

Myc increases self-renewal in neural progenitor cells through Miz-1

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Summary

The mechanisms underlying the decision of a stem or progenitor cell to either self-renew or differentiate are incompletely understood. To address the role of Myc in this process, we expressed different forms of the proto-oncogene *Myc* in multipotent neural progenitor cells (NPCs) using retroviral transduction. Expression of Myc in neurospheres increased the proportion of self-renewing cells fivefold, and 1% of the Myc-overexpressing cells, but none of the control cells, retained self-renewal capacity even under differentiation-inducing conditions. A Myc mutant (MycV394D) deficient in binding to Miz-1, did not increase the percentage of self-renewing cells but

was able to stimulate proliferation of NPCs as efficiently as wild-type Myc, indicating that these two cellular phenomena are regulated by at least partially different pathways. Our results suggest that Myc, through Miz-1, enhances self-renewal of NPCs and influences the way progenitor cells react to the environmental cues that normally dictate the cellular identity of tissues containing self-renewing cells.

Key words: Myc, Self-renewal, Miz-1, Neural progenitor, Stem cell, Oncogene

Introduction

Stem cells ensure the turnover capacity of a tissue throughout the lifespan of an individual. Mammalian neurogenesis takes place mostly during embryonic development and early childhood but some renewing activity is maintained through adulthood (Bhardwaj et al., 2006; Morshead et al., 1998; Suhonen et al., 1996; Taupin and Gage, 2002). Some of the developmental genes involved in self-renewal maintenance, differentiation and regeneration are also proto-oncogenes such as *Myc*.

The two cellular processes of self-renewal and differentiation are often seen as opposite events, both of which are needed in tissue homeostasis. These phenomena can be seen in normal development and function but also (although possibly unbalanced) in pathological conditions, such as malignant tumours, which contain a mixture of self-renewing cancer stem cells and their differentiated progeny (Huntly and Gilliland, 2005; Singh et al., 2004). In this work, we studied the effects of overexpression of the developmental and cancer protein Myc on the equilibrium of self-renewal and differentiation of neural progenitor cells (NPCs).

All genes of the Myc family (*Myc*, *n-Myc* and *l-Myc*) are expressed during foetal brain development (Hirvonen et al., 1990) and targeted loss of *n-Myc* in nestin-positive NPCs severely disrupts their ability to expand, differentiate and populate the brain even though the mice survive to adulthood (Knoepfler et al., 2002). *Myc* and *n-Myc* genes share substantial sequence homology and regulatory mechanisms (Malynn et al., 2000; Stanton et al., 1992) but have at least partially different expression patterns and thus different roles during development (Hirvonen et al., 1990; Zimmerman et al., 1986) as well as after birth (Bettess et al., 2005; Semsei et al., 1989). Homozygous mice lacking *Myc* or *n-Myc* both die around mid-gestation. The *Myc* knockout mice have a developmental failure of the heart and neural tube whereas lack of

n-Myc results in malformations of both the peripheral and central nervous system (CNS) as well as the heart (Charron et al., 1992; Davis et al., 1993; Stanton et al., 1992). Also, *n-Myc* amplification in neuroblastoma is associated with a poor prognosis (Schwab, 2004) and both Myc and *n-Myc* overexpression have been implicated in the pathogenesis of medulloblastoma and glioma (Collins, 1995; Eberhart et al., 2004; Stearns et al., 2006; Su et al., 2006).

The role of Myc in stem cell regulation differs between tissues and stem cell types (Murphy et al., 2005). In the epidermis and the bone marrow Myc mobilizes stem cells to exit from the stem cell compartment to become rapidly proliferating transit amplifying cells determined to differentiate (Arnold and Watt, 2001; Gandarillas and Watt, 1997; Wilson et al., 2004) although at the same time Myc also severely disrupts keratinocyte differentiation (Pelengaris et al., 1999).

Myc is a transcription factor that regulates gene expression through several mechanisms, including recruitment of histone acetylases and chromatin re-modelling factors, and interaction with basal transcriptional factors (Grandori et al., 2000; Levens, 2003). Myc is estimated to regulate as many as 15% of genes of the genome (Grandori et al., 2000) involved in cell cycle, growth, signalling, adhesion, differentiation and apoptosis (Coller et al., 2000; Dang et al., 2006). Together with its partner protein Max, Myc can either activate or repress transcription depending on the other DNA binding partners (Levens, 2003). Binding of Myc to the zinc finger protein Miz-1 represses gene expression of several cyclin-dependent kinase (CDK) inhibitors such as p15^{ink4b} and p21^{Cip1} (Seoane et al., 2002; Staller et al., 2001). Another group of genes repressed by Myc encode proteins involved in the cytoskeleton and cell adhesion (Coller et al., 2000; Staller et al., 2001), and binding to Miz-1 is required for the repression of β -1 integrin and other target genes

that regulate adhesive properties and stem cell exit in keratinocytes (Gebhardt et al., 2006). In order to investigate the role of Myc-induced signalling through Miz-1 in NPCs, we used the mutated form of Myc (MycV394D) that has lost its ability to repress Miz-1-activated transcription but functions normally in activation of target genes binding to E-boxes (Herold et al., 2002).

Stem cell maintenance is affected by the cues provided by the environment including the stem cell niche (Li and Neaves, 2006; Moore and Lemischka, 2006; Scadden, 2006). Normally, the stem cell pool is kept stable by asymmetric stem cell divisions. However, in case of an injury many tissues such as blood, liver, gut, skin and possibly brain (Zhang et al., 2004) respond by increasing the stem cell pool in similar self-renewing divisions to those during embryogenesis (Morrison and Kimble, 2006; Noctor et al., 2004; Taupin and Gage, 2002). We have studied embryonic neural stem cells and our results suggest that the proto-oncoprotein Myc, through the Miz-1 binding site, increases the pool of self-renewing cells by affecting the way that NPCs react to the signals they get from the microenvironment.

Results

Myc expression in NPCs

This study was carried out using NPCs in a neurosphere culture system (Gage, 2000; Reynolds and Weiss, 1992). Before starting with the viral transductions we verified that the NPCs we use are pluripotent by making clonal cultures of control neurospheres. All five neurosphere populations that had been initiated from single cells were able to differentiate into both neurons and glial cells when evaluated by immunolabelling (results not shown).

To study the function of Myc in self-renewal and differentiation, a tamoxifen-regulatable construct of human *MYC* (*MycER*) was transduced into NPCs. As the *MycER* turned out to be leaky, in this work we used constant tamoxifen administration in all *MycER* experiments, and an empty pBabe vector transduced to NPCs as a control.

The expression of the *MycER* transgene (hereafter called *Myc*) was confirmed by western blotting (WB; Fig. 1A) and fluorescence activated cell sorting (FACS), which showed that on average 91.4% of the transduced cells express Myc (Fig. 1B). The NPCs were found to express endogenous mouse *Myc* by using RT-PCR (Fig. 1C) and immunostaining on paraffin sections of embryonic mouse brain (Fig. 1D) as the protein was only faint in control NPC lysates when detected by WB (Fig. 1A). We also transduced a gene encoding a mutated form of Myc (MycV394D) unable to bind to Miz-1 (also known as ZBTB17 in mouse) into the NPCs and its expression was verified by WB (Fig. 1A). All the results shown in this article represent average numbers from experiments that were repeated at least three times and using cells from several independently transduced pools of NPCs.

Myc increases proliferation in NPCs

To study the function of Myc in NPCs, we first used BrdU labelling to measure the percentage of proliferating cells. During 24 hours of BrdU administration, 29% of the control NPCs and 77% of the NPCs expressing Myc entered S phase (Fig. 2A). There was no significant difference between the unmanipulated wild-type (WT) control cells with or without tamoxifen and the control cells transduced with an empty pBabe vector.

The BrdU proliferation analysis was confirmed by comparing the number of cells after 7 days in culture. The total number of Myc NPCs was ~70% higher than that of the WT NPCs (Fig. 2B).

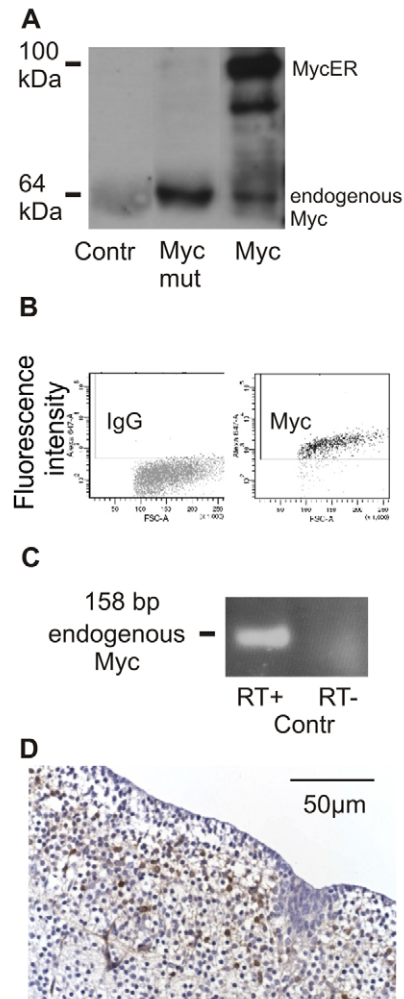


Fig. 1. Myc expression in NPCs. (A) *MycER* and the mutated *Myc* deficient in Miz-1 binding (MycV394D) were transduced to NPCs with retroviruses and the expression was analyzed by western blotting with an anti-Myc antibody. The 100 kDa transgene is not seen in the control NPCs. The endogenous 64 kDa Myc is only poorly visible in the control neurosphere lysates but increased in the Myc NPCs. The MycV394D mutant protein is of the same size as the endogenous mouse Myc. (B) 91.4% (s.d.=5.0, n=4) of the transduced NPCs express Myc as shown by flow cytometry. (C) RT-PCR shows the expression of the endogenous *Myc* in the mouse neurospheres (NPCs). (D) A sagittal section of an embryonic E16 mouse brain shows Myc immunoreactive cells in the lateral ventricle wall area.

Myc increases the proportion of self-renewing cells in the neurosphere tissue model of NPCs

Neurospheres, like tissues *in vivo*, consist of a heterogeneous population of stem cells, progenitors and differentiated cells, but the regulation of the balance between these cell types with different identity is poorly understood. We wanted to determine whether overexpression of Myc affects the proportion of self-renewing cells and measured the number of cells capable of forming a secondary neurosphere from a single cell (the assay setup is shown in Fig. 2C). In this assay the proportion of self-renewing cells in the neurospheres increased fivefold from 4.2% in the control to 24% in the Myc-overexpressing neurospheres (Fig. 2C). During further passaging up to passage 20 and beyond, these percentages of self-renewing cells remained the same.

Fig. 2. Myc overexpression increases proliferation and self-renewal in NPCs. (A) Overexpression of Myc increases proliferation as shown by the proportion of the BrdU-positive NPCs in neurosphere cultures.

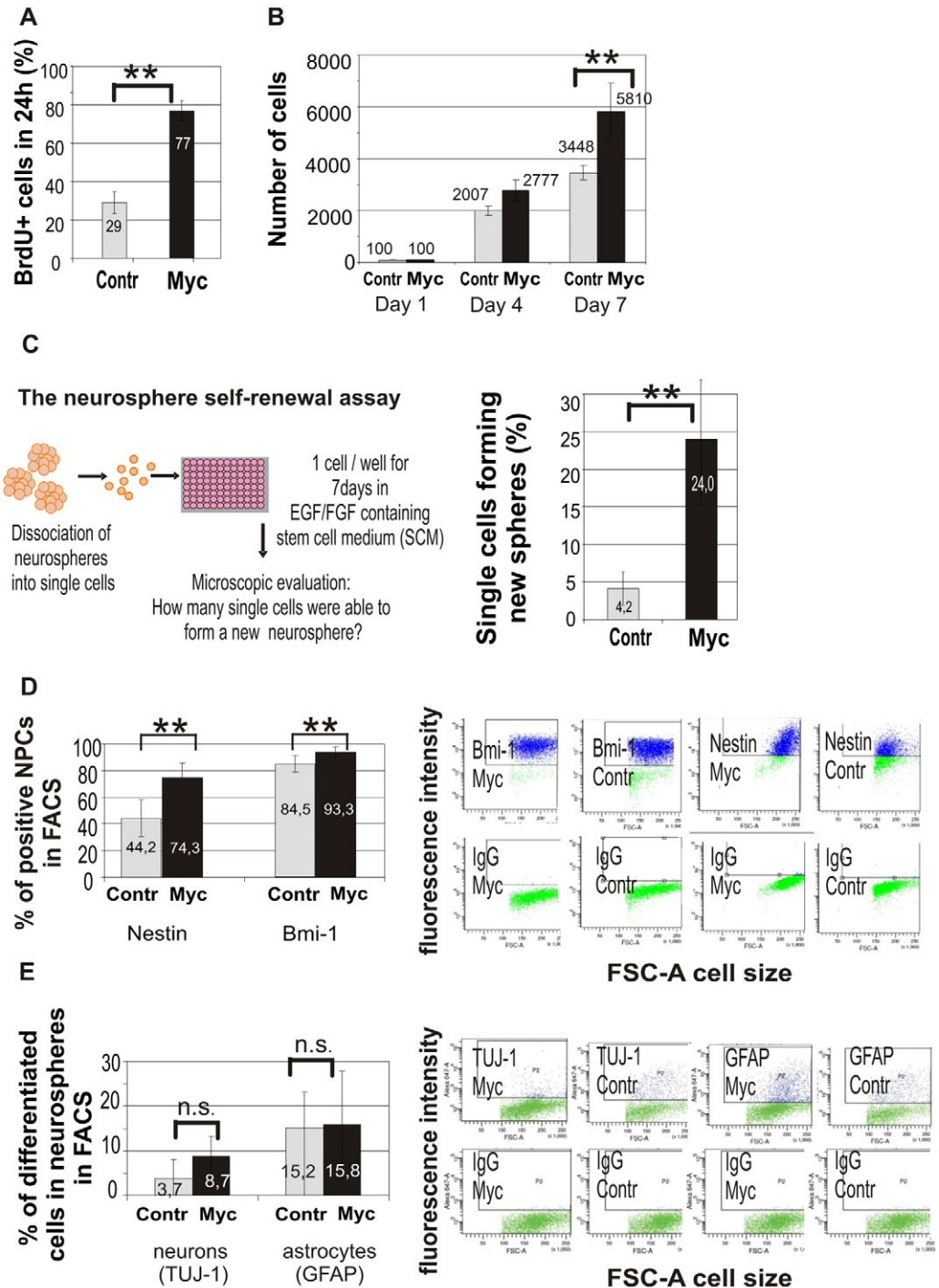
During 24 hours of exposure to BrdU, 28.9% of the control NPCs (s.d.=5.6, $n=4$) were in cell cycle whereas Myc overexpression increased the rate to 76.7% (s.d.=5.3, $n=5$, Student's t -test $**P=7.2E-06$).

(B) Myc increased the total cell number ~70% in 1 week (from 3448, s.d.=274, $n=3$ to 5810, s.d.=1004, $n=3$, Student's t -test $**P=0.00149$).

(C) Self-renewal assay set up and result in which Myc increases the proportion of self-renewing NPCs by fivefold from 4.2% (s.d.=2.2, $n=13$) in controls to 24.0% (s.d.=8.7, $n=10$, Student's t -test $**P=0.000104$) in the Myc-overexpressing neurospheres.

The ability of single cells to form new neurospheres was measured by microscopy after 7 days of culture in the EGF- and FGF-containing stem cell medium. (D) Flow cytometry shows quantitatively that Myc overexpression increases the expression of the neural progenitor markers nestin (74.3%, s.d.=11.3, $n=10$) and Bmi-1 (93.3%, s.d.=4.1, $n=11$) as compared with the control NPCs (nestin 44.2%, s.d.=13.9, $n=5$ and Bmi-1 84.5%, s.d.=6.2, $n=7$, Student's t -test for nestin $**P=0.0043$ and for Bmi-1 $**P=0.0085$).

(E) Even if the self-renewing proportion of the heterogenous neurosphere cell population is increased, Myc overexpression does not affect the expression of differentiation markers GFAP and TUJ-1 and thus the proportion of differentiated cells in the neurosphere (control TUJ-1 3.7%, s.d.=4.3, $n=6$; GFAP 15.2%, s.d.=7.9, $n=6$ and Myc TUJ-1 8.7%, s.d.=4.5, $n=10$; GFAP 15.8%, s.d.=12, $n=9$).



Myc increases the expression of early markers but does not affect the differentiation marker expression in neurospheres. To further analyze the effects of Myc overexpression on NPCs and the identity of cells in neurospheres we examined the neural progenitor marker nestin, the stem-cell-associated polycomb family protein Bmi-1 (Molofsky et al., 2005) and glial and neuronal markers glial fibrillary acidic protein (GFAP) and neuronal class III β -tubulin (TUJ-1), respectively, by FACS. The percentages of cells expressing nestin was increased significantly by Myc overexpression, from 44.2% to 74.3% (Fig. 2D). Expression of Bmi-1 was broader through the whole neurosphere population. Although

already 84.5% of the control cells were Bmi-1-positive, the expression was still significantly increased to 93.3% by Myc overexpression. All the neurospheres showed continuous heterogenic cell identity based on the expression of differentiation markers, and Myc overexpression did not significantly change the proportion of GFAP or TUJ-1-positive cells (Fig. 2E). Taken together, it seems that Myc-induced increase in the total number of cells in neurosphere cultures (Fig. 2B) is due to an increase in the proportion of self-renewing progenitors (Fig. 2C,D) that also proliferate more (Fig. 2A) rather than to a loss of differentiating cells (Fig. 2E).

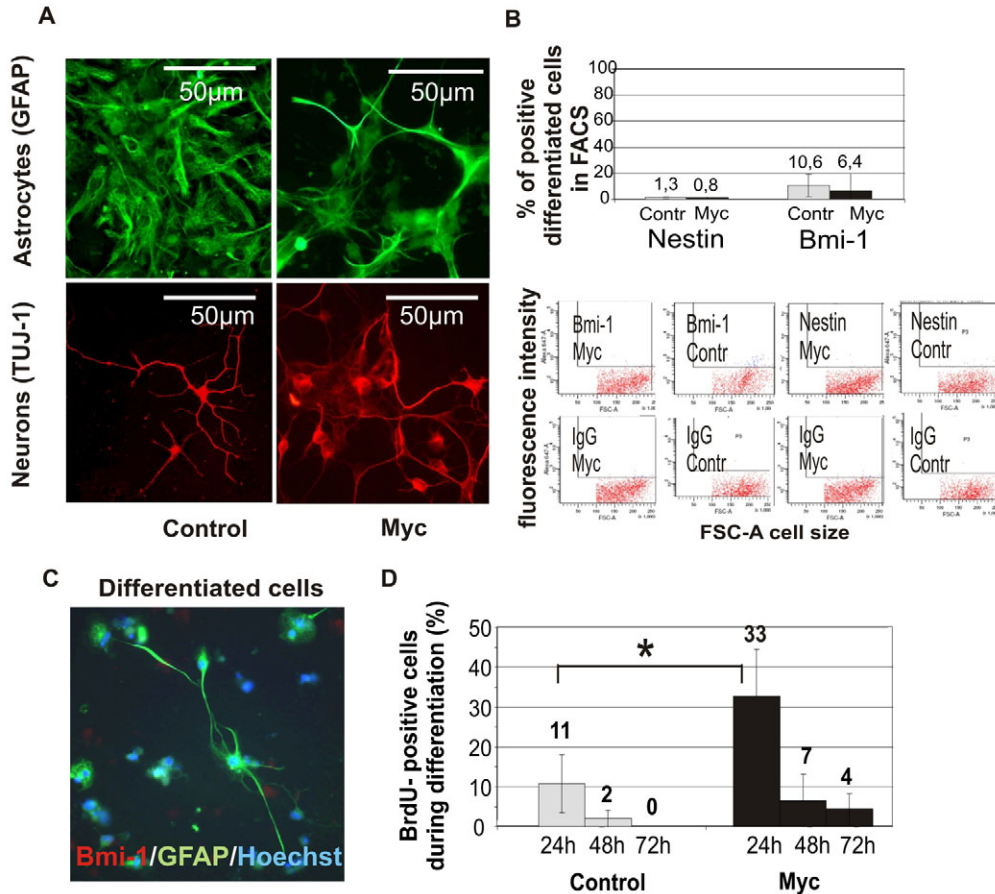


Fig. 3. Myc overexpression does not block differentiation but delays the cell cycle exit of neural cells. (A) After 4 days in 2% FCS both Myc-overexpressing and control cells expressed the differentiation markers GFAP and TUJ-1 although the morphology of the Myc NPCs varies. (B) The expression of the NPC markers Bmi-1 and nestin decrease dramatically during differentiation of NPCs. (C) Double staining shows that GFAP-positive Myc-overexpressing cells, with the morphology of differentiated cells, are not also positive for the progenitor marker Bmi-1. (D) Myc delays the cell cycle exit and increases the number of cells in S phase during differentiation-promoting conditions (2% FCS) for 3 days as demonstrated by BrdU labelling. During the first day 10.8% of the control cells (s.d.=7.2, $n=5$) and 32.8% of the Myc cells (s.d.=11.7, $n=5$) were still proliferating. During the second day, 2.1% (s.d.=2.1, $n=5$) of the control cells and 6.5% (s.d.=6.6, $n=5$) of the Myc cells had not exited the cell cycle. By day 3 almost none of the control cells (0.0%, s.d.=0.14, $n=6$) but 4.4% of the Myc-overexpressing NPCs (s.d.=3.9, $n=4$) were still proliferating (Student's t -test $*P=0.041$).

Myc does not block differentiation, but delays cell cycle exit and inhibits terminal mitosis in a small subpopulation of differentiating NPCs

NPCs that grow as neurospheres start to differentiate when the culture conditions are changed from EGF- and FGF- to a serum-containing medium. After 4 days in culture under differentiating conditions both the control and Myc NPCs resembled neurons and astrocytes and expressed markers for these cell types. However, the morphology of the Myc cells showed vast heterogeneity and some cells seemed less differentiated (Fig. 3A) but again others appeared normal after *in vitro* differentiation. Both the differentiated Myc and the control cells showed a markedly decreased expression of markers of primitive cells, nestin and Bmi-1, as detected by FACS (Fig. 3B) and also immunofluorescence (IF) in order to verify that differentiated Myc cells did not express both differentiation and progenitor cell markers in parallel (Fig. 3C). However, some cells in both Myc and control cultures continued to express primitive markers even after 4 days of differentiation (Fig. 3B).

To detect differences induced by Myc overexpression in populations of differentiating NPCs we studied the cell cycle exit to see whether NPCs expressing Myc are able to resist the differentiation

cues. The cells were differentiated as described and BrdU incorporation was measured during three time periods, 0-24, 24-48 and 48-72 hours. The results show that even though undifferentiated Myc-overexpressing neurospheres proliferated twice as much as the control NPCs, a clear majority of the cells (96%) was still able to respond to the surrounding cues and exit the cell cycle when transferred into differentiating culture conditions. However, a minority of the Myc-overexpressing cells (4%) continued to synthesise DNA even by day 3 when no control cells were seen in S phase (Fig. 3D).

Myc promotes self-renewal also during differentiation

Since a small fraction of the constitutively Myc-expressing NPCs did not exit the cell cycle during differentiation, we studied whether some of the cells also maintained the capacity to self-renew despite the culture conditions that promote differentiation. For this we used the same self-renewal assay as previously described for FGF- and EGF-containing stem cell culture conditions but now single cells were plated at different densities into 96-well plates to differentiate in a serum-containing medium (the experimental set up is shown in Fig. 4A). In the control cultures all NPCs in each experiment differentiated permanently and no clonally growing cells could ever

be detected. By contrast, in the cultures of Myc-overexpressing cells, new neurospheres appeared in the serum-containing medium in the middle of the differentiated cells (Fig. 4B). This phenomenon was called 're-sphering'. ~1-2% of the originally plated Myc-expressing cells were re-sphering after 2 weeks in culture, and the number of spheres remained similar after 3 and 4 weeks in culture (Fig. 4C) suggesting that the initial number of cells capable of re-sphering in the differentiating conditions was already present at the beginning of the experiment.

Re-sphering during differentiation is density dependent and the re-sphered cells resemble the primary Myc cells

Next we plated several thousand cells at different densities to study re-sphering more closely. Interestingly, re-sphering took place only when the cells were plated at a density of more than 200 cells/well. After reaching a seemingly critical number, higher cell densities did not increase the percentage of cells capable of re-sphering (Fig. 5A).

The re-sphered cells stayed attached to the bottom of the wells during the whole time of the differentiation experiment. However, after the initial re-sphering, the population could easily be expanded by detaching individual neurospheres from the wells and putting them back in the serum-free stem cell culture medium with EGF and FGF and culturing them again as a floating neurosphere population.

The re-sphered Myc NPC populations were analysed by a secondary neurosphere assay, cell counts and antibody stainings. First we wanted to rule out the possibility that the phenomenon of re-sphering was caused by a selection of clones expressing more Myc. The western blot analysis (Fig. 5B) showed similar expression levels of Myc and nucleolin, a known downstream target of Myc (Haggerty et al., 2003) in the re-sphered Myc populations as compared with the primary Myc-overexpressing NPCs. The percentage of the self-renewing cells was slightly higher (29%) in the re-sphered cell population as compared with the primary Myc NPCs but the difference was not statistically significant (Fig. 5C). The re-sphered cells also seemed to divide faster than the primary Myc NPCs but again, the difference was not statistically significant (Fig. 5D). The stainings and FACS analysis showed similar high percentages of Bmi-1 or nestin-positive cells as in the primary Myc NPCs (Fig. 5E). Finally, the progeny of the re-sphered cells were still capable of differentiation: when again recultured with serum, the cells attached to the culture dish surface and expressed the differentiation markers TUJ-1 or GFAP (although the morphology seems less differentiated) in a similar manner as in primary Myc cells (Fig. 5F and Fig. 3A).

A point mutant of Myc that does not bind to Miz-1 stimulates proliferation, but does not increase self-renewal in NPCs

Previous studies have shown that Myc regulates keratinocyte adhesion and differentiation in the epidermal stem cell niche via binding to Miz-1 (Gebhardt et al., 2006). We wanted to investigate whether the effects induced by Myc overexpression in NPCs are

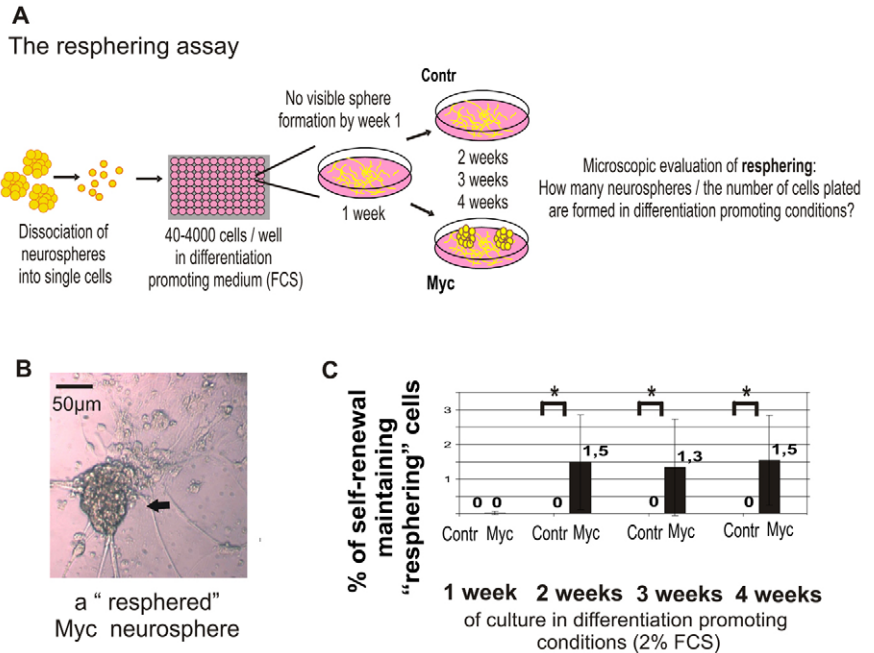


Fig. 4. Myc maintains self-renewal ability of differentiating NPCs. (A) Illustration of the experimental set-up for the re-sphering assay. (B) A light microscopy image of a 're-sphering' Myc neurosphere in 2% FCS after 4 weeks of culture. (C) During differentiation-promoting culture conditions Myc overexpression induced maintenance of self-renewal, seen as re-sphering, in 1-2% of the 76,800 plated cells (800 cells per well in 150 µl in a 96-well culture plate in each experiment) during differentiation in 2% FCS (Myc 1 week 0.0%, s.d.=0.05, n=4; Myc 2 weeks 1.5%, s.d.=1.4, n=7; Myc 3 weeks 1.3%, s.d.=1.3, n=7; Myc 4 weeks 1.5%, s.d.=1.3, n=7). Thus, re-sphering was visible after 2 weeks in culture. The number of formed neurospheres did not increase with time (when kept in 2% FCS). All the control cells differentiated and did not ever form neurospheres in differentiation-promoting conditions (contr 1-4 weeks 0%, s.d.=0, n=7, 2 weeks Student's *t*-test **P*=0.028; 3 weeks **P*=0.043; 4 weeks **P*=0.020).

mediated through the Miz-1 binding site. For this, a gene encoding a mutant form of Myc (MycV394D) that is unable to bind to Miz-1 (Herold et al., 2002) was transduced to NPCs and cell proliferation and self-renewal were measured using the same cell culture methods as described earlier in context of the Myc-overexpressing cells. The expression level of Myc was similar in the WT and mutated Myc populations, which was shown by Myc and nucleolin staining (Fig. 6A). Overexpression of the MycV394D increased proliferation of NPCs as much as WT Myc overexpression (Fig. 6B,C). Self-renewal, by contrast, as measured by the ability of single cells to form new neurospheres was not increased by the mutant MycV394D (Fig. 6D). The slight increase in the average number of self-renewal (from 4.2% in control cells to 9.8% in the mutants) is possibly due to the increased proliferation in the neurosphere cultures but the difference was not statistically significant. Furthermore, overexpression of the mutant MycV394D did not induce self-renewing divisions (the phenomenon of re-sphering) during differentiation-promoting conditions (Fig. 6E). In conclusion, these results suggest that Myc induces cell proliferation of NPCs in a Miz-1-independent manner, but that the increased self-renewal caused by Myc overexpression in NPCs requires complex formation of Myc with Miz-1.

Myc may cause polyploidy in NPCs but it does not increase apoptosis

Our analysis of the DNA content of the Myc NPC populations showed that one of the Myc-transduced NPC populations had

become tetraploid (Fig. 7A), potentially reflecting the ability of Myc to induce genomic instability (Wade and Wahl, 2006). However, all other NPC lines were diploid after 20 passages, before which all the experiments were performed. The FACS analysis showed also a subG1 population, potentially indicating Myc-induced apoptosis (Meyer et al., 2006). However, excess apoptosis of Myc NPCs could not be seen by Annexin staining (Vermees et al., 1995) and flow cytometry (Fig. 7B).

Discussion

In this work we have analysed the effects of overexpression of the proto-oncogene *Myc* on the regulation of stem cell properties in NPC cultures. Our results suggest that (1) *Myc* increases the pool of self-renewing cells and shifts the balance of the neurosphere tissue towards a more primitive cellular identity. (2) This increase requires the intact Miz-1 binding site of *Myc*. (3) As overexpression of the *Myc* mutant, which was unable to bind to Miz-1, increased proliferation but not self-renewal of the NPCs, the results strengthen the concept of these being two separately regulated cellular functions. (4) A small but significant fraction of all the *Myc* (but not the *MycV394D* mutant)-expressing cells is able to maintain the ability to self-renew (seen as re-sphering) under differentiating conditions. (5) In the vast majority of cells, *Myc* overexpression does not block the differentiation of NPCs towards neural or glial fate but causes cell cycle exit delay and can affect the morphology of the differentiated cells. Interestingly, also most of the daughter cells of the re-sphering NPCs are eventually able to differentiate, from which we conclude that (6) the effects of *Myc* on NPCs are influenced by the environment.

Regulation of the number of the self-renewing cells plays a key role in tissue homeostasis. Our results show that overexpression of *Myc* dramatically increases the proportion of cells that are able to self-renew in a neurosphere, thus changing the organization and cell-type balance of the neurospheres, which normally consist of a variety of cells at many stages of differentiation. About 4% of the cells in control neurospheres can self-renew, suggesting that most of the cells are more determined progenitors. Indeed, some of the cells in a neurosphere already express differentiation markers such as TUJ-1. Since both normal stem cell self-renewal and cancer cell proliferation are influenced by a balance between the activation of proto-oncogenes and tumour suppressors, our results with the classical oncoprotein *Myc* are consistent with previous reports by others and us on neural stem cell self-renewal (Molofsky et al., 2005; Pardal et al., 2005; Piltti et al., 2006; Zhang et al., 2004).

The role of *Myc* in controlling the balance of mammalian neural stem and progenitor cell identity or self-renewal has not been studied before, although *Myc* overexpression is known to promote

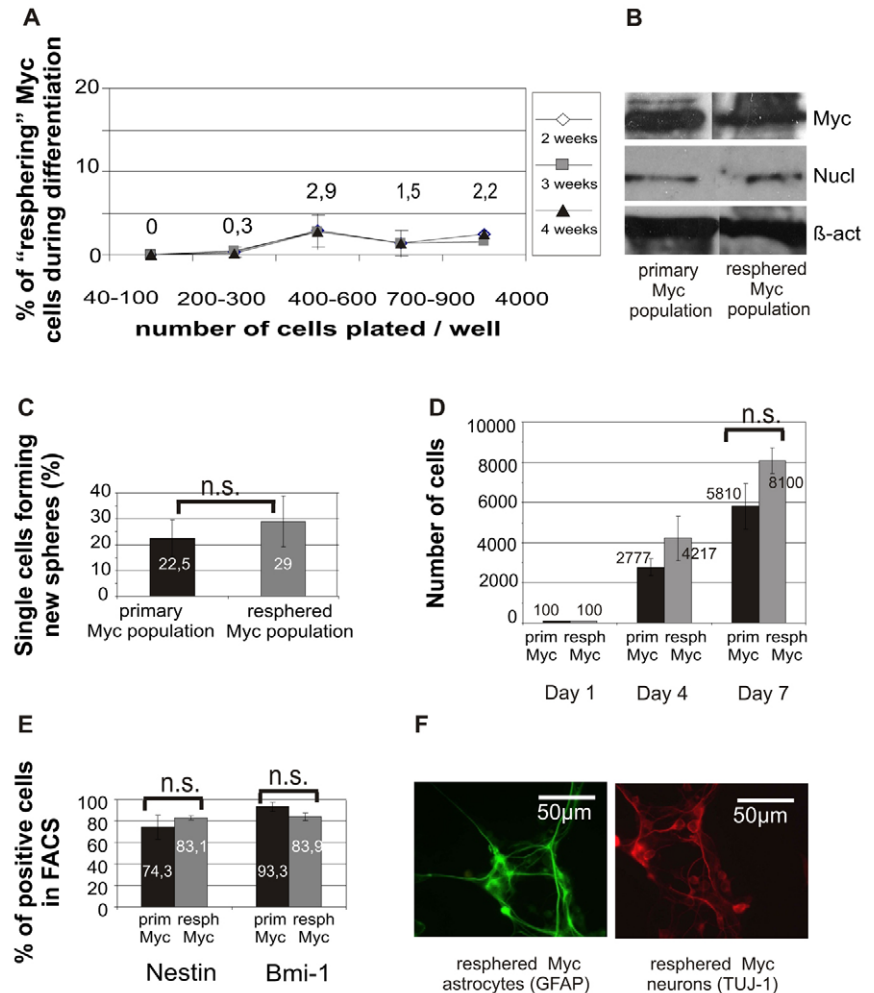


Fig. 5. The phenomenon of re-sphering is density dependent and the re-sphered cells resemble the primary *Myc* cells. (A) In 96-well plates (in 150 μ l of 2% FCS per 25 mm² well, one plate per experiment), no re-sphering was seen if less than 200 *Myc* NPCs were plated per well. Also, the percentage of re-sphering did not increase by adding more cells after 400 cells per well or by time. The numbers shown represent averages of the three counted time points. (40–100 cells per well 0%, $n=5$; 200–300 cells per well 0.3%, s.d.=0.4, $n=4$; 400–600 cells per well 2.9%, s.d.=1.9, $n=3$; 700–900 cells per well 1.5%, s.d.=1.5, $n=6$; 4000 cells per well 2.2%, $n=1$). (B) A western blot of primary and re-sphered *Myc* neurosphere populations shows that the level of *Myc* expression was approximately the same in both populations and thus was not increased in the re-sphered population. Staining for nucleolin verifies the same result. Staining for β -actin shows that the amount of the cell lysate per lane is similar. (C) When re-sphered single *Myc* neurospheres after 4 weeks of differentiation in FCS were changed back to FGF and EGF and expanded into a NPC population, the proportion of self-renewing cells increased from the initial 22.5% to 29% but the difference is not statistically significant (s.d.=9.7, $n=10$, Student's t -test $P=0.1$). (D) The re-sphered *Myc* cells proliferated fast but the increase in cell number is not statistically significant (re-sphered *Myc* 8100, s.d.=638, $n=3$, Student's t -test $P=0.0531$). (E) Staining and FACS analysis show no significant change in Bmi-1- or nestin-positive cells. (Re-sphered *Myc* nestin 83.1%, s.d.=2.2, $n=3$ and Bmi-1 83.9%, s.d.=3.5, $n=3$). (F) Re-sphered *Myc* NPCs can differentiate. Redifferentiated re-sphered *Myc* NPCs again attached to the culture well and expressed neuronal and astrocyte markers TUJ-1 and GFAP after 4 days culture in 2% FCS.

proliferation of NPCs in the mouse CNS (Fults et al., 2002). In the *Drosophila* neuroblast, *myc* does have a self-renewal-promoting role and mutants of the *Drosophila* *Myc* suppressor *brat* develop brain tumours (Betschinger et al., 2006). However, *Myc* is essential in the stem cell biology of various other mammalian tissues. In the epidermis and the bone marrow *Myc* drives proliferation of the transit amplifying progenitor cells and induces their exit from the stem cell niche towards differentiation and also triggers proliferation

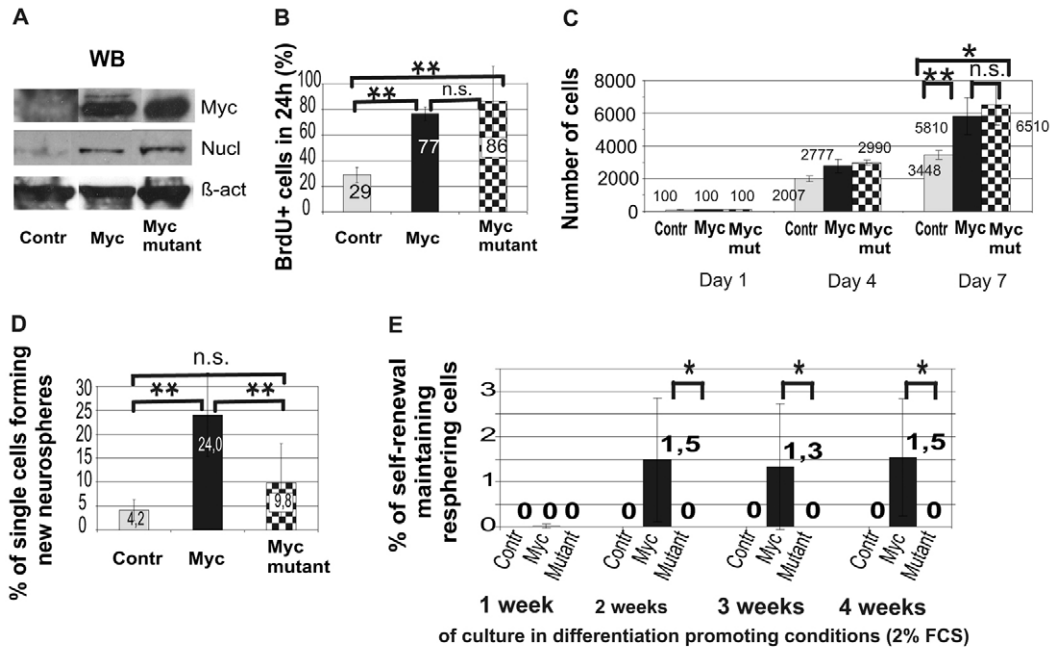


Fig. 6. Myc-induced effects on self-renewal are mediated through Miz-1 binding. (A) Western blot of the control, WT Myc and mutated MycV394D neurosphere lysates shows that the level of Myc expression is approximately the same in both Myc populations. Staining for nucleolin verifies the same result. Staining for β -actin shows that the amount of the cell lysate per lane is similar. (B) Overexpression of the Myc mutant, deficient in Miz-1 binding (MycV394D), increased proliferation of NPCs to the same level as wild-type Myc, as measured by BrdU labelling (mutant 86.4%, s.d.=27.2, $n=5$; Myc 76.7%, s.d.=5.3, $n=5$; control 28.9%, s.d.=5.6, $n=4$; Student's t -test for mutants vs control, $**P=0.008$). (C) The total number of cells was counted during 7 days of stem cell culture (mutant 6510, s.d.=1250, $n=3$, Student's t -test for mutants vs control, $*P=0.0457$). (D) Self-renewal is not increased by Myc overexpression if Miz-1 binding is inhibited. Self-renewal was measured by the ability of single cells to form new neurospheres in EGF- and FGF-containing stem cell culture medium. (MycV394D 9.8%, s.d.=8.2, $n=9$; wt Myc 24.0%, s.d.=8.7, $n=10$; control 4.2%, s.d.=2.2, $n=13$; Student's t -test for mutants vs Myc $**P=0.0021$). (E) Overexpression of Myc does not induce maintenance of self-renewal if Miz-1 binding is inhibited, as measured by the ability of cells to form neurospheres in conditions (2% FCS) that promote differentiation in the re-sphering assay (mycV394D mutant 1-4 weeks 0%, s.d.=0, $n=5$; control 1-4 weeks 0%, s.d.=0, $n=7$; Myc 1 week 0.0%, s.d.=0.05, $n=4$; Myc 2 weeks 1.5%, s.d.=1.4, $n=7$; Myc 3 weeks 1.3%, s.d.=1.3, $n=7$; Myc 4 weeks 1%, s.d.=1.3, $n=7$; Student's t -test for mutants vs Myc 2 weeks Student's t -test $*P=0.028$; 3 weeks $*P=0.043$; 4 weeks $*P=0.020$).

and disrupts differentiation if activated in postmitotic keratinocytes (Arnold and Watt, 2001; Wilson et al., 2004; Pelengaris et al., 1999). Our results show that Myc may change the morphology of differentiated neural cells but they still are able to exit cell cycle and express markers (TUJ-1 and GFAP) specific for differentiated cells although electrophysiological measurements would be needed to show the real functionality of these cells.

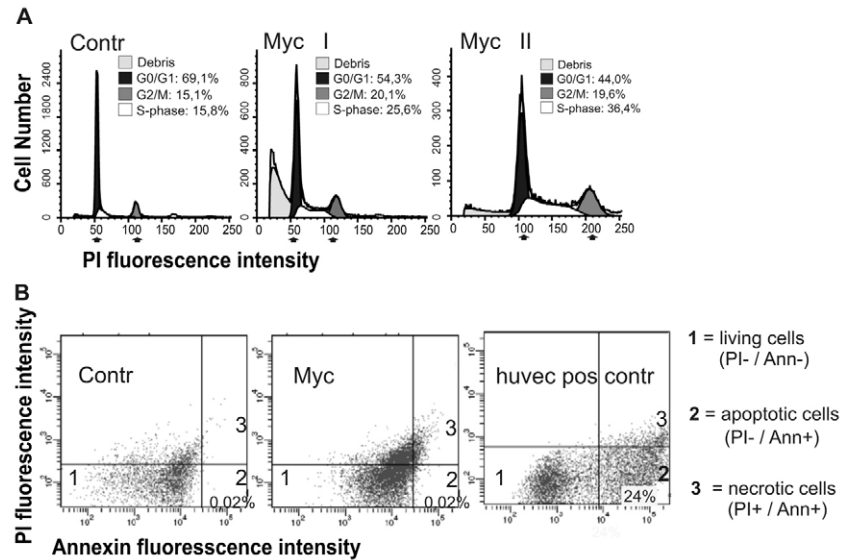
In the epidermal stem cells Myc binds to Miz-1, which in turn affects the adhesive properties of the niche by repression of β 1-integrin and other adhesive molecules and thereby induces stem cell exit (Gebhardt et al., 2006). Myc represses the cell cycle inhibitors p21Cip1, p15Ink4b and p57Kip2 via binding to Miz-1 (Adhikary et al., 2003; Seoane et al., 2002; Staller et al., 2001). A recent paper by Fasano et al. reveals that Bmi-1 induced inhibition of the p21Cip1 is essential for neural stem cell self-renewal during development (Fasano et al., 2007). Furthermore, Bmi-1 is also a direct transcriptional target of Myc (Guney et al., 2006). Our results shown here suggest that the Miz-1 binding site of Myc is needed for the increased self-renewal in NPCs but the exact identity of the downstream targets behind these effects remain speculative. Inhibition of Miz-1 binding to Myc had no effect on the Myc-induced increased proliferation in NPCs, which also emphasizes that self-renewal and proliferation are at least partly independently regulated cellular functions.

Our results suggest that overexpression of Myc changes the way NPCs interpret the microenvironmental cues controlling their self-

renewal and differentiation. This was seen both in the neurosphere stem cell cultures as well as in the re-sphering assay under differentiation-promoting culture conditions. Even though all the NPCs in the cultures survived the antibiotic selection and 91% also expressed a detectable level of Myc, only 24% of cells were able to initiate a new neurosphere. Also, only a constant 1-2% of them showed the abnormal capacity to maintain self-renewal properties (re-sphere) during differentiating conditions. A similar population level effect was detected when we analyzed the cell cycle exit during differentiation. Overall cell cycle exit was delayed by Myc overexpression but ultimately only 4% of the cells were in cycle at the time when all control NPCs had stopped proliferating, and the vast majority of the Myc cells showed some features of differentiated neural cells.

The re-sphering phenomenon was density dependent which further underlines the regulatory impact of the microenvironment: we postulate that the re-sphering cells do not represent cellular transformation, since a certain density of cells was needed in order for the re-sphering to take place. Repeatedly, no re-sphering occurred even if we seeded 20,000 cells but under 200 cells/well. Also, the re-sphered cells could not expand into a growing population as long as they were cultured in the differentiation-promoting serum-containing medium even if the cell density was increased or the culture time prolonged. This suggests that even in the re-sphered neurosphere most cells are still responding to environmental cues. Furthermore, the expression level of Myc was not increased in the progeny of the

Fig. 7. Myc may cause polyploidy in NPCs but it does not increase apoptosis. (A) Tetraploid Myc NPCs were found in one of the three transduced NPC progeny (Myc II) as shown by propidium iodide (PI) staining and flow cytometry. A higher rate of proliferation caused by Myc is seen as an increase in the percentages of cells in S and G2-M phases. The different amounts of debris shown can result from a number of factors: in addition to natural cell death, the mechanical dissociation of neurospheres into single cells causes debris and the amount varies somewhat between experiments. (B) Apoptosis is not changed by Myc overexpression in NPCs as analysed by Annexin staining and flow cytometry. Almost no apoptosis could be detected in NPCs (Contr 0.02%, $n=5$, s.d.=0.04 and Myc 0.18%, $n=6$, s.d.=0.35 Student's t -test $P=0.31$). Induced apoptosis in HU-VEC cells was used as a technical positive control for the Annexin staining.



re-sphered cells when, as single neurospheres, they were changed back to self-renewal-promoting conditions and expanded into a NPC population. The behaviour of the re-sphered cells in the self-renewal, proliferation and differentiation analysis was also similar to the cells in the primary Myc cultures.

It is not known how the Myc-induced self-renewal maintenance is enabled in only a small subset of differentiating cells. The first reports of induced pluripotent stem cells showed that coexpression of Myc together with three other factors (Oct3/4, Sox-2 and Klf4) is sufficient to induce pluripotency in adult fibroblasts (Okita et al., 2007; Takahashi and Yamanaka, 2006; Wernig et al., 2007) and self-renewal of embryonic stem cells is maintained by the LIF/STAT3 pathway via Myc (Cartwright et al., 2005). Interestingly, the reprogramming of fibroblasts into pluripotent embryonic stem-cell-like cells also happened only in a small percentage of the cells (Takahashi and Yamanaka, 2006). It seems that the outcome of Myc activation is determined by the developmental context of the target cells and perhaps by the position of the cell in relation to its microenvironment in the tissue.

Do our results reflect an oncogenic role of Myc overexpression? Myc overexpression alone is capable of causing neoplastic transformation in the skin (Pelengaris et al., 1999) and in hepatocytes in which the ability of Myc to induce tumorigenesis is inversely correlated with the developmental age of the host (Beer et al., 2004). Another study on GFAP-expressing astrocytes shows that overexpression of Myc drives the cell fate towards a less differentiated, nestin-expressing progenitor-like fate and that overexpression of Ras and Akt is insufficient to induce gliomas from astrocytes without coexpression of Myc (Lassman et al., 2004). Perhaps the increased number of self-renewing cells and the enhanced expression of nestin and Bmi-1 as well as the distorted capacity to read the environmental cues that we detected in the Myc overexpressing NPCs can, together with otherwise favourable conditions, contribute to oncogenesis.

Taken together, the shift in the tissue balance and cellular identity as well as the subpopulation effects of cell cycle exit delay and re-sphering make us hypothesize that the role of Myc is to enhance some of the already existing self-renewal promoting signals in the NPCs. This means that the presence of other factors, such as microenvironmental cues, is also needed, but perhaps amplified by Myc. The studies on the effects of Myc on self-renewal of some tissues have suggested its function to be involved in the remodelling

of the stem cell niche (Murphy et al., 2005). In the epidermis, for example, more than 40% of the target genes downregulated by Myc encode cell adhesion and cytoskeleton proteins (Frye et al., 2003). Our results are in accordance with this and suggest that Myc regulates the microenvironment of the NPC niche and also interferes with NPC terminal differentiation.

Our work is based on in vitro data of embryonic NPCs. However, one can speculate the possible in vivo relevance of *Myc* and *Miz-1* in NPCs based on their mRNA expression patterns in the adult mouse brain. Both of these genes are strongly expressed and co-localize in the hippocampus and the lateral ventricle wall where neural progenitors are known to be located (Fig. 8; data obtained from the Allen Brain Atlas). Also the expression pattern of *Zbtb4*, a new interactor of *Miz-1* (Weber et al., 2008) co-localizes clearly with *Myc* and *Miz-1* expression, which further strengthens the role of *Miz-1* in neural stem cells.

Materials and Methods

NPC collection and culture conditions

NPCs were collected from the lateral ventricle wall of embryonic day-12 mice (NMRI, The Jackson Laboratory, ME). NPCs were cultured as neurospheres in serum-free stem cell medium (SCM; DMEM supplemented with bFGF 40 ng/ml; F 0291 and EGF 20 ng/ml, E9644; Sigma-Aldrich) (Toma et al., 2001). For all the experiments, cell culture passages from 3-20 were used. For differentiation, small neurospheres or single cells were plated on poly-D-lysine (P0899, Sigma-Aldrich)-coated glass coverslips and cultured in DMEM supplemented with 2% foetal calf serum (FCS; C37360, PromoCell, Belgium).

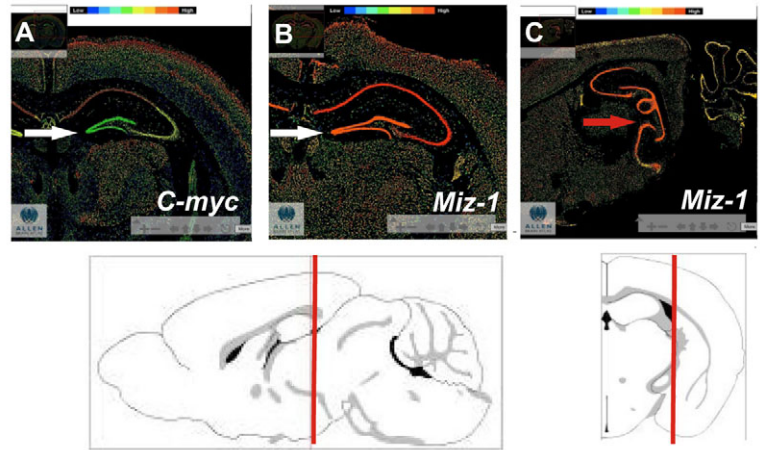
Retroviral transductions

The MycER fusion protein (a kind gift from Gerard Evan, University of California, CA) as well as the Myc V394D *miz-1* binding mutant were cloned into the pBabepuro vector and transfected into the Phoenix Amphi packaging cell line. Wild-type (WT) neurospheres were incubated with the viral supernatant for 3 hours at 37°C, washed and, starting the next day, selected with puromycin (P7255, Sigma), 2 µg/ml in SCM. As controls, we used neurospheres transduced with pBabe without a gene of interest as well as untransduced WT neurospheres. All these control data were pooled and averages were counted as the WT control results. To activate the MycER fusion protein the cells were treated with 100 nM 4-OH-tamoxifen (H7904, Sigma). The expression level of the transgene was verified by western blotting and FACS using an anti-Myc antibody (2 µg/ml; 9E10, Santa Cruz Biotechnology, Inc., CA) and Nucleolin (4 µg/ml; C-23, SC-8031, Santa Cruz Biotechnology).

RT-PCR

TRIzol reagent (Invitrogen, Carlsbad, CA) was used for total RNA extraction which was then treated with RQ1 DNase (Promega, Madison, WI) and incubated for 2 hours at +37°C with random primers and SuperScript II reverse transcriptase (Invitrogen). RNA without reverse transcriptase (RT-) was diluted to the same concentration as

Fig. 8. Myc and Miz-1 mRNA expressions are both localized in the same areas of the brain that are known to contain neural progenitors. A coloured 'heat map' from blue (low) to red (high) represents the intensity of the expression. The in situ hybridization images were obtained from the Allen Brain Atlas [Internet], Seattle, WA: Allen Institute for Brain Science. © 2006. Available from: <http://www.brain-map.org>. The red line in the sketches below the images illustrates the plane of the section in the mouse brain. (A,B) *Myc* (*C-Myc*) and *miz-1* mRNA expression in the hippocampus is especially concentrated to the dentate gyrus (white arrow) shown in coronal sections. (C) A sagittal section shows *miz-1* mRNA expression in the lateral ventricle zone (red arrow).



the cDNA and used as a control for possible genomic DNA contamination. AmpliTaq Gold PCR products (Roche, Nutley, NJ) and 1 μ l of each of the RT reactions and genomic DNA controls were used for 30-cycle PCR amplifications. Primers for mouse *Myc* were designed using the Primer3 program and are as follows: forward 5'-AGA-GCTCCTCGAGCTGTTG-3' and reverse 5'-TTCTCTTCCTCGTCGAGAT-3'.

Antibody staining for immunofluorescence (IF)

Differentiating neural cells were cultured on glass coverslips coated with poly-D-lysine. Neurosphere samples were prepared using a cytospin (Shandon, Runcorn, Cheshire, UK). Prior to staining, the cells were fixed with 4% PFA, washed with PBS and permeabilized and blocked with 0.2% Triton X-100, 5% FCS in PBS. Neurons were stained with monoclonal mouse anti-neuronal class III β -tubulin (TUJ-1; 2 μ g/ml, MMS-435P, BabCo, CA) and astrocytes with the rabbit polyclonal serum recognizing glial fibrillar acid protein (GFAP, 1:800; AB5804, Chemicon International, CA). The differentiated cells were stained with an antibody against the neural progenitor marker Bmi-1 (0.65 μ g/ml, ab 14389, Abcam plc, Cambridge, UK). The nuclei were stained with Hoechst (2.5 μ g/ml, B2261, Sigma). Negative controls were stained with an equal amount of mouse IgG (02-6502 Zymed, CA) or rabbit serum. As secondary antibodies, we used Rhodamine Red-X-conjugated goat anti-mouse IgG, 0.5 μ g/ml, or fluorescein-conjugated donkey anti-rabbit IgG, 3 μ g/ml (115-295-100 and 711-095-152, respectively; Jackson ImmunoResearch Laboratories, PA). The images were obtained with a Zeiss Axioplan 2 microscope, axiocam camera and the Axiovision 3.1 software (Carl Zeiss Microscopy, Jena, Germany).

Flow cytometry (FACS)

For the FACS assays the neurospheres were mechanically dissociated into single cells. Dead cells were excluded by using the LIVE/DEAD Fixable violet dead cell stain kit (L34955, Invitrogen, OR) and then fixed with 4% PFA, permeabilized with saponin (0.5%, S4521, Sigma) and blocked with 1% FCS. Differentiated cells were detached from the poly-D-lysine-coated culture flasks with 0.125% trypsin and Neurocult[®] (05707, StemCell Technologies, BC, Canada) together with mechanical dissociation. The cells were stained with primary antibodies on ice for 45 min (Nestin, 1 μ g/ml; Bmi-1, 0.65 μ g/ml; TUJ-1 2, μ g/ml; GFAP 1:800) or overnight (Myc, 20 μ g/ml), or with an equal amount of mouse IgG, washed three to five times with PBS and stained for 45 minutes on ice with the secondary antibody (Alexa Fluor 647, 2 μ g/ml; A21235, Invitrogen), washed three times with PBS and the minimum of 5000 living cells per experiment were analysed with FACSaria (BD Biosciences NJ).

BrdU cell proliferation assay

To measure the percentage of NPCs in S phase, small neurospheres were incubated with bromodeoxyuridine (BrdU, 1:1000; RPN 201, Amersham Biosciences UK Limited, Buckinghamshire, UK) for 12-16 hours, cytocentrifuged onto object glasses (5×10^4 cells per spot; cytospin 2, Shandon) fixed and stained with Hoechst and anti-BrdU antibody according to the instructions of the BrdU labelling kit (RPN 202, Amersham Biosciences). When cell proliferation was measured during differentiation, the protocol was otherwise identical but BrdU incorporation time was 24 hours. The percentage of proliferating cells was counted by dividing the number of BrdU-positive cells by the number of total nuclei. For this, 500-1000 cells from duplicate samples were counted per individual experiment.

Growth measurements

Neurospheres were dispersed into single cells, counted and then plated into 6-well culture plates (100,000 cells in 4 ml SCM per well). The total number of cells per well was counted on days 4 and 7.

Self-renewal assays

In order to evaluate the self-renewal capacity of the cultured NPCs, we used a clonal colony-forming assay to measure the proportion of cells that were able to make new neurospheres. Single cell-dissociated cultures of NPCs were plated into 96-well culture plates with 1-15 cells per well, and the number of newly formed neurospheres was counted after 7 days. The number of new neurospheres (>20 cells tightly attached to each other) was divided by the number of originally plated cells, which was then interpreted as the percentage of self-renewing cells (Fig. 2B).

We also measured the ability of the NPCs to retain their self-renewal capacity under differentiating culture conditions. Neurospheres were dispersed into single cells and plated as 40-4000 cells per well with 2% FCS on poly-D-lysine-coated 96-well plates for 4 weeks. Culture medium was partly changed once every 7 days and the 're-sphering' (number of emerging neurospheres) was counted after 1, 2, 3 and 4 weeks (Fig. 4A). Also, after 4 weeks, single 're-sphered' neurospheres were collected and placed in EGF- and FGF-containing SCM and expanded in order to evaluate the self-renewal and differentiation capacity of these cells.

Immunohistochemistry for paraffin sections

Paraformaldehyde-fixed, paraffin-embedded sections of an embryonic day-16 mouse brain were examined for Myc protein expression using immunohistochemistry. Sections were boiled at 120°C for 2 minutes in a 10 mM sodium citrate buffer (pH 6.0) and thereafter incubated with mouse monoclonal Myc antibody (0.2 μ g/ml) for 60 minutes in 23°C. Binding of the primary antibody was detected with a PowerVision + Poly-HRP histostaining kit (Immunovision Technologies & Co., CA). Tissue sections were counterstained with Haematoxylin.

Cell ploidy analysis with flow cytometry

To analyze ploidy of the Myc-overexpressing neurospheres, the cells were suspended into single cells and fixed with ice-cold 75% ethanol in PBS, stained with 50 mg/ml propidium iodide (PI; P5264, Sigma) and treated with RNase (40 μ g/ml; P5215, Sigma) for 30 min at 37°C. The cells were sorted with a FACSCalibur flow cytometer (BD) and analyzed with Sync Wizard Model ModFit LT software (BD).

Apoptosis

Apoptosis was measured using Annexin staining according to the protocol of the Annexin-V-FLUOS staining kit (1858777, Roche Diagnostics GmbH, Mannheim, Germany). Prior to staining, the cells were mechanically isolated into single cells. Negative controls were treated equally but without one of the appropriate stainings. Annexin labels both apoptotic and necrotic cells, while living cells remain unstained. Necrotic cells were excluded by PI staining. The number of labelled cells was measured by flow cytometry (FACSaria, BD Biosciences, NJ).

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