l**) Pan-neural progenitor cells (12 days under differentiation). n = 14; Magnification (Magn.): 100x.

To test the successful establishment of pnPCs from AG and GG ES cell cultures the neural differentiation capacity was analysed in subsequent experiments.

6.1.2 In Vitro Differentiation of pnPCs in Neuronal and Astroglial Cell Types

To assess the differentiation capability of AG and N ES cell-derived pnPCs, differentiated pnPCs cultures were analysed morphologically and by immunocytochemistry. PnPCs were subjected to neural differentiation conditions for up to 13 days, inducing neuronal and glial differentiation by withdrawing growth factors (EGF and bFGF) and by adding NeuroCult differentiation supplement. When adherent cells displayed elongated cell shapes, cells were immunocytochemically stained for neuronal and glial markers. As shown in Figure 7, AG, GG and N pnPCs differentiated into cells expressing the neuronal marker tubulin-ß-III (Figure 7, a, b, c; red) and the astroglial marker GFAP (glial fibrillary acidic protein) (Figure 7, d, e, f; green). The frequency of tubulin-ß-III$^+$ and GFAP$^+$ cells did not differ between AG cells and N cells (Tubulin-ß-III+ cells: AG 94 % ± 2.7 / GG 93 % ± 2.3 / N 95 % ± 2.1; GFAP+ cells: AG 3.4 % ± 1.9 / GG 4.9 % ± 2.4 / N 5.2 % ± 2.8).

A



B

	Frequency [%]		
	AG	GG	N
Neurons	94 ± 2,7	93 ± 2,3	95 ± 2,1
Astroglia	3,4 ± 1,9	4,9 ± 2,4	5,2 ± 2,8

Figure 7: (**A**) Immunostainings of pnPC-derived neural and glial cells. pnPCs were cultured for 13 days under neural differentiation conditions. (**a**)(**b**)(**c**) Tubulin-ß-III$^+$ neuronal cells (red). (**d**)(**e**)(**f**) GFAP$^+$ astroglial cells (green). Nuclei were counterstained with DAPI. A representative analysis is shown. Magn. 200x; n = 7. (**B**) Mean frequencies of neurons and astroglial cells with standard deviation are listed.

AG ES cells exhibited no detectable disadvantage to give rise to pnPCs *in vitro* and to differentiate into primitive neuronal and glial cell types when compared to GG and N ES cells. These findings suggested a wider neural developmental potential that was further investigated in the following *in vivo* experiments.

6.2 AG ES Cell Chimeric Embryos

Previous to this study, the contribution of uniparental AG cells to the brain has been assessed using aggregation chimaeras of embryonic cells from preimplantation stage AG embryos (ICM cells) and N biparental morulas (Barton *et al.*, 1991; Keverne *et al.*, 1996). AG ES cells were not considered in these studies and the contribution of AG ES cells to the developing brain is far less investigated than the contribution of GG and PG ES cells. Contrasting to the previous studies, here the capacity of AG ES cells to participate in neural development within a developing embryo was investigated: chimeric embryos were generated by blastocyst injection of AG or, as a control, N ES cells. Developing embryos were isolated at E12.5 and E14.5.

As expected for AG cells, the frequency of dead or absorbed embryos increased from E12.5 to E14.5 for AG ES cell-injected blastocysts. Following injection of AG ES cells, at E12.5 18/60 (30 %) and at E14.5 15/40 (38 %) dead or absorbed embryos were detected while following N ES cell injection at E12.5 6/36 (17 %) and at E14.5 7/25 (28 %) of embryos were dead or absorbed. In addition, size and appearance of the embryos were assessed. The consequences of abnormal genomic imprinting associated with the AG cells in E14.5 chimeric embryos included overall larger size as well as characteristic limb, head and trunk growth distortion and evidence of organomegaly (Mann *et al.*, 1990; McLaughlin *et al.*, 1997) (Figure 8).

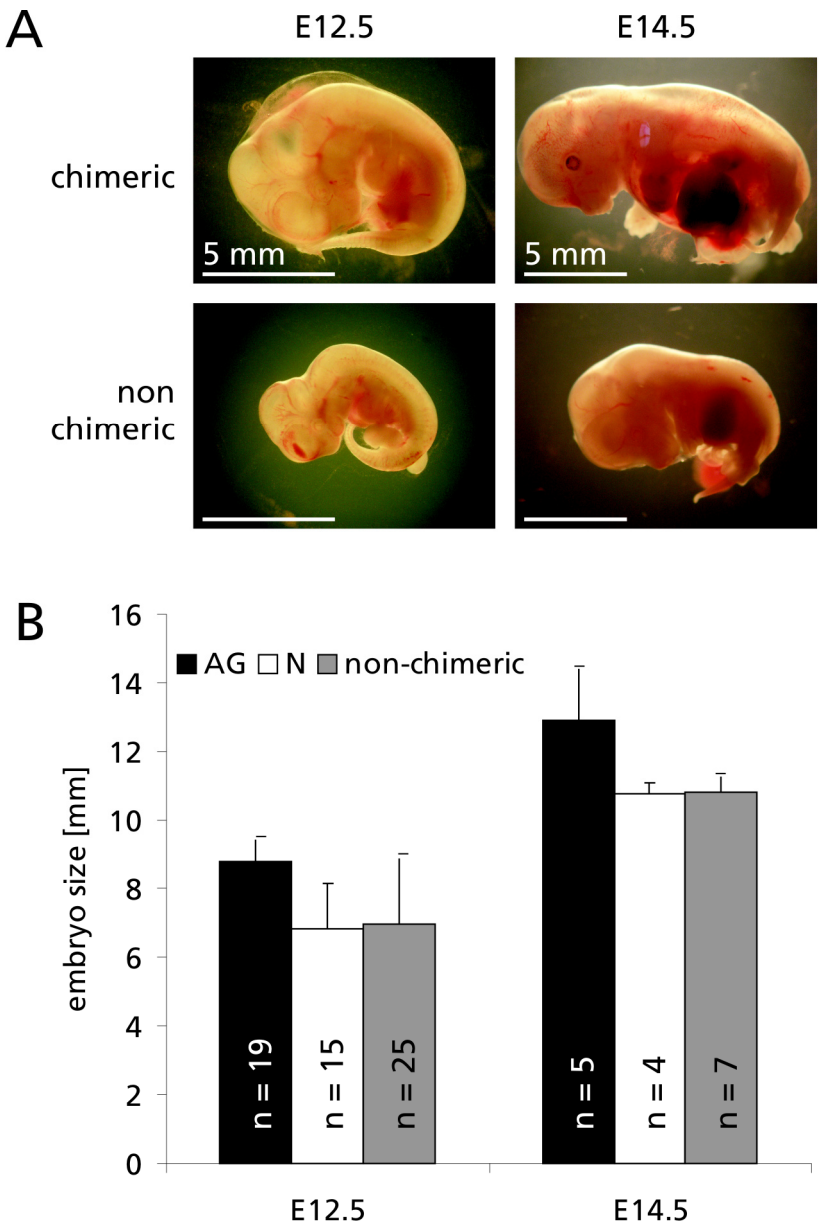


Figure 8: Size distribution of AG and N chimeric embryos. (**A**) Images of chimeric embryos and their non-chimeric littermates after blastocyst injection of AG or N ES cells at E12.5 and E14.5. Scale bar = 5 mm. Representative pictures are shown. (**B**) Embryo size of AG, N chimeric embryos and non-chimeric littermates at E12.5 and E14.5.

6.2.1 Chimerism in Foetal Liver and Brain of E12.5 and E14.5 Embryos

A recent study of the McLaughlin group showed that AG ES cells contribute to foetal livers. The contribution of eGFP-transgenic AG cells to chimeric foetuses was determined by flow cytometric analysis for eGFP expression in this organ (Eckardt *et al.*, 2007). Six independent injections of AG cells were performed with two individual AG ES cell line to exclude cell-line intrinsic results. 4 injections with AG ES cells A1 and two injections with AG ES cells A2 yielded in total 42 alive E12.5

embryos of which 22 (22 out of 42 (22/42); 52 %) were chimeric. Heads of 8/22 AG chimaeras were fixed in PFA-solution and used for immunohistochemical analyses of overall frequencies of $eGFP^+$ cells in the foetal brain and AG ES cell contribution to neural cell populations. The remaining foetal brains were isolated. Single-cell suspensions were prepared for further analysis of their proliferation and differentiation properties (see 6.4 and 6.5). In addition, of 30 living E12.5 embryos that developed out of four independent control injections of N ES cells, 24/30 (80 %) of the embryos showed $eGFP^+$ cells in the foetal liver. 15/24 E12.5 chimeric heads were processed further for immunohistochemistry, brain cell cultures were established from 9/24 embryos.

Further, 25 E14.5 embryos originating from eight independent injections of AG ES cells were analysed, 8/25 (32 %) embryos showed $eGFP^+$ cells in liver. As controls, 18 embryos were generated by blastocyst injection of N ES cells, producing 9/18 (50 %) chimaeras. All E12.5 or E14.5 embryos with $eGFP^+$ cells in liver also had $eGFP^+$ cells present in the brain. Due to the elevated death rate of AG chimaeras (Mann *et al.*, 1990; McLaughlin *et al.*, 1997), no advanced developmental stages were analysed. This study focused on E12.5 chimaeras instead.

Flow cytometric analysis revealed that AG ES cell derivatives contributed substantially to the brains of foetal chimaeras at E12.5 (Figure 9). Variation of ES cell contribution were detected between chimaeras, but no indication for exclusion of AG compared to N cells to foetal brain other than by stochastic variation was observed. For AG chimeric embryos, AG cell contribution ranged from 10 % to 76 % in the brain. Similarly, the chimerism in N chimeric embryos varied from 1 % to 38 % in the brain. In E14.5 foetal chimaeras also contribution to brain and liver was detected. In AG and N E14.5 chimaeras the contribution in the brain was higher than in the liver, ranging from 24 % to 86 % in AG chimaeras and from 12 % to 38 % in N chimaeras.

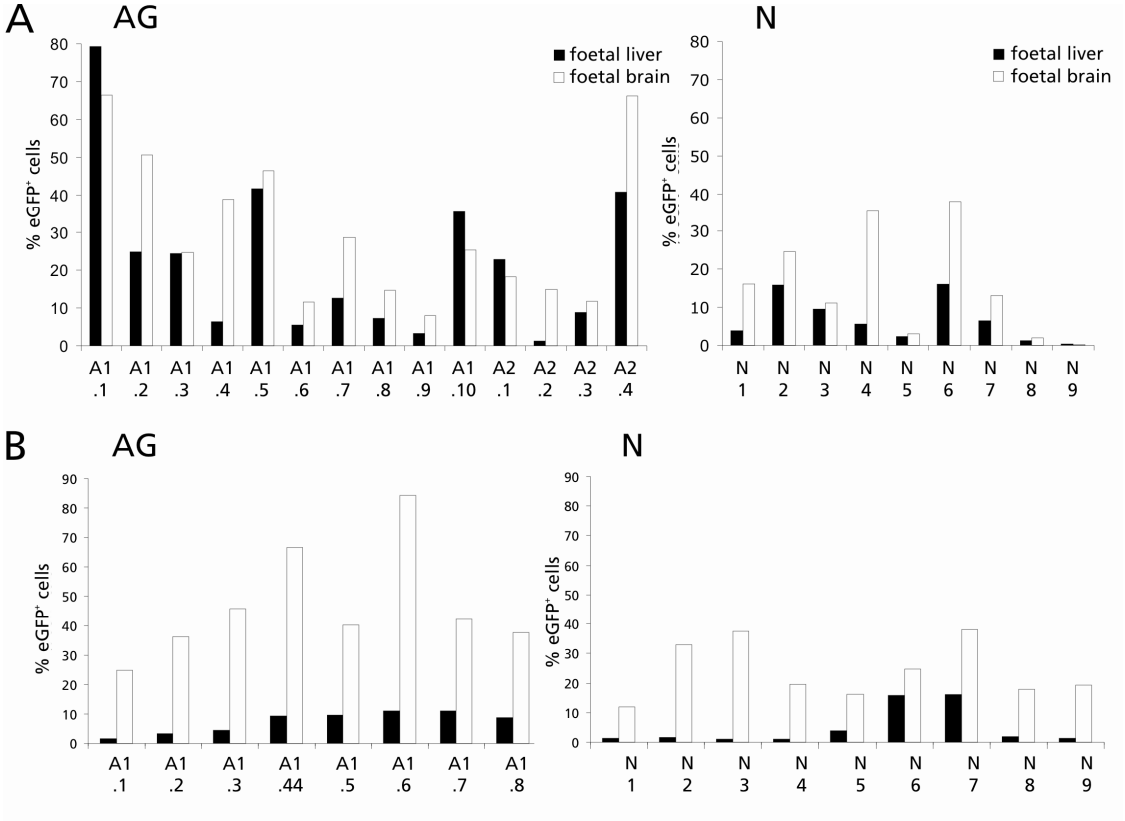


Figure 9: Percentage of eGFP⁺ AG- and N-derived cells in chimeric foetal livers and brains at E12.5 and E14.5 following blastocyst injection of AG and N ES cells. (**A**) Chimerism in foetal livers and brains of E12.5 embryos that develop upon blastocyst injection of two AG ES cell lines or one N ES cell line. Shown are percentages of eGFP⁺ ES cell-derived cells in single cell suspensions of freshly isolated brains as assessed by flow cytometry. Gates were defined by using foetal brain cells from eGFP-transgenic embryos (Okabe *et al.*, 1997) (not shown). (**B**) Chimerism in foetal livers and brains of E14.5 embryos from blastocyst injection of one AG ES cell line or one N ES cell line.

6.3 AG Cells Contribute to the Brain in Foetal AG ES Cell Chimaeras

AG ES cells showed *in vitro* neural developmental potential and generally contributed *in vivo* to E12.5 foetal brain. However, AG ES cells chimaeras exhibited overgrowth and deformation of the embryos like AG ICM chimaeras. This indicated on the one hand a wide neural developmental potential but on the other hand AG ES cells and their derivatives had not lost their uniparental identity during early development of chimeric embryos. Chimeric E12.5 were further analysed to investigate regional distribution and the contribution to neural cell populations of AG ES cell derivatives.

6.3.1 AG Cells Contribute to the Developing Brain and Form Neural Cell Types

To analyse the regional distribution of AG or N ES cell-derived eGFP⁺ cells in E12.5 chimeric foetal brains, transverse sections of AG and N chimeric brains were assessed for eGFP and tubulin-ß-III signals.

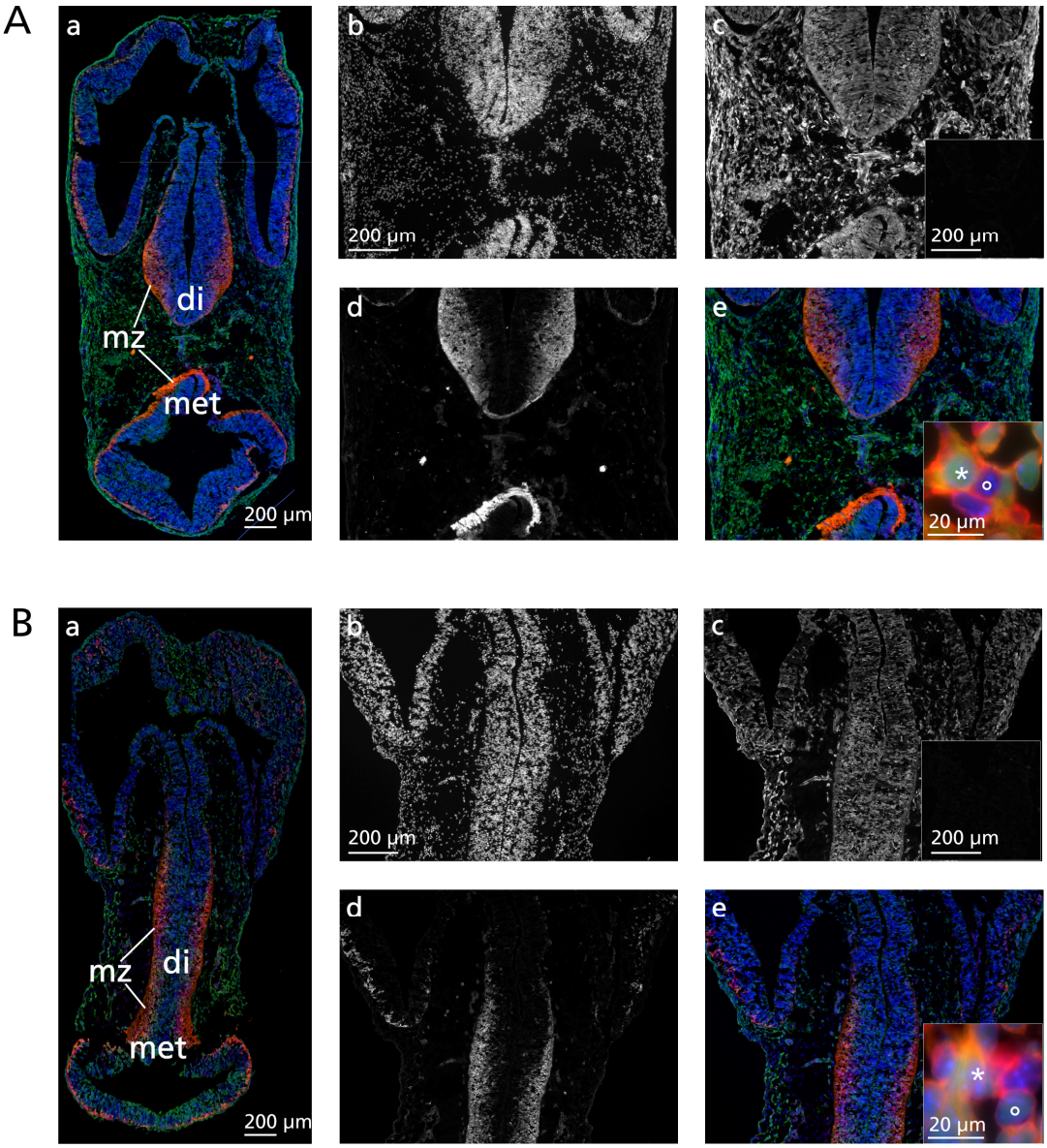


Figure 10: Distribution of AG- and N-derived cells in chimeric brains at E12.5. (**A**) Representative Immunostainings of cryosections of AG chimeric brains. Shown are: (**a**) Transversal section of an AG chimeric brain (image combined from five individual pictures). Indicated are diencephalon (di), metencephalon (met) and mantel zone (mz). (**b**) DAPI signals of metencephalon and diencephalon (ventral thalamus) (blue channel). (**c**) eGFP$^+$ ES cell-derived cells (green channel). Insert shows probes stained only with secondary Cy2-labelled antibody. (**d**) Tubulin-ß-III$^+$ neurons are displayed (red channel). (**e**) Overlay shows eGFP$^+$ ES cell-derived cells (green), tubulin-ß-III$^+$ neurons (red) and nuclei counterstained with DAPI (blue). Insert shows a tubulin-ß-III and eGFP double$^+$ ES cell-derived cell (*) and a tubulin-ß-III$^+$, eGFP$^-$ blastocyst-derived cell (°) from the mantel zone. n = 5 (**B**) Representative immunostainings of transversal cryosections from an N chimeric E12.5 brain; n = 4. Panels (**a**) – (**e**) are as described in Figure 10 (**A**).

As depicted in Figure 10A and B, AG and N ES cell-derived cells displayed similarly widespread distribution in all brain regions. Sections of the di- and metencephalon region, where neurons are formed, exhibited strong tubulin-ß-III$^+$ AG or N ES cell-derived contribution in the mantel zone (see Figure 10A, B, panels e, inserts). This result demonstrated that AG ES cell-derived cells are able to both

seed the developing brain and to contribute widespread and evenly distributed to foetal neurogenesis.

To further determine the regional distribution of AG or N ES cell-derived eGFP$^+$ cells in E12.5 chimeric foetal brains, sagittal sections of AG and N chimeric brains were assessed for eGFP and tubulin-ß-III signals. As presented in Figure 11A and 11B, AG and N ES cell-derived cells displayed similarly widespread distribution in all brain regions. Sections of the striatum, hypothalamus, cortex and brain stem region showed strong tubulin-ß-III$^+$ AG or N ES cell-derived contribution (see Figure 11A, B, panels b-e, inserts). These results indicated that AG ES cell-derived cells were not excluded from the developing foetal brain and took part in the neural development not different from the N ES cell-derived cells.

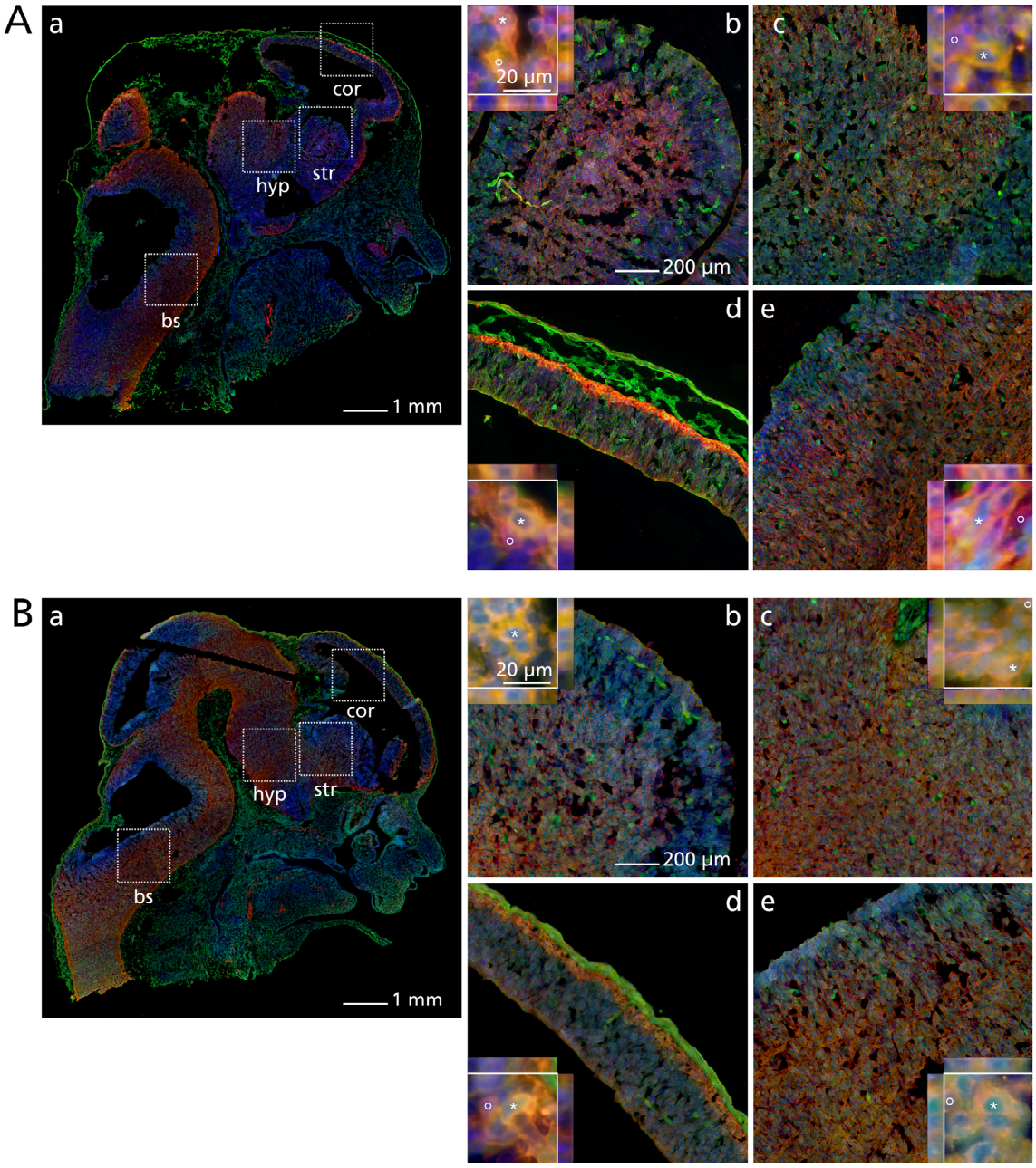


Figure 11: Analysis of AG and N cells in chimeric brains at E12.5 following blastocyst injection of AG and N ES cells. (**A**) Immunostaining of cryosections of AG ES cells chimeric brains. Representative samples shown are: (**a**) Sagittal section of an AG chimeric head (image combined from 19 individual pictures). Indicated are cortex (cor), striatum (str), hypothalamus (hyp) and brain stem (bs). Also shown are higher magnifications of (**b**) striatum, (**c**) hypothalamus, (**d**) cortex and (**e**) brain stem regions. Inserts in panels (**b**) – (**e**) show a 1 µm XY layer and orthogonal views in the Z direction of 10 µm cryosections to prove signal co-localisation. (*) marks co-localisation of eGFP and ß-III-tubulin in an individual cell; (°) marks an $eGFP^-$ and ß-III-$tubulin^+$ cell. All panels are overlays composed of green (α-eGFP, ES cell-derived cells), red (α-tubulin-ß-III, neurons) and blue signals (DAPI, nuclei). n = 3. (**B**) Immunostaining of representative sagittal cryosections from an N chimeric E12.5 brain; n = 3. Panels (**a**) – (**e**) as described in Figure 11(**A**).

To ascertain the neural differentiation capacity of the AG and N ES cell derivatives stainings of sagittal brain sections of AG and N chimaeras for eGFP and neuronal nuclei (NeuN) as an additional neuronal marker were performed. The NeuN also showed widespread NeuN$^+$ AG or N ES cell-derived cells contributing to cortex, striatum, hypothalamus and brain stem (Figure 12 A, B).

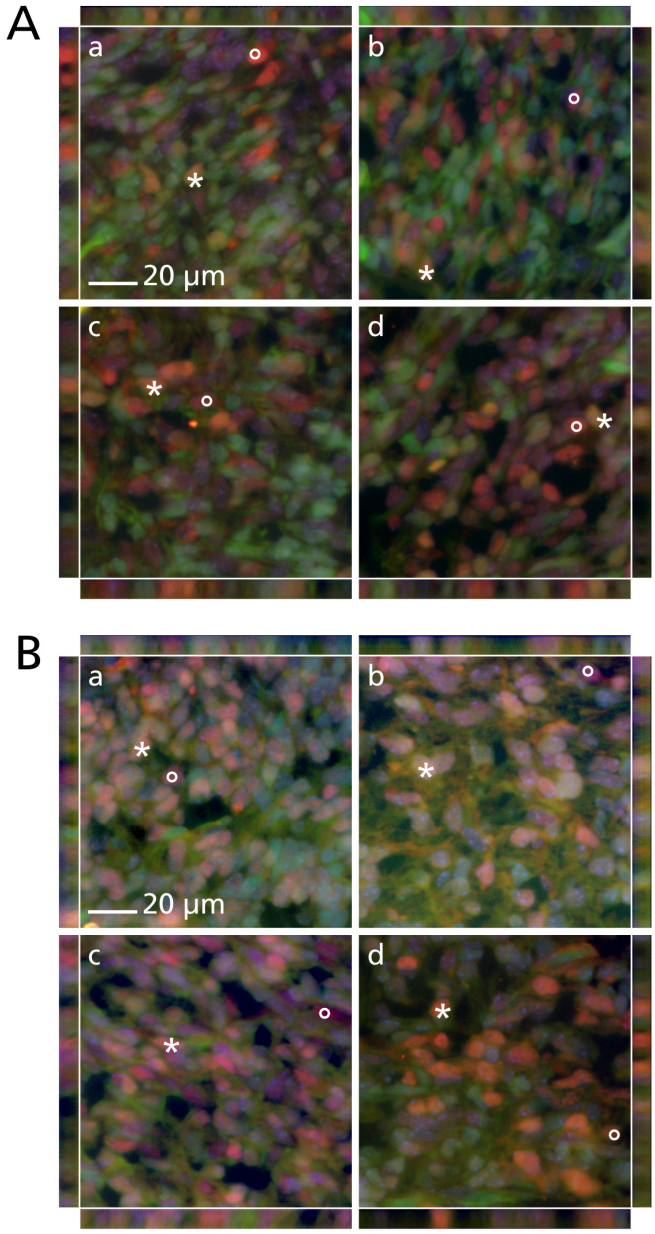


Figure 12: AG- and N-derived cells in E12.5 chimeric brains. (**A**) Immunostainings of a representative sagittal section of an AG chimeric head with the immature neuronal marker NeuN. Shown are: (**a**) striatum, (**b**) hypothalamus, (**c**) cortex and (**d**) brain stem. Panels are overlays depicting ES cell-derived cells (α-eGFP, green), neurons (α-NeuN, red) and nuclei (DAPI, blue). To proof signal co-localisation, a 1 µm XY layer from a 10 µm Z-Stack together with orthogonal views in the Z direction are shown. (*) marks an eGFP$^+$ / NeuN$^+$ cell, showing co-localisation of red and green signals, (°) marks a eGFP$^-$ / NeuN$^+$ cell, with no green signal co-localising with red signals. n = 3 (**B**) Immunostainings of a representative sagittal cryosection from an E12.5 N chimeric brain; n = 3. Panels (**a**) – (**d**) are as described in (**A**).

To evaluate the distribution of eGFP+ ES cell-derived cells in distinct brain regions , the percentage of eGFP+ cells in striatum, hypothalamus, cortex and brain stem was determined for AG and N chimeric foetal brains (Figure 13). Comparison of the percentages of eGFP+ cells for striatum, hypothalamus and cortex relative to brain stem revealed no significant differences in the distribution of eGFP+ cells in these brain regions and no significant distribution differences when comparing AG to N chimeric brains. These results show that AG ES cell-derived cells can evenly seed the developing brain and contribute to early foetal neurogenesis.

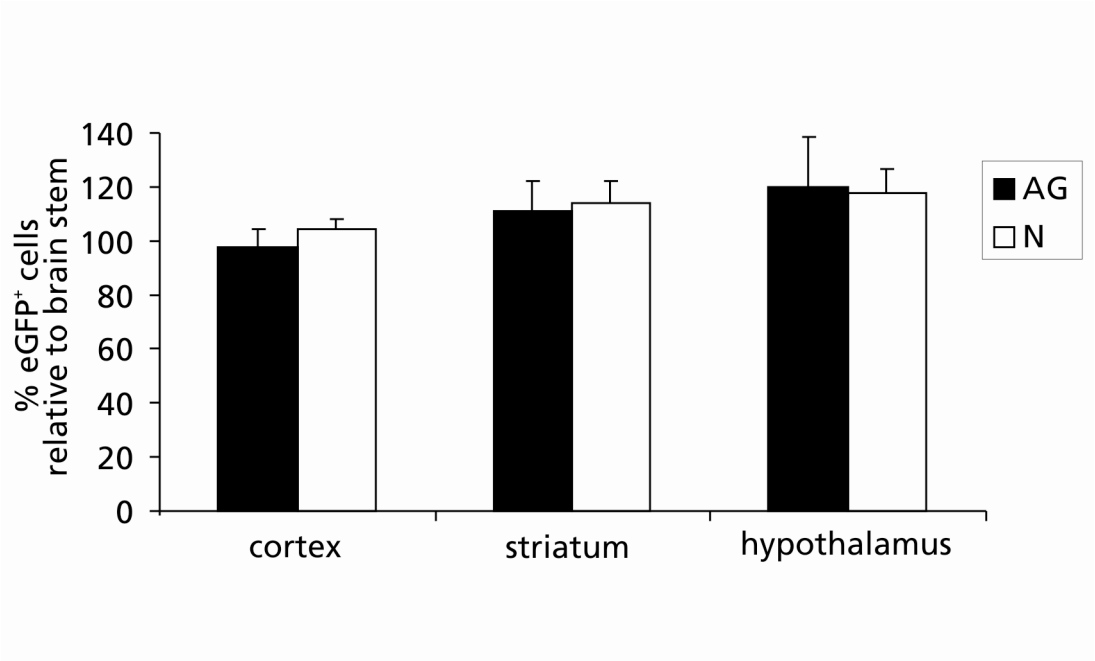


Figure 13: Contribution of eGFP+ ES cell-derived cells to different brain regions. Bar graphs depict frequencies of eGFP+ ES cell-derived cells in cortex, striatum and hippocampus relative to brain stem for AG and N chimeric brains. n = 3.

6.3.2 Cell Proliferation and Apoptosis in AG Chimeric Foetal Brains

To assess the proliferative and apoptotic properties of AG cells in chimeric brains, transverse brain sections were analysed for the expression of proliferating cell nuclear antigen (PCNA) and cleaved-caspase-3, a crucial component of the apoptosis pathway (Namura *et al.*, 1998; Slee *et al.*, 2001) (Figure 14A, B). As expected at this early stage of embryonic development, brain tissues are highly proliferative with almost all cells positive for PCNA including eGFP+ AG ES cell-derived cells (see Figure 14A, panel d, insert). No co-localisation of eGFP+ AG ES cell-derived signals with cleaved-caspase-3 staining (see Figure 14B, panel d, insert), and overall a very low number of apoptotic cells was detected.

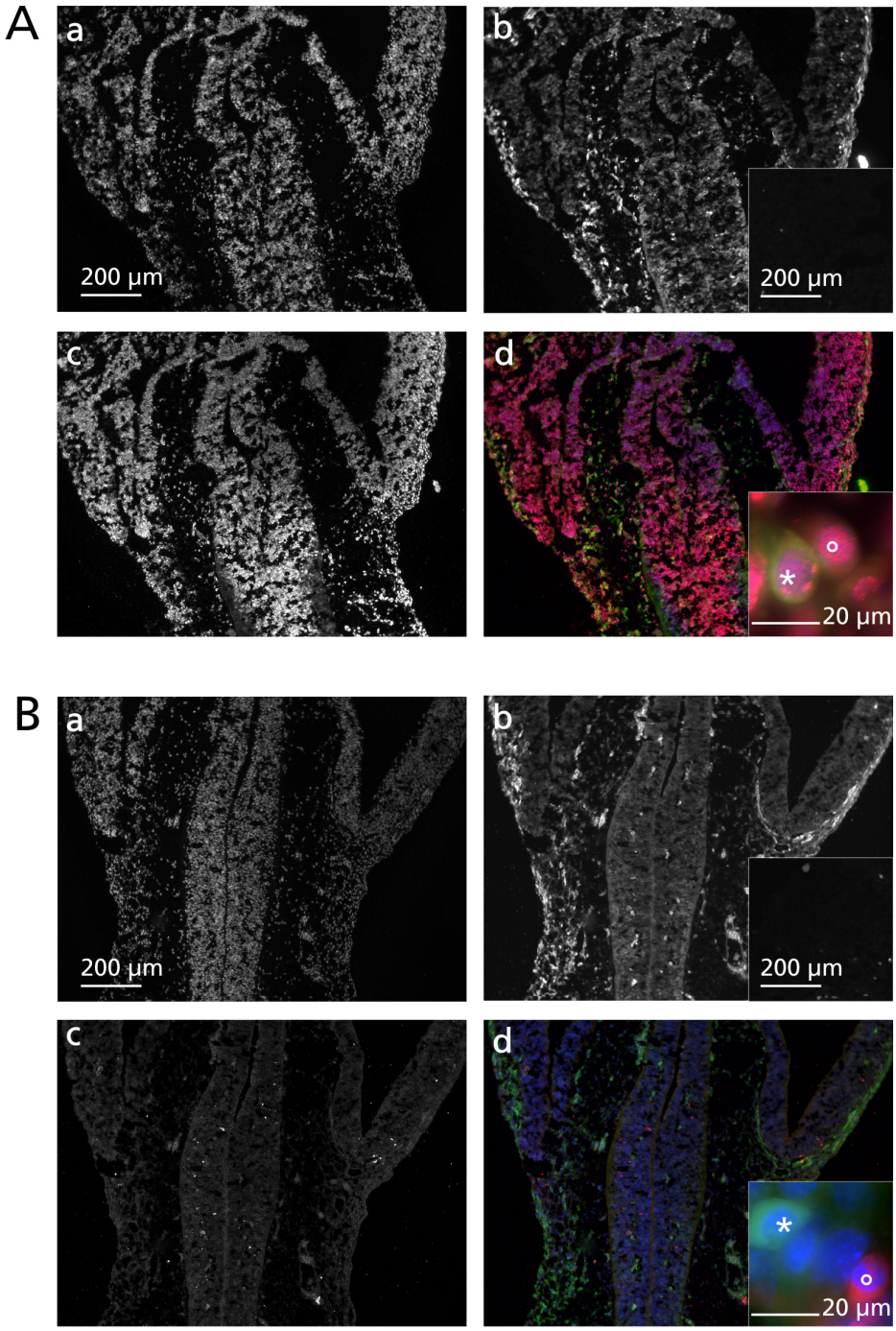


Figure 14: Cell proliferation and cell death in AG chimeric foetal E12.5 brains (**A**) PCNA-specific immunostainings of a transversal AG brain cryosection. Shown are: (**a**) DAPI stainings (blue channel); (**b**) eGFP$^+$ ES cell-derived cells (green channel); (**c**) PCNA$^+$ cells (red channel). Insert shows control staining without primary but with secondary Cy2-labelled antibody. (**d**) Overlay of eGFP (green), PCNA (red) and DAPI (blue) signals. Insert shows a PCNA and eGFP double$^+$ ES cell-derived cell (*) and a PCNA$^+$ and eGFP$^-$ blastocyst-derived cell (°).n = 3 (**B**) Cleaved caspase 3-specific immunostainings of a transversal cryosection from an AG chimeric foetal brain. Panels show: (**a**) DAPI stainings (blue channel), (**b**) eGFP$^+$ ES cell-derived cells (green channel) Control staining with only secondary Cy2-labelled antibody shown in the insert. (**c**) Cleaved caspase 3$^+$ cells (red channel). (**d**) Overlay of eGFP (green), cleaved caspase 3 (red) and DAPI (blue) signals. Insert shows a cleaved caspase 3$^-$ and eGFP$^+$ ES cell-derived cell (*) and a cleaved caspase 3$^+$ and eGFP$^-$ blastocyst-derived cell (°). n = 3.

6.4 AG Chimeric Foetal Brain Cells in Mixed Neurosphere Cultures

The results from the cryosections showed that AG ES cell derived cells were capable to contribute to the developing E12.5 brain and to take part in neural differentiation. To further assess the developmental potential of the AG ES cell-derived cells, brain cells from chimeric embryos were expanded and analysed *in vitro*.

Single-cell suspensions of AG chimeric brains were cultured under neurosphere growth conditions to determine AG neural stem / progenitor cell formation when co-developing *in vivo* with blastocyst-derived cells. As chimeric brains consisted of AG ES cell- and blastocyst-derived cells, brain cell cultures were a mixture of cells of these two origins. After isolation of the foetal brains, before culture (d0), after five days (d5) and after ten days (d10) of culture, the percentage of $eGFP^+$ AG cells in neurosphere cultures was measured by flow cytometry. In total, 14 AG and, as control, nine N E12.5-derived chimeric brain cultures were analysed (Figure 15). The frequencies of $eGFP^+$ cells in individual AG and N brain-derived neurosphere cultures displayed variations in the proportion of $eGFP^+$, AG or N ES cell-derived cells, particularly in AG cultures at day 5. However, on average, AG and N neurosphere cultures exhibit similar frequencies of $eGFP^+$ ES cell-derived cells. Thus, in chimeric brain cultures of AG- mixed with blastocyst-derived cells, AG cells continued to grow and form neurosphere cells.

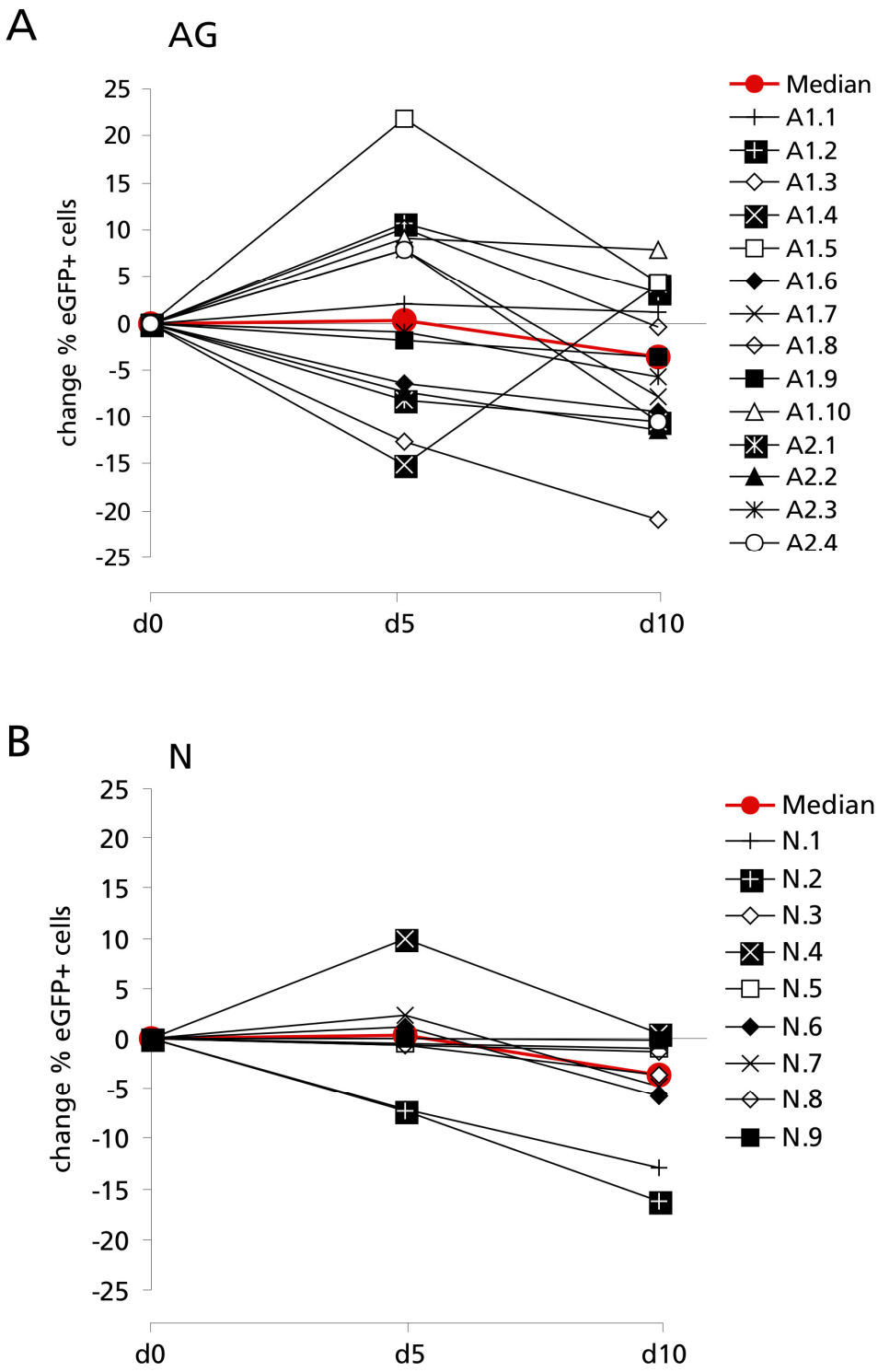


Figure 15: Proliferation of (**A**) AG or (**B**) N ES cell-derived and blastocyst-derived cells in competitive E12.5 brain cell cultures. Proportions of eGFP$^+$ cells in neurosphere cultures established from AG or N chimeric foetal brains. Percentage eGFP$^+$ ES cell-derived cells in freshly isolated foetal brains and in corresponding five and ten day neurosphere cultures. Percentages eGFP$^+$ ES cell-derived cells were assessed by flow cytometry. Shown are changes in eGFP levels during ten days of *in vitro* culture. Percentages of the starting populations were set to 0. Brain cell cultures are from embryos analysed in Figure 9A.

6.5 Stem Cell Properties of AG Neurospheres

Brain cell cultures from AG and N chimaeras displayed the formation of neurospheres. As the portion of $eGFP^+$ AG and N ES cell derived cells in these neurosphere cultures did vary between individual isolation during the first 10 days of culture but on average no elimination of $eGFP^+$ cells was detected, the progenitor / stem cell properties of the AG and N ES cell derived neurosphere cells was examined.

6.5.1 Neurosphere Initiating Number

To further investigate the properties of AG-derived neurosphere cells and to estimate progenitor cell frequencies and differentiation capabilities of cells in brain cultures, neurosphere cultures originating from chimeric brains were separated by FACS into $eGFP^+$ AG or N ES cell-derived and $eGFP^-$ blastocyst-derived cells. Sorted neurosphere cells were cultured for two to four passages, followed by analyses of neurosphere initiating cell frequencies and neural *in vitro* multi-lineage differentiation potential. To assess the frequency of neurosphere initiating cells in neurosphere cultures, 5000 sorted cells obtained from $eGFP^+$ AG, control N and $eGFP^-$ blastocyst-derived neurosphere cells were plated under neurosphere growth conditions. After five days of culture the number of newly formed neurospheres was counted. As shown in Figure 16A, sorted $eGFP^+$ AG as well as N brain cells formed neurospheres. The frequencies of neurosphere initiating cells in sorted AG brain cells were similar to the frequencies of neurosphere initiating cells in sorted N and blastocyst-derived brain cells (Figure 16B).

The levels of ES cell contribution to chimeric liver and brains (Figure 9), the growth behaviour in cultures grown in mixed cultures (Figure 15A, B) and the neurosphere initiating cell frequencies (Figure 16B) did not correlate for individual embryos. Thus, the level of brain chimerism was not predictive of neurosphere initiating cell frequencies.

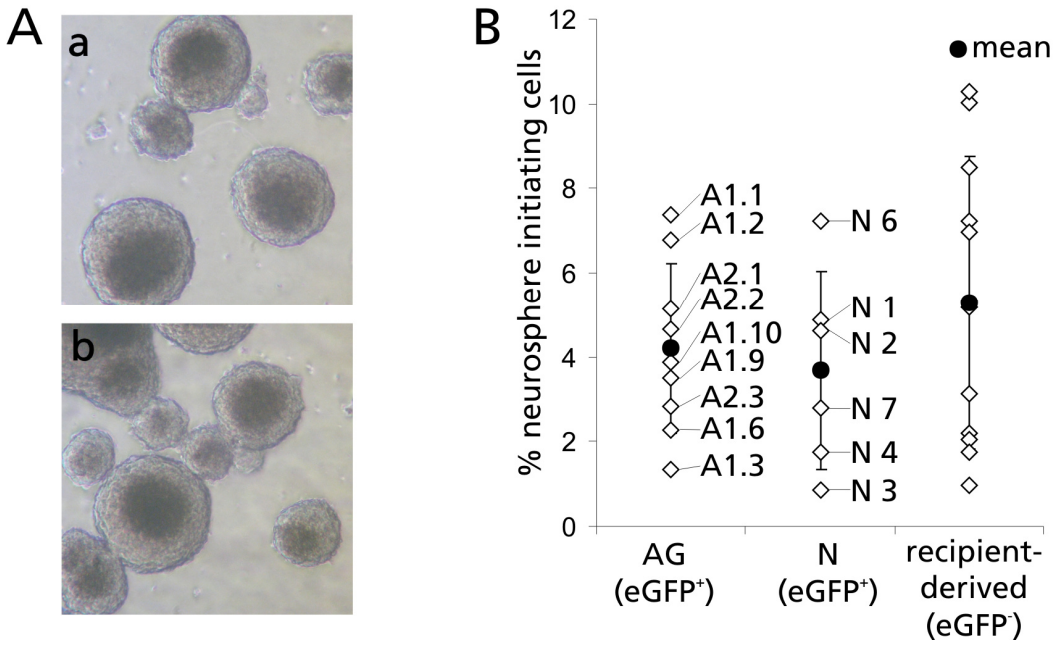


Figure 16: Self-renewal of AG and N foetal brain-derived neurosphere cells. (**A**) Phase contrast images of free floating neurospheres originating from single cell suspensions of E12.5 chimeric brains. ES cell-derived cells were isolated by sorting of eGFP$^+$ cells from single cell suspensions of chimeric E12.5 brains by FACS. Cells were cultures for two - four passages. Neurospheres originating from sorted AG (**a**) and N (**b**) brain cells are shown. Magn. 20x; n = 5 for sorted AG neurosphere cells/ n = 3 for sorted N neurosphere cells (**B**) Quantification of neurosphere initiating cells of AG and N brain-derived neurosphere cultures. Also shown are neurosphere initiating cell frequencies of foetal brain-derived eGFP$^-$ sorted blastocyst-derived neurosphere cultures. 5000 single cells from dissociated neurospheres in 500 µL medium per well were cultured for five days and newly formed neurospheres were counted. Plotted are the frequencies of newly formed neurospheres per 100 cells (percentage of neurosphere initiating cells) from nine AG chimeric embryos (2 individual cell lines), six N chimeric embryos and from 11 eGFP$^-$ blastocyst-derived neurosphere cultures. n = 4

6.5.2 Quantitative RT-PCR of Imprinted Genes

Using quantitative RT-PCR, the expression levels for several imprinted genes were assessed in eGFP$^+$ AG-derived and eGFP$^-$ blastocyst derived neurosphere cells (Figure 17). While in AG-derived neurosphere cells of embryo 1 the two genes with a paternal expression bias, *Igf2* and *Impact*, exhibited approximately five-fold and two-fold higher expression levels in AG- compared to blastocyst-derived neurosphere cells (N), indicating conservation of genomic imprinting, in embryo 2 and 3 the expression of *Igf2* was reduced to normal level and only *Impact* retained its parental biased expression. Silencing of the maternally expressed genes with brain-specific imprinting patterns, *Igf2r*, *Ube3a Zim1* and *H19*, was preserved in AG-derived neurosphere cells of embryo 3. Embryo 1 showed an approximately

three-fold higher expression of H19 while conserving the maternally silencing of *Igf2r*, *Ube3a and Zim1*. Embryo 2 showed an approximately two-fold higher expression of *Ube3a* while conserving the maternally silencing of *Igf2r*, *Zim1 and H19*.

Giving no overall equal pattern, the expression analysis of genes with parent-of-origin-specific expression pattern in the brain in neurosphere cultures revealed that genomic imprinting or the imprint-readout of these genes is altered during embryonic development, establishment and culture of neurospheres or both.

Cell culture of sorted neurospheres, RNA isolation and reverse transcription into cDNA, selection of primers and data interpretation was done by T. C. Dinger; quantitative RT-PCR was performed by S. W. Choi.

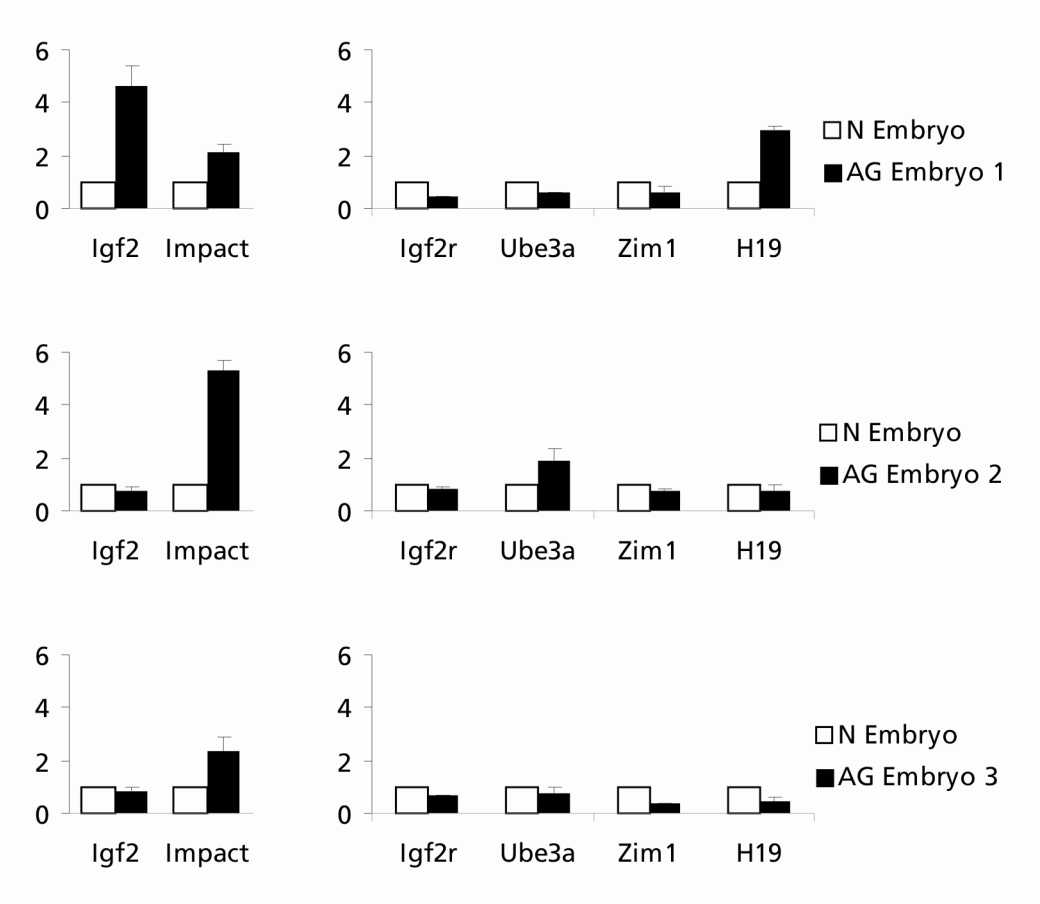
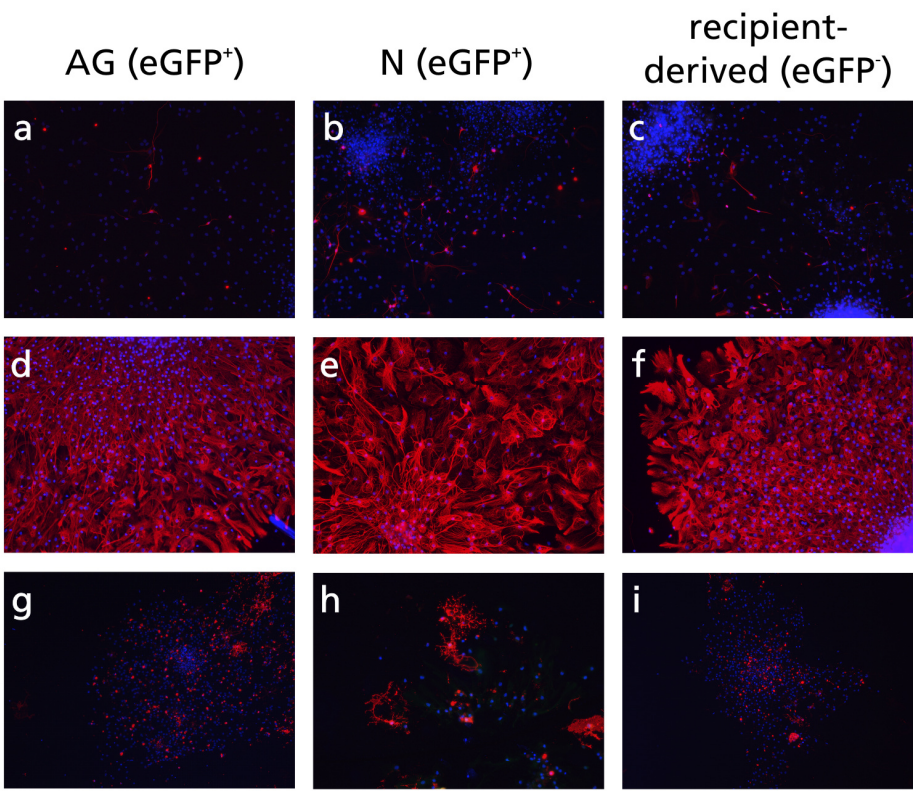


Figure 17: Gene expression of imprinted brain genes in sorted neurosphere cell cultures. Relative expression of imprinted genes was compared in eGFP$^+$ (AG) and eGFP$^-$ (N) neurosphere cells sorted from chimeric embryos. Shown are quantitative RT-PCR data from 3 representative individual embryos. The relative expression represents the fold change of gene expression in AG to N cells. Fold change was calculated by the $2^{-\Delta\Delta Ct}$ method. Expression levels of N cells were set to 1. In brain *Igf2* and *Impact* are preferentially paternally expressed, *Igf2r*, *Ube3a, Zim1* and *H19* are preferentially maternally expressed. n = 3. Cell culture, RNA isolation, cDNA reverse transcription, primer selection and data interpretation: T. C. Dinger; quantitative RT-PCR: S. W. Choi.

6.5.3 Neural In Vitro Differentiation of Neurosphere Cells

Sorted AG, N and blastocyst-derived cells were cultured under neural differentiation conditions for analysis of multi-lineage neural differentiation potential. Following the culture under neural differentiation conditions, cells were stained with tubulin-ß-III (neuronal cells)-, GFAP (glial fibrillary acidic protein, astroglial cells) - and O4 (oligodendroglial cells) -specific antibodies (Figure 18).

A



B

	Frequency [%]		
	AG	N	blastocyst-derived
Neurons	1,5 ± 0,2	1,0 ± 0,8	1,3 ± 0,4
Astroglia	98 ± 3,3	99 ± 0,9	97 ± 1,3
Oligodendroglia	1,0 ± 0,7	1,0 ± 0,3	1,5 ± 0,8

Figure 18: Neural differentiation of AG and N foetal brain-derived neurosphere cells. (**A**) Immunostainings of differentiating foetal brain-derived AG and N neurospheres. Also shown are neural and glial cells originating from eGFP$^-$ blastocyst-derived neurospheres. Neurosphere cells were cultured for 13 days under neural differentiation. (**a**) AG, (**b**) N and (**c**) blastocyst-derived tubulin-ß-III$^+$ neuronal cells (red), (**d**) AG, (**e**) N, and (**f**) blastocyst-derived GFAP$^+$ astroglial cells (red), (**g**) AG, (**h**) N and (**i**) blastocyst-derived O4$^+$ oligodendroglial cells (red). Cells were subjected to nuclear counterstaining (DAPI; blue). Magn. 100x; n = 6 for AG neurosphere cells / n = 3 for N neurosphere cells / n = 9 for eGFP$^-$ blastocyst-derived neurosphere cells. (**B**) Shown are mean frequencies of neurons, astroglial and oligodendroglial cells with standard deviation.

Analysis of the frequencies of neuronal and glial cell types revealed no difference between AG compared to N or blastocyst-derived cells. Frequencies for tubulin-ß-III$^+$ cells were: AG 1.5 % ± 0.2; N 1.0 % ± 0.8 and eGFP$^-$ sorted blastocyst-derived 1.3 % ± 0.4. Frequencies for GFAP$^+$ cells were for AG 98 % ± 3.3; N 99 % ± 0.9 and eGFP$^-$-sorted blastocyst-derived 97 % ± 1.3. Frequencies for O4$^+$ cells were for AG 1 % ± 0.7; N 1 % ± 0.3 and eGFP$^-$-sorted blastocyst-derived 1.5 % ± 0.8. As a result, AG like N and blastocyst-derived neurosphere cultures contained cells that formed new neurospheres with similar frequencies and differentiated into neuronal and glial cell types.

7 DISCUSSION

7.1 Differentiation Potential of AG ES Cells

Uniparental cells of AG ES cell origin like N ES cells can generate adult-transplantable hematopoietic stem cells that can repopulate the hematopoietic system of adult transplant recipients (Eckardt *et al.*, 2007). Likewise, *in vitro* differentiation of AG ES cells leads to neuronal and glial cell frequencies similar to N ES cells (Okabe *et al.*, 1996). Further, proliferation and differentiation properties of foetal brain-derived AG and N neurosphere cells did not differ. This indicates that, outside the normal developmental paradigm, the differentiation potential of uniparental ES cells may be much less restricted than that of uniparental cells in chimaeras.

7.2 One in Many: Alternatives to Normal Embryonic Stem Cells

Pluripotent ES cells can be expanded to almost unlimited numbers and have the potential to differentiate into all cell types *in vitro*. This renders ES cells an attractive donor source for transplantation. Thereby ES cells could revolutionise regenerative medicine (Lerou and Daley, 2005). However, a potential ES cell-based therapy faces several potential difficulties. Firstly, immune rejection due to immunological incompatibility between patient and donor ES cells has to be dealt with. The successful generation of cloned animal ES cells (Evans and Kaufman, 1981; Robertson *et al.*, 1986; Doetschman *et al.*, 1987; Thomas and Capecchi, 1987) and cloned animals by somatic cell nuclear transfer (SCNT) created the possibility to overcome this problem (Wilmut *et al.*, 1997). Genetically identical patient-specific SCNT-ES cells could in principal be generated by using nuclei from donor cells from a patient, transferring them into enucleated oocytes (Hochedlinger and Jaenisch, 2003; Jaenisch, 2004). Cloned human cells by SCNT have not been established so far. This strategy would eliminate the requirement for immune suppression. Secondly, despite successful application of SCNT-ES cells in animal disease models, the practical realisation of ''therapeutic cloning'' in human remains ethically questionable (www.isscr.org/public/ethics.htm; www.bioethik-kommission.bayern.de/stellungnahmen/stellungnahme1.htm). The necessity of oocyte donation, the "copying" of human beings and the destruction of viable human embryos leaves the generation of SCNT-ES cells not applicable for humans (Wakayama *et al.*, 2001; Rideout *et al.*, 2002; Barberi *et al.*, 2003; Weissman, 2006). Therefore, alternative routes to pluripotent cells are needed.

7.3 Induced Pluripotent Stem Cells and Other Alternative Strategies

Pluripotent cells have been isolated from several tissues such as bone marrow (MAPCs: multipotent adult progenitor cells (Jiang *et al.*, 2002)) and adult testes (spermatogonial stem cells (Guan *et al.*, 2006; Seandel *et al.*, 2007)). Only recently, human spermatogonial stem cells have been described (Conrad *et al.*, 2008; Kossack *et al.*, 2008). Both cell types are rare and while human spermatogonial stem cells are considered pluripotent *in vitro* and *in vivo* with their full potential remaining unknown, the pluripotency of MAPC is currently under investigation since promising initial results could not or only in part be reproduced (see Table 1). A promising approach for producing autologous pluripotent cells is the reprogramming of somatic cells to ES cell-like cells by the expression of defined factors *in vitro* (iPS, induced pluripotent stem cells). The iPS reprogramming strategy has been successfully demonstrated in mouse (Takahashi and Yamanaka, 2006) and also in human (Takahashi *et al.*, 2007; Yu *et al.*, 2007). In the mouse iPS cell derivatives have been successfully used for hematopoietic tissue replacement in a humanised sickle cell anaemia mouse model (Hanna *et al.*, 2007) and have been shown to function in a rat Parkinson model (Wernig *et al.*, 2008). The developmental and therapeutic potential of human iPS cells is uncertain, in particular as some human iPS cell lines exhibit limitations in their neural differentiation potential (Yu *et al.*, 2007) (Table 1).

7.4 Uniparental Cells: A Candidate for the Generation of Pluripotent Patient-Specific Stem Cells?

Uniparental ES cells may represent alternative sources for patient-specific pluripotent stem cells and are derived from gametic rather than somatic genomes. Both in mouse and in human, PG ES cell lines with a full MHC complement of the oocyte donor have been derived (Kim *et al.*, 2007; Revazova *et al.*, 2007). Generated without using fertilised eggs and without destroying fertilised human embryos, human parthenotes have shown a similar differentiation capability compared to ES cells derived from human embryos (Revazova *et al.*, 2007) and human PG ES cells are considered pluripotent. While the generation of uniparental ES cells may circumvent the destruction of viable embryos, it still requires the manipulation and destruction of donated oocytes. Furthermore, the parent-of-origin specific-imprinting in uniparental cells is not erased. Although the number of imprinted genes is small, many of them are expressed in the brain, affecting neurodevelopment and thereby influence brain function and behaviour

(Wilkinson *et al.*, 2007). Lastly, while human PG ES cells show promising results, the derivation of human AG ES cells remains to be demonstrated. However, the formation of hydatidiform moles provides some evidence for the early developmental potential of human AG conceptuses (Kajii and Ohama, 1977; Slim and Mehio, 2007).

	iPS	Spermatogonial stem cells	MAPCs	Uniparental cells
Availability	mouse and human	mouse and human (male only)	mouse and human, currently under investigation	AG: mouse, NOT human PG/GG: mouse primates, human
Ethical status	safe	safe	safe	questionable
Application	Successful in mice, retroviral overexpression of oncogenes still problematic.		Pluripotent state unclear. Only limited application due to the rarity of the cells.	- Hematopoietic reconstitution in mice for AG/GG. - Improvement of Parkinson's disease symptoms in rats for primate PG cells.

Table 1: Availability, ethical status and level of application of alternative sources of pluripotent cells.

7.5 The Full Therapeutic Circle Using Uniparental Cells: Chances and Hurdles

Using uniparental cells in therapy is likely to be a complicated process. Given the possibility, that a protocol for the generation of human AG ES cells will be developed, patient-specific AG ES cells could be produced for male patients (Figure 19). However, the process would still have impediments at crucial points. For the generation of AG zygotes it is necessary to have a large number of donated human oocytes available. This prerequisite is currently and in the future most likely difficult to fulfil. The process of pronuclear exchange has to be performed, bearing the risk of accidently producing normal fertilised, fully viable embryos. Furthermore, uniparental zygotes and blastocysts are considered not-viable, but their ethical and legal status still remains to be clearly defined. Once AG blastocysts have been produced, patient-specific AG ES cells would be generated and selected for MHC-compatibility with the patient, to avoid graft

rejection. MHC-matched AG ES-cells could then be differentiated into the required cell types. Procedures for genetically modifying the cells prior to differentiation and transplantation could be used to repair genetic defects, as described (Rideout *et al.*, 2002). Furthermore, the functionality and differentiation status of the transplanted cells has to be closely monitored. In addition, transplanting partial- or undifferentiated cells bears the risk of graft overgrowth and teratoma formation (Roy *et al.*, 2006; Sonntag *et al.*, 2007).

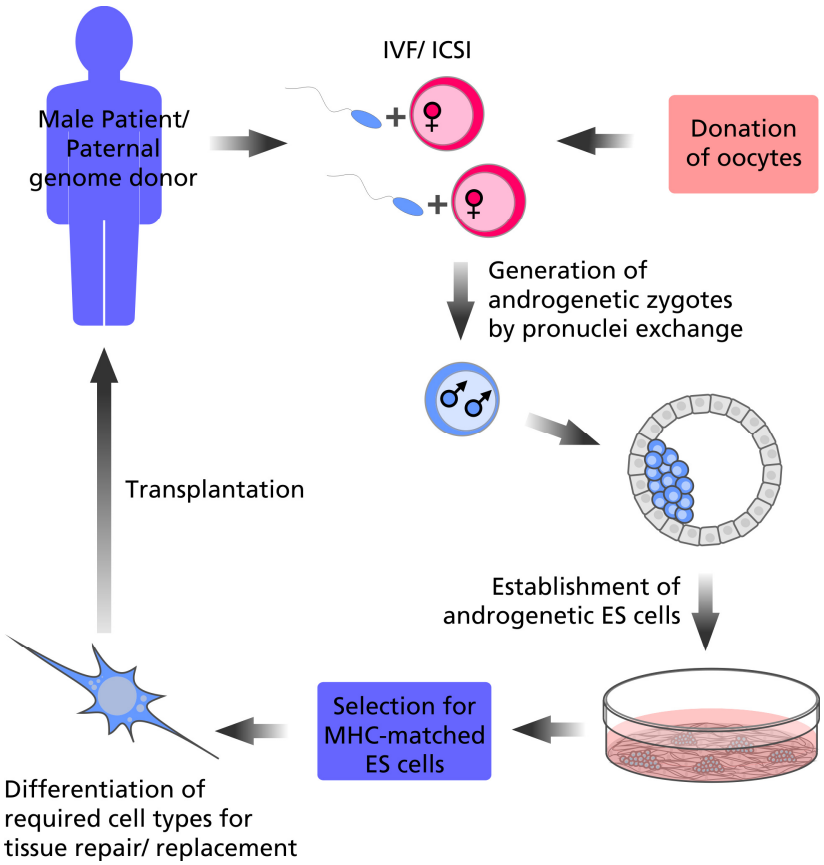


Figure 19: Potential therapeutic circle utilising AG stem cells. Donated oocytes are fertilised via IVF or ICSI with sperms from a male patient. By pronuclear exchange uniparental AG zygotes are formed. These zygotes develop to blastocyst stage and AG ES cell are established, containing only genetic material of the male patient. AG ES cells are expanded on clonal level and selected for MHC compatibility with the male patient. MHC-matched AG ES cells then are subject to differentiation into the required cell types prior to transplantation.

If the trapdoors mentioned above can be eliminated or adequately addressed to make the process save, AG ES cells can be a powerful source for patient-specific pluripotent cells and make a wide range of transplantable cells available.

7.6 Uniparental Cells in Therapies for Neural Diseases

A widely used model neural disease, Parkinson's disease, is considered for stem cell therapy because of the selective nature of the degenerative process and the possibility of functional cell-replacement. This has been shown by foetal tissue transplants in some Parkinson's disease patients (Mendez *et al.*, 2005). In addition, mouse ES cells can *in vitro* and *in vivo* differentiate into functional dopaminergic neurons and upon transplantation improve motor deficits in animal models of Parkinson's disease (Bjorklund *et al.*, 2002; Kim *et al.*, 2002; Rodriguez-Gomez *et al.*, 2007).

A recent publication in 2008 by Sanchez-Pernaute *et al.* showed the restoration of function in a Parkinson's disease rat model by dopaminergic neurons derived from primate PG ES cells. These neurons displayed constant expression of midbrain region- and cell-specific transcription factors. Upon transplantation of PG dopamine neurons the authors observed the restoration of motor function in Parkinson's disease model rats. Treatment with defined growth factors during the differentiation process and transplanting only differentiated, post-mitotic neurons eliminated the risk of tumour formation (Sanchez-Pernaute *et al.*, 2008). This study further advances the concept that uniparental stem cells are a potential source of functional neural cells for further therapeutic applications.

7.7 Neural Differentiation Potential of Murine AG ES Cells

In the experiments of this thesis the neural developmental potential of murine AG ES cells *in vitro* and *in vivo* was investigated. In summary, it was observed that AG ES cells exhibit *in vitro* and early *in vivo* neural developmental potential similar to N ES cells. AG cells contribute to developing brains in early foetal stages and show widespread and balanced distribution in chimeric brains. AG brain cells form neurospheres with self-renewal and neural differentiation capacity similar to N ES cells. These results on early foetal brain seeding contrast the findings for AG ICM cells by Keverne *et al.* The *in vitro* analyses of AG ES cells and AG ES cell-derive neurosphere cells exceed the study from 1996.

Under *in vitro* neural differentiation conditions, AG cells formed pnPCs that differentiated into neuronal and astroglial cells at frequencies comparable to N ES cells. *In vivo* differentiation of AG ES cells in chimaeras revealed unrestricted contribution of AG cells to developing foetal brains with widespread distribution of proliferating AG neurons. When cultured together, AG-derived neurosphere

cultures isolated from chimeric foetal brains showed similar growth properties compared to blastocyst derived (N) cultures indicating that the paternal origin AG cells in E12.5 brains do not have a proliferative disadvantage. Finally, it was observed that AG and control N neurosphere cells from chimeric brains displayed similar self-renewal capacity and neural multi-lineage differentiation potential. Therefore, both *in vitro* and *in vivo* at early foetal stages, ES cell-derived AG cells appear to be fully potent.

7.8 Neural *In Vitro* Differentiation of AG ES Cells

As shown for N ES cells, AG ES cells gave rise to pnPCs and differentiated into neural and glial cells, when subjected to appropriate culture conditions (Brüstle *et al.*, 1997; Barberi *et al.*, 2003; Haupt *et al.*, 2007). The frequencies of early neurons and astroglial cells did not significantly differ between AG- and N-derived pnPCs. These results demonstrate that the uniparental, paternal-only setup of the AG cells leads to no disadvantage in neural *in vitro* differentiation in the utilised assay system.

7.9 *In vivo* Contribution of AG ES Cells to Early Foetal Brains

The brain seeding capacity of AG ICM cells in E10-12 embryos was previously described (Barton *et al.*, 1991). However, as whole brain extracts were analysed for donor glucose-6-phosphate isomerase-1 (Gpi1) isoform contribution, this did not include analysis of regional distribution. In a second study, AG cells arising either from aggregation of AG and N morulae or from injection of AG ICM cells into N blastocysts were found to substantially contribute to the developing E13 and E17 hypothalamus but not to the cortex, and AG cell contribution was associated with smaller brain size (Keverne *et al.*, 1996). The limited contribution of AG ICM cells to brain establishes an expectation that AG cells would show restricted neural differentiation potential compared to N cells. ES cell contribution displayed stochastic variation between chimaeras, but no indication for exclusion to foetal brain was observed for AG compared to N cells. Stochastic variation of ES cells contributing to different tissues in chimeric embryos has been described earlier (Beddington and Robertson, 1989). Despite the apparent phenotype and lethality in AG chimaeras, the analyses shown here of AG contribution to early brain development at E12.5 revealed a widespread and balanced distribution of AG ES cell-derived cells to forebrain regions including striatum, hypothalamus and cortex. This suggests that during establishment and culturing AG ES cells overcome

limitations which are still active in ICM cells (Dean *et al.*, 1998; Humpherys *et al.*, 2001; Jiang *et al.*, 2007).

7.10 Neurosphere Cells from AG Chimeric Foetal Brains

Analyses of AG- compared to N-derived brain cells revealed no differences in neurosphere initiating cell and neural differentiation frequencies. Although the ability of ICM derived AG cells in foetal chimaeras to form neurospheres is unknown, the findings of this study indicate that AG ES cells and ICM cells differ in their developmental potential *in vivo* (Keverne *et al.*, 1996). One reason for this apparent discrepancy may be that during the establishment of ES cells from ICM cells, regulators of differentiation are modified or the epigenome undergoes changes (Dean *et al.*, 1998; Humpherys *et al.*, 2001; Jiang *et al.*, 2007).

Gene expression analysis of a selected set of imprinted genes expressed in brain tissue (Wilkinson *et al.*, 2007) revealed no overall equal pattern in neurospheres from three individual foetal brains. The paternally expressed genes *Igf2* and *Impact* were not equally upregulated in AG derived neurosphere cells. The maternally expressed genes *Igf2r* and *Zim1* were downregulated while the maternally expressed genes *Ube3A* and *H19* exhibited no uniform expression pattern in the analysed embryos, even showing overexpression in single embryos (see 6.5.2). The expression analysis of genes with parent-of-origin-specific expression pattern in the brain in neurosphere cultures suggests that genomic imprinting of these genes is altered during embryonic development, establishing and culturing of neurospheres or both. Imprinted gene expression is not static during development (Mitalipov, 2006). For the brain it is described as spatiotemporally dynamic (Wilkinson *et al.*, 2007), suggesting that depending on when and where the cell is located in the developing organism imprinted gene expression can be altered, imprinting can be relaxed or erased. Furthermore, the mechanism recognising imprinting can be inactive and the genetic imprint is ignored (Solter, 1998). The underlying mechanisms for the dynamic nature of imprinted gene expression are not yet understood. However, these mechanisms could be activated during AG ES cell establishment or during NSC culture.

Differences were observed in the neuronal lineage differentiation frequencies between ES cell- and *in vitro* expanded foetal brain-derived cells, respectively. The different neuronal capacity is probably caused by the *in vitro* expansion of neurosphere cells over four - six passages. It was previously reported that ES cell-derived neural progenitor cells maintain their neuronal potential during *in vitro*

expansion, whereas E12.5 foetal brain-derived neural progenitor / stem cells do not (Chung *et al.*, 2006). In mixed whole brain cell cultures of AG and N chimeric embryos we detected on average a minor growth disadvantage both for AG- and N neurosphere cells in comparison to the blastocyst-derived cells. As both AG and N cells exhibit this feature, it is likely that this is caused by the different mouse strain backgrounds of ES cells and blastocysts or due to eGFP transgene expression (Badrian and Bogoyevitch, 2007).

7.11 Conclusion

The results of this study show that the *in vitro* and *in vivo* early neural developmental potential of AG ES cells in early embryos is similar to N ES cells, in contrast to the limitations of AG ICM cells. Together with the normal hematopoietic reconstitution with AG- / GG-derived cells, the successful transplantation and functional integration of primate PG neurons in rat Parkinson's disease brains and the generation of MHC-matched human PG ES cells, the results shown here argue that uniparental ES cells are a promising source for pluripotent cells. However, now it is necessary to test AG-derived neurons for their functionality as well as to generate human AG ES cells and to analyse these cells as potential sources of patient-specific pluripotent cells.

8 LITERATURE

Albrecht, U., Sutcliffe, J. S., Cattanach, B. M., Beechey, C. V., Armstrong, D., Eichele, G. and Beaudet, A. L. (1997). Imprinted expression of the murine Angelman syndrome gene, Ube3a, in hippocampal and Purkinje neurons. *Nat Genet* **17**, 75-8.

Allen, N. D., Barton, S. C., Hilton, K., Norris, M. L. and Surani, M. A. (1994). A functional analysis of imprinting in parthenogenetic embryonic stem cells. *Development* **120**, 1473-82.

Allen, N. D., Logan, K., Lally, G., Drage, D. J., Norris, M. L. and Keverne, E. B. (1995). Distribution of parthenogenetic cells in the mouse brain and their influence on brain development and behavior. *Proc Natl Acad Sci U S A* **92**, 10782-6.

Badrian, B. and Bogoyevitch, M. A. (2007). Changes in the Transcriptional Profile of Cardiac Myocytes Following Green Fluorescent Protein Expression. *DNA Cell Biol*.

Balakier, H. and Tarkowski, A. K. (1976). Diploid parthenogenetic mouse embryos produced by heat-shock and Cytochalasin B. *J Embryol Exp Morphol* **35**, 25-39.

Barberi, T., Klivenyi, P., Calingasan, N. Y., Lee, H., Kawamata, H., Loonam, K., Perrier, A. L., Bruses, J., Rubio, M. E., Topf, N. *et al.* (2003). Neural subtype specification of fertilisation and nuclear transfer embryonic stem cells and application in parkinsonian mice. *Nat Biotechnol* **21**, 1200-7.

Barton, S. C., Ferguson-Smith, A. C., Fundele, R. and Surani, M. A. (1991). Influence of paternally imprinted genes on development. *Development* **113**, 679-87.

Beddington, R. S. and Robertson, E. J. (1989). An assessment of the developmental potential of embryonic stem cells in the midgestation mouse embryo. *Development* **105**, 733-7.

Bianco, P., Robey, P. G. and Simmons, P. J. (2008). Mesenchymal stem cells: revisiting history, concepts, and assays. *Cell Stem Cell* **2**, 313-9.

Bjorklund, L. M., Sanchez-Pernaute, R., Chung, S., Andersson, T., Chen, I. Y., McNaught, K. S., Brownell, A. L., Jenkins, B. G., Wahlestedt, C., Kim, K. S. *et al.* (2002). Embryonic stem cells develop into functional dopaminergic neurons after transplantation in a Parkinson rat model. *Proc Natl Acad Sci U S A* **99**, 2344-9.

Bradley, A., Evans, M., Kaufman, M. H. and Robertson, E. (1984). Formation of germ-line chimaeras from embryo-derived teratocarcinoma cell lines. *Nature* **309**, 255-6.

Brüstle, O., Jones, K. N., Learish, R. D., Karram, K., Choudhary, K., Wiestler, O. D., Duncan, I. D. and McKay, R. D. (1999). Embryonic stem cell-derived glial precursors: a source of myelinating transplants. *Science* **285**, 754-6.

Brustle, O., Spiro, A. C., Karram, K., Choudhary, K., Okabe, S. and McKay, R. D. (1997). In vitro-generated neural precursors participate in mammalian brain development. *Proc Natl Acad Sci U S A* **94**, 14809-14.

Brüstle, O., Spiro, A. C., Karram, K., Choudhary, K., Okabe, S. and McKay, R. D. (1997). In vitro-generated neural precursors participate in mammalian brain development. *Proc Natl Acad Sci U S A* **94**, 14809-14.

Christoforou, N. and Gearhart, J. D. (2007). Stem cells and their potential in cell-based cardiac therapies. *Prog Cardiovasc Dis* **49**, 396-413.

Chung, S., Shin, B. S., Hwang, M., Lardaro, T., Kang, U. J., Isacson, O. and Kim, K. S. (2006). Neural precursors derived from embryonic stem cells, but not those from fetal ventral mesencephalon, maintain the potential to differentiate into dopaminergic neurons after expansion in vitro. *Stem Cells* **24**, 1583-93.

Cibelli, J. B., Grant, K. A., Chapman, K. B., Cunniff, K., Worst, T., Green, H. L., Walker, S. J., Gutin, P. H., Vilner, L., Tabar, V. *et al.* (2002). Parthenogenetic stem cells in nonhuman primates. *Science* **295**, 819.

Colombo, E., Giannelli, S. G., Galli, R., Tagliafico, E., Foroni, C., Tenedini, E., Ferrari, S., Corte, G., Vescovi, A., Cossu, G. *et al.* (2005). ES derived vs. somatic neural stem cells: a comparative analysis of their developmental potential and molecular phenotype. *Stem Cells*.

Conrad, S., Renninger, M., Hennenlotter, J., Wiesner, T., Just, L., Bonin, M., Aicher, W., Buhring, H. J., Mattheus, U., Mack, A. *et al.* (2008). Generation of pluripotent stem cells from adult human testis. *Nature* **456**, 344-9.

Constancia, M., Angiolini, E., Sandovici, I., Smith, P., Smith, R., Kelsey, G., Dean, W., Ferguson-Smith, A., Sibley, C. P., Reik, W. *et al.* (2005). Adaptation of nutrient supply to fetal demand in the mouse involves interaction between the Igf2 gene and placental transporter systems. *Proc Natl Acad Sci U S A* **102**, 19219-24.

Conti, L., Pollard, S. M., Gorba, T., Reitano, E., Toselli, M., Biella, G., Sun, Y., Sanzone, S., Ying, Q. L., Cattaneo, E. *et al.* (2005). Niche-independent symmetrical self-renewal of a mammalian tissue stem cell. *PLoS Biol* **3**, e283.

Davies, W., Isles, A., Smith, R., Karunadasa, D., Burrmann, D., Humby, T., Ojarikre, O., Biggin, C., Skuse, D., Burgoyne, P. *et al.* (2005a). Xlr3b is a new imprinted candidate for X-linked parent-of-origin effects on cognitive function in mice. *Nat Genet* **37**, 625-9.

Davies, W., Isles, A. R. and Wilkinson, L. S. (2005b). Imprinted gene expression in the brain. *Neurosci Biobehav Rev* **29**, 421-30.

Dean, W., Bowden, L., Aitchison, A., Klose, J., Moore, T., Meneses, J. J., Reik, W. and Feil, R. (1998). Altered imprinted gene methylation and expression in completely ES cell-derived mouse fetuses: association with aberrant phenotypes. *Development* **125**, 2273-82.

Delaval, K. and Feil, R. (2004). Epigenetic regulation of mammalian genomic imprinting. *Curr Opin Genet Dev* **14**, 188-95.

Doetsch, F. (2003). The glial identity of neural stem cells. *Nat Neurosci* **6**, 1127-34.

Doetsch, F., Caille, I., Lim, D. A., Garcia-Verdugo, J. M. and Alvarez-Buylla, A. (1999). Subventricular zone astrocytes are neural stem cells in the adult mammalian brain. *Cell* **97**, 703-16.

Doetschman, T., Gregg, R. G., Maeda, N., Hooper, M. L., Melton, D. W., Thompson, S. and Smithies, O. (1987). Targetted correction of a mutant HPRT gene in mouse embryonic stem cells. *Nature* **330**, 576-8.

Doetschman, T. C., Eistetter, H., Katz, M., Schmidt, W. and Kemler, R. (1985). The in vitro development of blastocyst-derived embryonic stem cell lines: formation of visceral yolk sac, blood islands and myocardium. *J Embryol Exp Morphol* **87**, 27-45.

Eckardt, S., Leu, N. A., Bradley, H. L., Kato, H., Bunting, K. D. and McLaughlin, K. J. (2007). Hematopoietic reconstitution with androgenetic and gynogenetic stem cells. *Genes Dev* **21**, 409-19.

Efstratiadis, A. (1994). Parental imprinting of autosomal mammalian genes. *Curr Opin Genet Dev* **4**, 265-80.

Evans, M. J. and Kaufman, M. H. (1981). Establishment in culture of pluripotential cells from mouse embryos. *Nature* **292**, 154-6.

Falls, J. G., Pulford, D. J., Wylie, A. A. and Jirtle, R. L. (1999). Genomic imprinting: implications for human disease. *Am J Pathol* **154**, 635-47.

Fundele, R., Norris, M. L., Barton, S. C., Reik, W. and Surani, M. A. (1989). Systematic elimination of parthenogenetic cells in mouse chimeras. *Development* **106**, 29-35.

Fundele, R. H., Norris, M. L., Barton, S. C., Fehlau, M., Howlett, S. K., Mills, W. E. and Surani, M. A. (1990). Temporal and spatial selection against parthenogenetic cells during development of fetal chimeras. *Development* **108**, 203-11.

Gough, N. M., Williams, R. L., Hilton, D. J., Pease, S., Willson, T. A., Stahl, J., Gearing, D. P., Nicola, N. A. and Metcalf, D. (1989). LIF: a molecule with divergent actions on myeloid leukaemic cells and embryonic stem cells. *Reprod Fertil Dev* **1**, 281-8.

Graham, C. F. (1970). Parthenogenetic mouse blastocysts. *Nature* **226**, 165-7.

Graham, C. F. (1974). The production of parthenogenetic mammalian embryos and their use in biological research. *Biol Rev Camb Philos Soc* **49**, 399-424.

Gritti, A., Frolichsthal-Schoeller, P., Galli, R., Parati, E. A., Cova, L., Pagano, S. F., Bjornson, C. R. and Vescovi, A. L. (1999). Epidermal and fibroblast growth factors behave as mitogenic regulators for a single multipotent stem cell-like population from the subventricular region of the adult mouse forebrain. *J Neurosci* **19**, 3287-97.

Guan, K., Nayernia, K., Maier, L. S., Wagner, S., Dressel, R., Lee, J. H., Nolte, J., Wolf, F., Li, M., Engel, W. *et al.* (2006). Pluripotency of spermatogonial stem cells from adult mouse testis. *Nature* **440**, 1199-203.

Hanna, J., Wernig, M., Markoulaki, S., Sun, C. W., Meissner, A., Cassady, J. P., Beard, C., Brambrink, T., Wu, L. C., Townes, T. M. *et al.* (2007). Treatment of sickle cell anemia mouse model with iPS cells generated from autologous skin. *Science* **318**, 1920-3.

Haupt, S., Edenhofer, F., Peitz, M., Leinhaas, A. and Brustle, O. (2007). Stage-specific conditional mutagenesis in mouse embryonic stem cell-derived neural cells and postmitotic neurons by direct delivery of biologically active Cre recombinase. *Stem Cells* **25**, 181-8.

Hernandez, L., Kozlov, S., Piras, G. and Stewart, C. L. (2003). Paternal and maternal genomes confer opposite effects on proliferation, cell-cycle length, senescence, and tumor formation. *Proc Natl Acad Sci U S A* **100**, 13344-9.

Hochedlinger, K. and Jaenisch, R. (2003). Nuclear transplantation, embryonic stem cells, and the potential for cell therapy. *N Engl J Med* **349**, 275-86.

Hubner, K., Fuhrmann, G., Christenson, L. K., Kehler, J., Reinbold, R., De La Fuente, R., Wood, J., Strauss, J. F., 3rd, Boiani, M. and Scholer, H. R. (2003). Derivation of oocytes from mouse embryonic stem cells. *Science* **300**, 1251-6.

Humpherys, D., Eggan, K., Akutsu, H., Hochedlinger, K., Rideout, W. M., 3rd, Biniszkiewicz, D., Yanagimachi, R. and Jaenisch, R. (2001). Epigenetic instability in ES cells and cloned mice. *Science* **293**, 95-7.

Ivanova, N. B., Dimos, J. T., Schaniel, C., Hackney, J. A., Moore, K. A. and Lemischka, I. R. (2002). A stem cell molecular signature. *Science* **298**, 601-4.

Jaenisch, R. (1997). DNA methylation and imprinting: why bother? *Trends Genet* **13**, 323-9.

Jaenisch, R. (2004). Human cloning - the science and ethics of nuclear transplantation. *N Engl J Med* **351**, 2787-91.

Jagerbauer, E. M., Fraser, A., Herbst, E. W., Kothary, R. and Fundele, R. (1992). Parthenogenetic stem cells in postnatal mouse chimeras. *Development* **116**, 95-102.

Jiang, H., Sun, B., Wang, W., Zhang, Z., Gao, F., Shi, G., Cui, B., Kong, X., He, Z., Ding, X. *et al.* (2007). Activation of paternally expressed imprinted genes in newly derived germline-competent mouse parthenogenetic embryonic stem cell lines. *Cell Res* **17**, 792-803.

Jiang, Y., Jahagirdar, B. N., Reinhardt, R. L., Schwartz, R. E., Keene, C. D., Ortiz-Gonzalez, X. R., Reyes, M., Lenvik, T., Lund, T., Blackstad, M. *et al.* (2002). Pluripotency of mesenchymal stem cells derived from adult marrow. *Nature* **418**, 41-9.

Kajii, T. and Ohama, K. (1977). Androgenetic origin of hydatidiform mole. *Nature* **268**, 633-4.

Keverne, E. B., Fundele, R., Narasimha, M., Barton, S. C. and Surani, M. A. (1996). Genomic imprinting and the differential roles of parental genomes in brain development. *Brain Res Dev Brain Res* **92**, 91-100.

Kim, J., Lu, X. and Stubbs, L. (1999). Zim1, a maternally expressed mouse Kruppel-type zinc-finger gene located in proximal chromosome 7. *Hum Mol Genet* **8**, 847-54.

Kim, J. H., Auerbach, J. M., Rodriguez-Gomez, J. A., Velasco, I., Gavin, D., Lumelsky, N., Lee, S. H., Nguyen, J., Sanchez-Pernaute, R., Bankiewicz, K. *et al.* (2002). Dopamine neurons derived from embryonic stem cells function in an animal model of Parkinson's disease. *Nature* **418**, 50-6.

Kim, K., Lerou, P., Yabuuchi, A., Lengerke, C., Ng, K., West, J., Kirby, A., Daly, M. J. and Daley, G. Q. (2007). Histocompatible embryonic stem cells by parthenogenesis. *Science* **315**, 482-6.

Kossack, N., Meneses, J., Shefi, S., Nguyen, H. N., Chavez, S., Nicholas, C., Gromoll, J., Turek, P. J. and Reijo-Pera, R. A. (2008). Isolation and Characterisation of Pluripotent Human Spermatogonial Stem Cell-Derived Cells. *Stem Cells*.

Lagutina, I., Lazzari, G., Duchi, R. and Galli, C. (2004). Developmental potential of bovine androgenetic and parthenogenetic embryos: a comparative study. *Biol Reprod* **70**, 400-5.

Lee, S. H., Lumelsky, N., Studer, L., Auerbach, J. M. and McKay, R. D. (2000). Efficient generation of midbrain and hindbrain neurons from mouse embryonic stem cells. *Nat Biotechnol* **18**, 675-9.

Lerou, P. H. and Daley, G. Q. (2005). Therapeutic potential of embryonic stem cells. *Blood Rev* **19**, 321-31.

Mann, J. R. (2001). Deriving and propagating mouse embryonic stem cell lines for studying genomic imprinting. *Methods Mol Biol* **181**, 21-39.

Mann, J. R., Gadi, I., Harbison, M. L., Abbondanzo, S. J. and Stewart, C. L. (1990). Androgenetic mouse embryonic stem cells are pluripotent and cause skeletal defects in chimeras: implications for genetic imprinting. *Cell* **62**, 251-60.

Mann, J. R. and Stewart, C. L. (1991). Development to term of mouse androgenetic aggregation chimeras. *Development* **113**, 1325-33.

Martin, G. R. (1981). Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc Natl Acad Sci U S A* **78**, 7634-8.

Mayer-Proschel, M., Kalyani, A. J., Mujtaba, T. and Rao, M. S. (1997). Isolation of lineage-restricted neuronal precursors from multipotent neuroepithelial stem cells. *Neuron* **19**, 773-85.

McGrath, J. and Solter, D. (1983). Nuclear transplantation in the mouse embryo by microsurgery and cell fusion. *Science* **220**, 1300-2.

McGrath, J. and Solter, D. (1984). Completion of mouse embryogenesis requires both the maternal and paternal genomes. *Cell* **37**, 179-83.

McLaughlin, K. J., Kochanowski, H., Solter, D., Schwarzkopf, G., Szabo, P. E. and Mann, J. R. (1997). Roles of the imprinted gene Igf2 and paternal duplication

of distal chromosome 7 in the perinatal abnormalities of androgenetic mouse chimeras. *Development* **124**, 4897-904.

Mendez, I., Sanchez-Pernaute, R., Cooper, O., Vinuela, A., Ferrari, D., Bjorklund, L., Dagher, A. and Isacson, O. (2005). Cell type analysis of functional fetal dopamine cell suspension transplants in the striatum and substantia nigra of patients with Parkinson's disease. *Brain* **128**, 1498-510.

Mitalipov, S. M. (2006). Genomic imprinting in primate embryos and embryonic stem cells. *Reprod Fertil Dev* **18**, 817-21.

Moore, K. A. and Lemischka, I. R. (2006). Stem cells and their niches. *Science* **311**, 1880-5.

Morison, I. M. and Reeve, A. E. (1998). A catalogue of imprinted genes and parent-of-origin effects in humans and animals. *Hum Mol Genet* **7**, 1599-609.

Morrison, S. J., Hemmati, H. D., Wandycz, A. M. and Weissman, I. L. (1995). The purification and characterisation of fetal liver hematopoietic stem cells. *Proc Natl Acad Sci U S A* **92**, 10302-6.

Morshead, C. M. and van der Kooy, D. (2004). Disguising adult neural stem cells. *Curr Opin Neurobiol* **14**, 125-31.

Mujtaba, T., Mayer-Proschel, M. and Rao, M. S. (1998). A common neural progenitor for the CNS and PNS. *Dev Biol* **200**, 1-15.

Müller, A. M., Medvinsky, A., Strouboulis, J., Grosveld, F. and Dzierzak, E. (1994). Development of hematopoietic stem cell activity in the mouse embryo. *Immunity* **1**, 291-301.

Nagy, A., Gocza, E., Diaz, E. M., Prideaux, V. R., Ivanyi, E., Markkula, M. and Rossant, J. (1990). Embryonic stem cells alone are able to support fetal development in the mouse. *Development* **110**, 815-21.

Nagy, A., Sass, M. and Markkula, M. (1989). Systematic non-uniform distribution of parthenogenetic cells in adult mouse chimaeras. *Development* **106**, 321-4.

Namura, S., Zhu, J., Fink, K., Endres, M., Srinivasan, A., Tomaselli, K. J., Yuan, J. and Moskowitz, M. A. (1998). Activation and cleavage of caspase-3 in apoptosis induced by experimental cerebral ischemia. *J Neurosci* **18**, 3659-68.

Narasimha, M., Barton, S. C. and Surani, M. A. (1997). The role of the paternal genome in the development of the mouse germ line. *Curr Biol* **7**, 881-4.

Newman-Smith, E. D. and Werb, Z. (1995). Stem cell defects in parthenogenetic peri-implantation embryos. *Development* **121**, 2069-77.

Okabe, M., Ikawa, M., Kominami, K., Nakanishi, T. and Nishimune, Y. (1997). 'Green mice' as a source of ubiquitous green cells. *FEBS Lett* **407**, 313-9.

Okabe, S., Forsberg-Nilsson, K., Spiro, A. C., Segal, M. and McKay, R. D. (1996). Development of neuronal precursor cells and functional postmitotic neurons from embryonic stem cells in vitro. *Mech Dev* **59**, 89-102.

Paldi, A., Nagy, A., Markkula, M., Barna, I. and Dezso, L. (1989). Postnatal development of parthenogenetic in equilibrium with fertilized mouse aggregation chimeras. *Development* **105**, 115-8.

Pham, N. V., Nguyen, M. T., Hu, J. F., Vu, T. H. and Hoffman, A. R. (1998). Dissociation of IGF2 and H19 imprinting in human brain. *Brain Res* **810**, 1-8.

Pollard, S. M., Conti, L., Sun, Y., Goffredo, D. and Smith, A. (2006). Adherent neural stem (NS) cells from fetal and adult forebrain. *Cereb Cortex* **16 Suppl 1**, i112-20.

Qian, X., Shen, Q., Goderie, S. K., He, W., Capela, A., Davis, A. A. and Temple, S. (2000). Timing of CNS cell generation: a programmed sequence of neuron and glial cell production from isolated murine cortical stem cells. *Neuron* **28**, 69-80.

Ramalho-Santos, M., Yoon, S., Matsuzaki, Y., Mulligan, R. C. and Melton, D. A. (2002). "Stemness": transcriptional profiling of embryonic and adult stem cells. *Science* **298**, 597-600.

Rao, M. S. and Mayer-Proschel, M. (1997). Glial-restricted precursors are derived from multipotent neuroepithelial stem cells. *Dev Biol* **188**, 48-63.

Reik, W. and Walter, J. (2001). Genomic imprinting: parental influence on the genome. *Nat Rev Genet* **2**, 21-32.

Reubinoff, B. E., Pera, M. F., Fong, C. Y., Trounson, A. and Bongso, A. (2000). Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro. *Nat Biotechnol* **18**, 399-404.

Revazova, E. S., Turovets, N. A., Kochetkova, O. D., Kindarova, L. B., Kuzmichev, L. N., Janus, J. D. and Pryzhkova, M. V. (2007). Patient-specific stem cell lines derived from human parthenogenetic blastocysts. *Cloning Stem Cells* **9**, 432-49.

Rideout, W. M., 3rd, Hochedlinger, K., Kyba, M., Daley, G. Q. and Jaenisch, R. (2002). Correction of a genetic defect by nuclear transplantation and combined cell and gene therapy. *Cell* **109**, 17-27.

Robertson, E., Bradley, A., Kuehn, M. and Evans, M. (1986). Germ-line transmission of genes introduced into cultured pluripotential cells by retroviral vector. *Nature* **323**, 445-8.

Robertson, E. J., Evans, M. J. and Kaufman, M. H. (1983). X-chromosome instability in pluripotential stem cell lines derived from parthenogenetic embryos. *J Embryol Exp Morphol* **74**, 297-309.

Rodriguez-Gomez, J. A., Lu, J. Q., Velasco, I., Rivera, S., Zoghbi, S. S., Liow, J. S., Musachio, J. L., Chin, F. T., Toyama, H., Seidel, J. *et al.* (2007). Persistent dopamine functions of neurons derived from embryonic stem cells in a rodent model of Parkinson disease. *Stem Cells* **25**, 918-28.

Rougeulle, C., Glatt, H. and Lalande, M. (1997). The Angelman syndrome candidate gene, UBE3A/E6-AP, is imprinted in brain. *Nat Genet* **17**, 14-5.

Roy, N. S., Cleren, C., Singh, S. K., Yang, L., Beal, M. F. and Goldman, S. A. (2006). Functional engraftment of human ES cell-derived dopaminergic neurons

enriched by coculture with telomerase-immortalized midbrain astrocytes. *Nat Med* **12**, 1259-68.

Runte, M., Kroisel, P. M., Gillessen-Kaesbach, G., Varon, R., Horn, D., Cohen, M. Y., Wagstaff, J., Horsthemke, B. and Buiting, K. (2004). SNURF-SNRPN and UBE3A transcript levels in patients with Angelman syndrome. *Hum Genet* **114**, 553-61.

Sanchez-Pernaute, R., Lee, H., Patterson, M., Reske-Nielsen, C., Yoshizaki, T., Sonntag, K. C., Studer, L. and Isacson, O. (2008). Parthenogenetic dopamine neurons from primate embryonic stem cells restore function in experimental Parkinson's disease. *Brain* **131**, 2127-39.

Sanchez-Pernaute, R., Studer, L., Ferrari, D., Perrier, A., Lee, H., Vinuela, A. and Isacson, O. (2005). Long-term survival of dopamine neurons derived from parthenogenetic primate embryonic stem cells (cyno-1) after transplantation. *Stem Cells* **23**, 914-22.

Schmittwolf, C., Kirchhof, N., Jauch, A., Durr, M., Harder, F., Zenke, M. and Muller, A. M. (2005). In vivo haematopoietic activity is induced in neurosphere cells by chromatin-modifying agents. *Embo J* **24**, 554-66.

Schuldiner, M., Yanuka, O., Itskovitz-Eldor, J., Melton, D. A. and Benvenisty, N. (2000). Effects of eight growth factors on the differentiation of cells derived from human embryonic stem cells. *Proc Natl Acad Sci U S A* **97**, 11307-12.

Seandel, M., James, D., Shmelkov, S. V., Falciatori, I., Kim, J., Chavala, S., Scherr, D. S., Zhang, F., Torres, R., Gale, N. W. *et al.* (2007). Generation of functional multipotent adult stem cells from GPR125+ germline progenitors. *Nature* **449**, 346-50.

Slee, E. A., Adrain, C. and Martin, S. J. (2001). Executioner caspase-3, -6, and -7 perform distinct, non-redundant roles during the demolition phase of apoptosis. *J Biol Chem* **276**, 7320-6.

Slim, R. and Mehio, A. (2007). The genetics of hydatidiform moles: new lights on an ancient disease. *Clin Genet* **71**, 25-34.

Solter, D. (1998). Imprinting. *Int J Dev Biol* **42**, 951-4.

Sonntag, K. C., Pruszak, J., Yoshizaki, T., van Arensbergen, J., Sanchez-Pernaute, R. and Isacson, O. (2007). Enhanced yield of neuroepithelial precursors and midbrain-like dopaminergic neurons from human embryonic stem cells using the bone morphogenic protein antagonist noggin. *Stem Cells* **25**, 411-8.

Stevens, L. C. (1978). Totipotent cells of parthenogenetic origin in a chimaeric mouse. *Nature* **276**, 266-7.

Stevens, L. C., Varnum, D. S. and Eicher, E. M. (1977). Viable chimaeras produced from normal and parthenogenetic mouse embryos. *Nature* **269**, 515-7.

Surani, M. A. and Barton, S. C. (1983). Development of gynogenetic eggs in the mouse: implications for parthenogenetic embryos. *Science* **222**, 1034-6.

Surani, M. A., Barton, S. C. and Kaufman, M. H. (1977). Development to term of chimaeras between diploid parthenogenetic and fertilised embryos. *Nature* **270**, 601-3.

Surani, M. A., Barton, S. C. and Norris, M. L. (1984). Development of reconstituted mouse eggs suggests imprinting of the genome during gametogenesis. *Nature* **308**, 548-50.

Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K. and Yamanaka, S. (2007). Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* **131**, 861-72.

Takahashi, K. and Yamanaka, S. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* **126**, 663-76.

Thomas, K. R. and Capecchi, M. R. (1987). Site-directed mutagenesis by gene targeting in mouse embryo-derived stem cells. *Cell* **51**, 503-12.

Thomson, J. A., Itskovitz-Eldor, J., Shapiro, S. S., Waknitz, M. A., Swiergiel, J. J., Marshall, V. S. and Jones, J. M. (1998). Embryonic stem cell lines derived from human blastocysts. *Science* **282**, 1145-7.

Toyooka, Y., Tsunekawa, N., Akasu, R. and Noce, T. (2003). Embryonic stem cells can form germ cells in vitro. *Proc Natl Acad Sci U S A* **100**, 11457-62.

Vescovi, A. L., Parati, E. A., Gritti, A., Poulin, P., Ferrario, M., Wanke, E., Frolichsthal-Schoeller, P., Cova, L., Arcellana-Panlilio, M., Colombo, A. *et al*. (1999). Isolation and cloning of multipotential stem cells from the embryonic human CNS and establishment of transplantable human neural stem cell lines by epigenetic stimulation. *Exp Neurol* **156**, 71-83.

Vescovi, A. L. and Snyder, E. Y. (1999). Establishment and properties of neural stem cell clones: plasticity in vitro and in vivo. *Brain Pathol* **9**, 569-98.

Wakayama, T., Tabar, V., Rodriguez, I., Perry, A. C., Studer, L. and Mombaerts, P. (2001). Differentiation of embryonic stem cell lines generated from adult somatic cells by nuclear transfer. *Science* **292**, 740-3.

Walter, J. and Paulsen, M. (2003). Imprinting and disease. *Semin Cell Dev Biol* **14**, 101-10.

Watt, F. M. (1998). Epidermal stem cells: markers, patterning and the control of stem cell fate. *Philos Trans R Soc Lond B Biol Sci* **353**, 831-7.

Weiss, S., Dunne, C., Hewson, J., Wohl, C., Wheatley, M., Peterson, A. C. and Reynolds, B. A. (1996a). Multipotent CNS stem cells are present in the adult mammalian spinal cord and ventricular neuroaxis. *J Neurosci* **16**, 7599-609.

Weiss, S., Reynolds, B. A., Vescovi, A. L., Morshead, C., Craig, C. G. and van der Kooy, D. (1996b). Is there a neural stem cell in the mammalian forebrain? *Trends Neurosci* **19**, 387-93.

Weissman, I. L. (2006). Medicine: politic stem cells. *Nature* **439**, 145-7.

Wenger, R. H., Kopf, M., Nitschke, L., Lamers, M. C., Kohler, G. and Nielsen, P. J. (1995). B-cell maturation in chimaeric mice deficient for the heat stable antigen (HSA/mouse CD24). *Transgenic Res* **4**, 173-83.

Wernig, M., Zhao, J. P., Pruszak, J., Hedlund, E., Fu, D., Soldner, F., Broccoli, V., Constantine-Paton, M., Isacson, O. and Jaenisch, R. (2008). Neurons derived from reprogrammed fibroblasts functionally integrate into the fetal brain and improve symptoms of rats with Parkinson's disease. *Proc Natl Acad Sci U S A* **105**, 5856-61.

Wilkinson, L. S., Davies, W. and Isles, A. R. (2007). Genomic imprinting effects on brain development and function. *Nat Rev Neurosci* **8**, 832-43.

Williams, R. L., Hilton, D. J., Pease, S., Willson, T. A., Stewart, C. L., Gearing, D. P., Wagner, E. F., Metcalf, D., Nicola, N. A. and Gough, N. M. (1988). Myeloid leukaemia inhibitory factor maintains the developmental potential of embryonic stem cells. *Nature* **336**, 684-7.

Wilmut, I., Schnieke, A. E., McWhir, J., Kind, A. J. and Campbell, K. H. (1997). Viable offspring derived from fetal and adult mammalian cells. *Nature* **385**, 810-3.

Wobus, A. M., Grosse, R. and Schoneich, J. (1988). Specific effects of nerve growth factor on the differentiation pattern of mouse embryonic stem cells in vitro. *Biomed Biochim Acta* **47**, 965-73.

Wu, Q., Kumagai, T., Kawahara, M., Ogawa, H., Hiura, H., Obata, Y., Takano, R. and Kono, T. (2006). Regulated expression of two sets of paternally imprinted genes is necessary for mouse parthenogenetic development to term. *Reproduction* **131**, 481-8.

Ying, Q. L., Stavridis, M., Griffiths, D., Li, M. and Smith, A. (2003). Conversion of embryonic stem cells into neuroectodermal precursors in adherent monoculture. *Nat Biotechnol* **21**, 183-6.

Yu, J., Vodyanik, M. A., Smuga-Otto, K., Antosiewicz-Bourget, J., Frane, J. L., Tian, S., Nie, J., Jonsdottir, G. A., Ruotti, V., Stewart, R. *et al.* (2007). Induced pluripotent stem cell lines derived from human somatic cells. *Science* **318**, 1917-20.

9 ABBREVIATIONS

AG	Androgenetic
BSA	Bovine Serum Albumin
bFGF	Basic fibroblast growth factor
cm	Centimetre
DAPI	4,6-diamidino-2-phenylindole
°C	Degree Celsius
DMEM	Dulbecco's modified Eagle's medium (
DEPC	Diethylpyrocabonate
DMSO	Dimethylsulphoxide
DNA	Desoxyribonucleic acid
dNTPs	Desoxyribonucleosidtriphosphates
dT	Desoxythymidine
dpc	Days post coitum
DTT	Dithiothreitol
EB	Embryoid body
eGFP	Enhanced green fluorescent protein
ES cells	Embryonic Stem Cell
EGF	Epidermal growth factor
FACS	Fluorescence Activated Cell Sorting
g	gram
g	acceleration of gravity on Earth (9.8 m/s^2)
GG	Gynogenetic
HBSS	Hank's balanced salt solution
hCG	Human chorionic gonadotropin
ICM	Inner cell mass
ICSI	Intracytoplasmatic sperm injection
iPS cell	Induced pluripotent stem cell

IVF	*In vitro* fertilisation
kU	Kilounit
L	Litre
m	metre
M	Mol per litre
MAPC	Multipotent adult progenitor cell
max	Maximum
MEF	Murine Embryonic Fibroblast
MHC	Major histocompatibility complex
µg	Microgram
µL	Microlitre
µm	Micrometre
min	Minimum
mL	Millilitre
mm	Millimetre
mM	Millimol per litre
MSZ	Institut für Medizinische Strahlenkunde und Zellforschung
N	Normal fertilised
NEAA	Non essential amino acids
NeuN	Neuronal Nuclei
NSC	Neural stem cell
PBS	Phosphate buffered Saline
PCNA	Proliferating cell nuclear antigen
PFA	Paraformaldehyde
PMSG	Pregnant mare’s serum
pnPC	Pan-neural progenitor cells
RCF	Relative centrifugal force
RNA	Ribonucleic acid

RT-PCR	Reverse transcriptase polymerase chain reaction
s	Second
SCNT	Somatic cell nuclear transfer
SDS	Sodiumdodecylsulfate
U	Enzyme unit (Amount of enzyme converting 1 µmol substrate per minute)

10 PUBLICATIONS

10.1 Articles

Dinger, T. C., Eckardt, S., Choi, S. W., Camarero, G., Kurosaka, S., Hornich, V., McLaughlin, K. J. and Müller, A. M. (2008). *Androgenetic Embryonic Stem Cells form Neural Progenitor Cells In Vivo and In Vitro.* Stem Cells 26, 1474-83.

Eckardt, S., **Dinger, T. C.**, Kurosaka, S., Leu, N. A., Müller, A. M. and McLaughlin, K. J. (2008). *In vivo and in vitro differentiation of uniparental embryonic stem cells into hematopoietic and neural cell types.* Organogenesis 4, 31-41.

Ruau, D., Ensenat Waser, R., **Dinger, T. C.**, Vallabhapurapu, D. S., Rolletschek, A., Hacker, C., Hieronymus, T., Wobus, A. M., Müller, A. M. and Zenke, M. (2008). *Pluripotency Associated Genes are Reactivated by Chromatin Modifying Agents in Neurosphere Cells.* Stem Cells 26, 920-6.

Volkenstein, S., Brors, D., Hansen, S., Mlynski, R., **Dinger, T. C.**, Müller, A. M. and Dazert, S. (2008). *Investigation of Neural Stem Cell-derived Donor Contribution in the Inner Ear Following Blastocyst Injection.* Laryngorhinootologie 87, 168-73.

Vukicevic, V., **Dinger T. C.**, Jauch, A., Gebauer, L., Ehrhart-Bornstein, M. and Müller, A. M. *Telomere Length Erosion and Karyotype Instability in Long-Term Cultured Mouse Neurosphere cells*. (Manuscript in preparation).

10.2 Book Chapters

Müller, A. M., Obier, N., Choi, S. W., Li, X., **Dinger, T.C.** and Brousos, N. (2009). *Möglichkeiten und Chancen der Stammzellforschung: Stammzellen für Alle?* In: Hilpert, K. (Hrsg.). *Streitfall Stammzellforschung – Ethische Konflikte um den Lebensschutz in der frühen Entwicklung*. Quaestiones Disputatae, Herder Verlag (in press).

10.3 Poster Presentations / Oral Presentations

Dinger, T. C., Eckardt, S., Hornich, V., McLaughlin, K. J. and Müller, A. M. *Neural Differentiation Potential of Androgenetic and Gynogenetic Murine ES Cells*, 2nd International Conference of the Würzburg Initiative Tissue Engineering (WITE) "Strategies in Tissue Engineering", Würzburg, 31.05.-02.06.2006
(Poster presentation).

Dinger, T. C., Eckardt, S., Hornich, V., McLaughlin, K. J. and Müller, A. M. *Neural Differentiation Potential of Androgenetic Murine ES Cells*, 3rd International PhD Student Symposium „Horizons in Molecular Biology", Göttingen, 14.-16.09.2006 (Poster presentation).

Dinger, T. C., Eckardt, S., Hornich, V., McLaughlin, K. J. and Müller, A. M. *Neural Differentiation of Androgenetic Murine Embryonic Stem Cells*, 2nd Congress of the German Society for Stem Cell Research, Würzburg, 04.-06.10.2007
(Oral presentation).

11 CURRICULUM VITAE

Diplom-Biochemiker
Timo Christoph Dinger

geboren am 27.12.1976
in Hilden
verheiratet

2004 - 2008	Promotion am Institut für Medizinische Strahlenkunde und Zellforschung (MSZ) der Universität Würzburg Arbeitsgruppe „Stammzellbiologie“, Prof. Dr. Albrecht M. Müller Thema der Dissertation: „Neural Differentiation Potential of Murine Androgenetic Embryonic Stem Cells“ Stipendiat im DFG-Graduiertenkolleg 1048 „Molekulare Grundlagen der Organentwicklung in Wirbeltieren“; Student an der International Graduate School (IGS) der Universität Würzburg, Class of Biomedicine
1997 – 2003	Studium Biochemie, Fakultät für Chemie, Universität Bielefeld Abschluss: Diplom-Biochemiker Diplomarbeit an der Fakultät für Biologie, Universität Bielefeld, Lehrstuhl für Entwicklungsbiologie und Molekulare Pathologie, Prof. Dr. Harald H. Jockusch, Thema der Diplomarbeit: „Biochemische Untersuchung von *Kiaa*0903 als Kandidatengen für die *wobbler*-Mutation der Maus“
1996 – 1997	Zivildienst als Laborhilfskraft Biopsie-Labor, Pathologisches Institut Städtisches Klinikum Solingen
1983 – 1996	Schulausbildung in Solingen Abschluss: Abitur (Gymnasium), Leistungskurse Biologie und Mathematik

Timo C. Dinger

Würzburg, 16.12.2008

ACKNOWLEDGEMENTS

I thank Prof. Dr. Albrecht Müller for the opportunity to work in his stem cell biology laboratory. I owe him my deepest gratitude for his advice, input, discussion and constant encouragement during the course of this study. I am most grateful for his patient guidance as well as for the plethora of chances to learn. I will always treasure the insights in science that he gave me.

I also thank the supervisors of my thesis committee, Prof. Dr. Thomas Brand and Prof. Dr. Manfred Gessler for their support and guiding advice.

To Prof. Dr. K. John McLaughlin and Dr. Sigrid Eckhardt I express my greatest thanks and gratefulness. Only due to this productive collaboration, their support, their adding of ideas and scientific input and especially their friendly helpfulness this study has been possible.

Dr. Guadelupe Camarero, Prof. Dr. Thomas Raabe and Dr. Rudolf Götz have my deep gratitude for letting me benefit from their broad experience and their profound scientific and technical knowledge as well as from the microscopy equipment of their labs. To Dr. Frank Edenhofer and Prof. Dr. Verdon Taylor I give great thanks for providing protocols for pnPC-establishment and neurosphere analyses, and for the opportunity to discuss my experiments as well as for their trouble-shooting.

I am greatly indebted to Vroni Hornich and Soon Won Choi. Without their constant support, excellent work, friendly help and technical expertise major parts of this study would have been impossible. I thank Christian Linden for doing excellent work on cell sorting and Sabine Lorenz for her help during the first stages of this project.

I am very grateful for the excellent time I had in the Müller lab. And this is due to the people who make it an extraordinary inspiring and pleasant place to work. These thanks go to Soon Won Choi, Nikos Brousos, Vroni Hornich, Nadine Obier and all members of the Müller lab. Among numerous other things I am very much obliged for the valuable discussions, the scientific input and constructive criticisms of my work. Further I thank all my previous lab-mates in the Müller group, especially Sandra Trüpschuch, Andrea Reusch, Dr. Michael Dürr and Dr. Kallal Pramanik for generously providing help and assistance and Dr. Matthias Porsch for taking me under his wings in my initial days in the lab.

I thank all members, fellows and associates of the DFG research training group GRK 1048 “Molecular Basis of Organ Development in Vertebrates” for broadening my horizon in developmental biology and the helpful discussions and suggestions on my project. Financial support from GRK 1048 during the course of my studies is duly acknowledged.

I want to thank my long-time companions in biochemistry, Dr. Thorsten Krause and Alexander Trampe for advice and discussion on all aspects of a Ph.D. thesis, but most of all for their friendship.

I am deeply indebted to my parents, my parents-in-law and my whole family whose never-ceasing support is always with me. Finally, I thank my loving wife, Kristina, for her advice, support, encouragement and blessings in every step of my life.