Pitx2 expression induces cell cycle exit and p21 expression in neural stem cells

Nina Heldring[1], Bertrand Joseph[2], Ola Hermanson[1, 4], Chrissa Kioussi[3, 4]

[1]Department of Neuroscience, Karolinska Institutet, Retzius väg 8, S-171177 Stockholm, Sweden

[2]Department of Oncology-Pathology, Cancer Centrum Karolinska, R8:03 Karolinska Institutet, S-17176, Stockholm, Sweden

[3]Department of Pharmaceutical Sciences, College of Pharmacy, Oregon State University, Corvallis, OR 97331-3507, USA

[4]Corresponding authors:
chrissa.kioussi@oregonstate.edu, phone: +1 5417372179;
ola.hermanson@ki.se; phone:+46 8 52487477

ABSTRACT

Cortical development is a complex process that involves many events including proliferation, cell cycle exit, and differentiation that need to be appropriately synchronized.. Neural stem cells (NSCs) isolated from embryonic cortex are characterized by their ability of self-renewal under continued maintenance of multipotency. The G1 phase of the cell cycle is mostly associated with cell cycle arrest and cell differentiation. Cell cycle progression and exit during development is regulated by numerous factors, including cyclins, cyclin dependent kinases and their inhibitors. In this study, we exogenously expressed the homeodomain transcription factor Pitx2, usually expressed in postmitotic neurons of the embryonic cortex, in NSCs with low expression of endogenous Pitx2, and found that Pitx2 expression induced a rapid decrease in proliferation associated with an accumulation of NSCs in G1 phase. A search for potential cell cycle inhibitors responsible for such cell cycle exit of NSCs revealed that Pitx2 expression caused a rapid and dramatic (≈20-fold) increase in expression of the cell cycle inhibitor p21Cip. In addition, Pitx2 bound directly to the p21Cip promoter as assessed by chromatin immunoprecipitation (ChIP) in NSCs. Surprisingly, Pitx2 expression was not associated with an increase in differentiation markers, but instead the expression of nestin, associated with undifferentiated NSCs, was maintained. Our results suggest that Pitx2 directly regulates p21Cip expression and induces cell cycle exit in neural progenitors.

INTRODUCTION

The development of the nervous system requires a sophisticated gene network that regulates each stage of the development. During this process, cells exit the cell cycle and become specialized to support distinct tissue and organ functions. In the developing cortex, multipotent neural progenitors, referred to as neural stem cells (NSCs) cells, migrate from the ventricular and subventricular zones and from the ganglionic eminences to undergo cell cycle exit and adopt a terminal phenotype. As development proceeds, NSCs can be divided into two major cell populations, one that generates additional NSCs (expansion) and one that generates committed progenitors or post-mitotic cells (differentiation). Cortical NSCs undergoing mitosis in the ventricular zone will generate the apical progenitors, the neuroepithelial and radial glia cells, while the subventricular zone will generate the basal progenitors, the intermediate or abventricular progenitors {Gotz, 2005 #171}. The progression of NSCs from proliferative to neurogenic is associated with an increased cell cycle during neurogenesis due to the lengthening of the G1 phase and inhibition of the G1-S progression {Takahashi, 1995 #112}. The length of the cell cycle will determine the symmetrical vs. asymmetrical differentiation of the daughter cells {Calegari, 2003 #116}.

Cell cycle progression is regulated by cyclins, cyclin-dependent kinases (CDK) and their inhibitors (CDKI) {Morgan, 1995 #26;Lees, 1995 #28}. CDKIs act as initiators of cell differentiation {Marx, 1995 #41}. CDKIs are represented by two families the INK4 proteins ($p16^{INK4A}$, $p15^{INK4B}$, $p19^{INK4C}$) that interfere with the cyclin D binding to inhibit CDK4 and CDK6 {Hunter, 1994 #43}, and the CIP/KIP proteins ($p21^{Cip1}$, $p27^{Kip1}$, $p57^{Kip2}$)

that inhibit CDK2 {Harper, 1995 #47;Matsuoka, 1995 #48}**[Hermanson 2009, 2010]**. Reduced expression levels of CDK2 trigger the G1 checkpoint through the activation of the ATM-CHK2-p53-p21 pathway. In NSCs $p21^{Cip1}$ is required for self-renewal and its loss results in exhaustion of the proliferation capacity {Kippin, 2005 #20}.

Pitx2 is a homeodomain transcription factor that is expressed in the lateral plate mesoderm during gastrulation, and in diencephalon mesencephalon and ventral spinal cord during embryogenesis {Kitamura, 1997 #55;Lindberg, 1998 #56;Muccielli, 1996 #57}. In humans, PITX2 haploinsufficiency results to Axenfeld-Rieger syndrome type 1 (RIEG1, MIM180500, 4q25), defined by ocular dysgenesis, umbilical abnormalities and tooth agenesis {Semina, 1996 #108}. Rare RIEG1 individuals exhibit mental retardation and developmental delay {De Hauwere, 1973 #64;Summitt, 1971 #65}. Pitx2 deficient mice also exhibit ocular, craniofacial and abdominal wall malformations similar to humans {Gage, 1999 #246;Kitamura, 1999 #249;Lin, 1999 #222;Lu, 1999 #230}. Pitx2 is expressed in the hypothalamic $Nestin^{+}$ neural progenitor cells, in the diencephalic g-aminoburyric (GABA) producing neurons {Martin, 2002 #1;Waite, 2011 #77} and is required for the development and migration of the subthalamic nuclei {Martin, 2002 #86;Skidmore, 2008 #85}. At postnatal stages, Pitx2 is present in multiple brain nuclei including the subthalamic, posterior, hypothalamic, red and reticular thalamic nucleus {Lindberg, 1998 #56;Muccielli, 1996 #57;Smidt, 1997 #60}. Pitx2 appears to be a downstream target of general growth factor signaling pathways that mediate cell-type specific control of proliferation. For example, the Wnt1/ß-catenin signaling pathway regulates Pitx2 expression in pituitary cardiac and skeletal muscles and controls their

proliferation by regulating the expression of critical G1 cell cycle control genes. Growth factor-dependent signaling results in release of Pitx2-associated co-repressors and mediates recruitment of specific co-activator complexes {Kioussi, 2002 #154}. Pitx2 is a positive regulator of FGF8 and a repressor of BMP4 signaling during craniofacial development, and Pitx2 mutant cells exhibit misdirected or arrested migration {Liu, 2003 #90} maybe due to the lack of active Rho GTPase during planar cell polarity {Wei, 2002 #91}.

The aim of this study was to investigate a putative role for Pitx2 in NSCs. We examined the fate of cortical NSCs by examining their proliferation and differentiation fate after exogenous expression of Pitx2. We have demonstrated that overexpression of Pitx2 in NSCs resulted in activation of Nestin expression, arrest in G1 phase and increased levels of $p21^{Cip1}$. All these three events collectively suggest that Pitx2 induce cell cycle exit in NSC through regulation of the cyclin kinase inhibitor $p21^{Cip1}$. Pitx2 might act as a NSC gatekeeper by controlling their entry to cell cycle or their exit towards commitment and differentiation.

MATERIAL AND METHODS

Embryonic Neural Stem Cell Cultures. Rat embryonic neural stem cells (NSCs) were derived as previously described {Johe, 1996 #17}. Briefly, rat cortical tissue from embryonic day 15.5 was dissociated and $1000x10^3$ cells were plated per 10cm dish, previously coated with poly-L-ornithine (15μg/ml) and fibronectin (1μg/ml) (cat no P3655 and F1141, Sigma-Aldrich, St. Louis, MO, USA). NSCs were then expanded in N2 media with 10ng/ml of basic fibroblastic growth factor (FGF) (R&D Systems, Inc, Minneapolis, MN, USA) and splited by light dissociation in the presence of HBSS (Gibco cat no 14180, Life technologies, Grand Island, NY, USA) NaHCO3 and HEPES (S5761 and H4034, Sigma-Aldrich, St. Louis, MO, USA). FGF were added daily and media was changed every second day until cells reach confluency. Animals were treated in accordance with institutional and national guidelines (Ethical permit no. N284/11).

Nucleofections. Nucleofections were performed according to the supplier's recommendations (Rat NSC kit, program A-33, AMAXA-Lonza). 3μg of plasmid DNA and 3-4 $x10^6$ cells were used for each nucleofection. Cells were collected for FACS or ChIP analysis at the time points stated.

Immunocytochemistry and Antibodies. NSCs were cultured on 3.5 cm well plates were fixed with 4%paraformaldehyde for 20 min and washed and permeated with 0.2% Triton-x100/PBS and blocked with 3% BSA. Cells were incubated with mouse anti-Nestin (1:500 Dako Cytomation M0760), chicken anti-EGFP (1:500 Chemicon International AB16901)

at 40^{o}C overnight. After washing with 0.2% Triton-x100/PBS, cells were incubated with chick-Alexa488 and mouse Alexa594 (1:500; Invitrogen, Carlsbad USA) for 2 hrs at room temperature. Cells were photographed in a Zeiss Axioskop 2 fluorescence microscope.

Proliferation Assay. Click-iT EdU assay (Invitrogen, Carlsbad CA USA) was used to detect the NSC proliferation index. Briefly, EdU (5-ethynyl-2'deoxyuridine) was incorporated into NSC DNA during DNA synthesis, during incubation with 10μM EdU for 2hrs, washed with PBS and fixed with 3.7% formaldehyde for further immunocytochemistry.

Quantitative Real - Time PCR (qPCR) - cDNA or Immunoprecipitated (IP) DNA from NSCs were analyzed by qPCR on the ABI 7500 machine using SYBR Green 1 methodology (Invitrogen, Carlsbad CA USA). Samples were run in technical triplicates from pooled tissue preparations in three independent experiments. Expression analysis was normalized against transcriptional basic protein (TBP) expression levels, while IPs were normalized against input. All primers were tested for specificity with standard PCR. Rat primers used in qPCR: TBP (F) GGGGAGCTGTGATGTGAAGT, TBP (R) CCAGGAAATAATTCTGGCTCA, EGFP (F) CACATGAAGCAG CACGACTTCT, EGFP (R) AACTCCAGCAGGACCATGTGAT, Nestin (F) GCTGGAACAGAGATTGGAAGG, Nestin (R) CCAGGATCTGAGCGATCTGAC, Acta2 (F) GTCCCAGACACCAGGGAGTAT, Acta2 (R) TCGGATACTTCAGCCTCAGGA, $p21^{Cip1}$(F) CGAGAACGGTGGAACTTTGAC, $p21^{Cip1}$(R) CAAGATCTACCTGAAGCCCTG, $p27^{Kip1}$(F)

TCAAACGTGAGAGTGTCTAACG, $p27^{Kip1}$(R) CAGAAACTCTTCGGCCGGTCAT, $p57^{Kip2}$(F) CGAGGAGCAGGACGAGAATC, $p57^{Kip2}$(R) GATCGCACCGTTCGCATTGGC, Cyclin D1(F) GCGTACCCTGACACCAATCTC, Cyclin D1(R) CTCCTCTTCGCACTTCTGCTC. Rat primers used in ChIP qPCR: $p21^{Cip1}$ (F) GACCTGAGGAGAGCCAACTG, $p21^{Cip1}$(R) GGTTATCTGGGGTCTCTGC, $p21^{Cip1}$ negative region (F) CTCACAGCGCCCAATAATCT, $p21^{Cip1}$ negative region (R) AGACTTGGAGGGGGTAAGG

Flow Cytometry for Determination of Cell Cycle Distribution. The distribution of cells in the cell cycle phases was determined by DNA flow-cytometry as described earlier {Castro, 2001 #3}. 24-h post-nuleofection, cells were harvested, washed in PBS, fixed in 70% ice-cold ethanol, and stored at -20 °C. NSCs were stained with propidium iodine (PI) (50 µg/ml PI, 10 mM Tris pH 7.5, 5 mM $MgCl_2$, 10 µg/ml RNaseA (50 mg/ml, DNase free) at 37 ˚C for 30 min. A. Flow cytometric analysis was carried out on 10,000 gated EGFP-expressing cells using a FACSCalibur flow cytometer equipped with CellQuest software (Becton Dickinson).

Chromatin Immunoprecipitation (ChIP). Embryonic NSCs nucleofected with Pitx2 or empty vector were fixed in 1% formaldehyde for 10 min, 48h after nucleofection. Chromatin immunoprecipitation was performed using the "LowCell# kch-maglow-G48 ChIP" kit (Diagenode, Liège, Belgium) using chromatin from 8000 cells per reaction (50ng). For the immunoprecipitation step, antibody against PITX2 (Cat #sc-8748; Santa Cruz Biotechnology Inc, CA, USA), were used. IgG (Diagenode, Liège, Belgium) was

used as control. The ChIP was evaluated by qPCR using “platinum SYBR Green qPCR SuperMix-UDG” (cat no 11733-038, Life technologies, Grand Island, NY, USA). Results are presented as fold over background antibody and error bars represent standard error of the means from three biological replicates.

RESULTS

Pitx2 Promotes Nestin Expression in NSCs

In early neural development, the homeodomain transcription factor Pitx2 is expressed in nestin-positive neural progenitors and in postmitotic developing neurons, TuJ1-positive cells, in diencephalon, mesencephalon and rhombencephalon {Martin, 2002 #1}. In the developing telencephalon and neocortex, Pitx2-positive cells are located just outside and bordering the zones of proliferating cells, and thus increased levels of cortical Pitx2 correlates nicely with cell cycle exit.

To examine the role of Pitx2 during neural differentiation, we used a telencephalic neural stem cell system, (NSCs) {Hermanson, 2002 #191}**(Johe)**. Pitx2 expression was not detected in NSCs grown in stem cell maintaining conditions (with FGF2) or in differentiating conditions (without FGF2, with serum, in the presence of BMP4 or CNTF; data not shown). Thus, we concluded that the cell system was ideal for investigating the effects of increased levels of Pitx2 by exogenous delivery. Pitx2 overexpression vector {Shih, 2007 #14} was nucleofected into NSCs and cells were allowed to grow up to 4 days in presence of FGF2 {Hermanson, 2002 #191}. Cells were tested for nucleofection efficiency and cell characteristics by immunostaining for EGFP and for nestin, the intermediate filament associated with undifferentiated NSCs, respectively. All nucleofected cells were $EGFP^{+}/Nestin^{+}$ at day 1 to day 3 (Fig 1A-F). No obvious phenotypic changes were observed in cells with Pitx2 overexpression compared to the control NSCs nucleofected with vector only (Fig. 1A-F).

The relative expression levels of nestin was calculated by qRT-PCR assays (Fig 1G). Nestin expression levels were increased by 2-fold in presence of Pitx2 compared to empty vector at day 1 whereas at days 2 or 3, no difference was detected between Pitx2-transfected and control NSCs. As comparison the expression levels of Acta2, smooth muscle actin, were tested and no differences were detected during the 3-day period of the experiment (Fig 1H).

Exogenous Pitx2 expression inhibits NSC proliferation

Due to the lack of signs of differentiation after Pitx2 overexpression as assessed by morphology and nestin expression, we next decided to investigate the effects on proliferation by EdU incorporation. NSCs were cultured in presence of FGF2 for one and three days. Immunocytochemistry against EGFP was used to determine the nucleofection efficiency ($EGFP^+$) and EdU staining to determine the fraction of proliferating cells (Fig 2A-D). Three independent sets of experiments were performed and cells were counterstained with DAPI. Cells were counted for expression of EGFP, EdU and EGFP/EdU after one and three days in culture (Fig 2E). In control experiments, 30% of the cell population was EdU^+ both days 1 and 3. However, when Pitx2 was overexpressed the $EGFP^+/EdU^+$ cells were reduced to 12% at day 1, i.e. less than half of the NSCs compared to the control cultures. The proliferation rate of Pitx2-transfected cells recovered and at day 3, it was up to 30%, similar to the control cells. These results showed that increased levels of Pitx2 in NSCs reduced proliferation significantly.

Pitx2 expression leads to an accumulation of NSCs in G1 phase

To elucidate candidate factors mediating the effects of Pitx2 on NSC proliferation, we decided to analyze which phase of the cell cycle that the Pitx2-expressing NSCs were arrested in. NSCs were therefore stained with propidium iodine and subsequently analyzed by flow cytometry. FACS analysis revealed that only 5% of the Pitx2-overexpressed NSCs entered the S-phase (Fig 3B) compared to 25% of the control cells (Fig 3A). The majority of the Pitx2-overexpressed NSCs were arrested at G1 (79% versus 62% of the control cell; Fig 3C). No difference was observed in G2/M between control and Pitx2 overexpressed NSCs. The switch from expansion to differentiation in NSCs is controlled by the duration of G1 {Salomoni, 2010 #157}. Thus, Pitx2 might control the length of G1 and thereby influence the switch from expansion to differentiation of the NSCs.

Exogenous Pitx2 expression promotes a dramatic increase in $p21^{Cip1}$ expression

We next aimed at identifying the factor/s mediating the decrease in proliferation and G1 accumulation of Pitx2-expressing NSCs. Members of the Cip/Kip family regulate cell-cycle exit by regulating the activity of cyclin-CDK complexes (**Hermanson 2009, 2010**). To test if the levels of Cip/Kip family members were affected by Pitx2 overexpression, we analyzed by qRT-PCR the gene expression of p21Cip, p27, Kip1, and p57Kip2 at one, two, or three days after the nucleofection. These experiments revealed that $p21^{Cip1}$ and $p27^{Kip1}$ expression levels in NSCs showed differences in the presence of Pitx2, whereas the expression of p57Kip2 remained unaffected (Fig 4A-C). Most notably, a dramatic increase of ≈20 fold in $p21^{Cip1}$ expression was observed at day one after Pitx2 nucleofection (Fig. 4A). The p21Cip1 levels returned to control levels or even decreased at day two and three

(Fig 4A). In contrast, p27Kip1 expression was moderately up-regulated at day one, decreased at day two, but then showed a 3-fold increase at day three after Pitx2-transfection (Fig. 4B). These data suggest that Pitx2 specifically promotes the activation of $p21^{Cip1}$. This could be linked to the decreased proliferation of NSCs by Pitx2 overexpression, as this was most obvious at day one after Pitx2 transfection.

Pitx2 occupies the $p21^{Cip1}$ promoter

Possible cis-regulatory modules (CRMs) that could mediate the observed Pitx2-dependent expression were searched for in genomic sequences surrounding the $p21^{Cip1}$ gene. The mouse genomic sequences (mouse build 37) encompassing $p21^{Cip1}$ transcript was downloaded using the genomic representative sequences link in the mouse genome informatics (MGI) web site. Genomic sequences 20kb upstream and downstream of the transcription unit were included in the download to give the genomic loci to be searched for the optimal consensus Pitx2 binding motif TAATCY {Amendt, 1998 #51;Vadlamudi, 2005 #54;Wilson, 1996 #52}, (Fig 5A). ChIP assays of control and Pitx2-overexpressed NSCs were performed to determine the Pitx2 occupancy on identified sites of the $p21^{Cip1}$ locus *in vivo*. Test genomic amplicons were analyzed to determine if any enrichment of Pitx2 occupancy could be demonstrated in NSCs. Pitx2 occupied $p21^{Cip1}$ (Fig 5B) indicating its physical presence in the vicinity of the gene and thus that Pitx2 may directly influence the expression of $p21^{Cip1}$ at the transcriptional level. We conclude that increased levels of Pitx2 may regulate mammalian NSC cell cycle by occupying $p21^{Cip1}$ promoter region, activate its expression, and inhibit cell cycle in G1.

DISCUSSION

The length of the G1 phase of the cell cycle is a key player for the expansion/differentiation switch {Salomoni, 2010 #157}. In this study, we have shown that activation of the homeodomain transcription factor Pitx2 extends the time of G1 phase of mammalian cortical NSCs and thereby exiting the cell cycle. This negative control of the S phase of the NSCs is mediated by the activation of the CDKI, $p21^{Cip1}$.

Inhibition of the G1 to S progression is sufficient to increase neurogenesis and to inhibit neuroepithelial cell proliferation. G1 arrest is associated with increased expression of $p21^{Cip1}$, inhibition of stem cell/progenitors transcription factors and activation of pro-neuronal transcription factors. The $p21^{Cip1}$ is a molecular switch that governs the entry of stem cells into the cell cycle, regulates stem cell and progenitor pool size and protects them from exhaustion {Cheng, 2000 #184}. Here we show that a homeobox gene by regulating $p21^{Cip1}$ activation in the NSC pool can maintain the cells in the G1 phase thereby limiting NSC proliferation.

It has been demonstrated that in the absence of the orphan nuclear receptor TLX the cell cycles is prolonged and cell cycle exit is increased in NSCs which is associated with increased expression of $p21^{Cip1}$ and decreased expression of cyclin D1 {Li, 2008 #144}. The orphan nuclear receptor TLX is expressed in periventricular embryonic NSCs and TLX is known to interact with HDACs that represses $p21^{Cip1}$ and thereby promotes NSC proliferation {Sun, 2007 #200}. Absence of HDAC1 and HDAC2 in primary fibroblast and B cells also results in G1 arrest, which is the result of an increased transcriptional

activity of $p21^{Cip1}$ {Yamaguchi, 2010 #254}. In addition, HDAC inhibition results in G1 arrest of NSCs and transcriptional changes that lead to activation of neuronal fate and reduction of the stem cell fate {Zhou, 2011 #255}. Interestingly, we and others have shown that Pitx2 interacts with HDAC1 and HDAC3 during embryogenesis and regulates cell proliferation by activating cyclin D2 and cyclin D1 {Kioussi, 2002 #154;Hilton, 2010 #152}. In addition, absence of Pitx2 during development results in an arrest of organ development {Lu, 1999 #230;Lin, 1999 #239;Gage, 1999 #246;Kitamura, 1999 #249}. It has also been demonstrated that in the Wnt1/ß-catenin pathway, the TCF factor mediates the activation of the downstream genes upon recruitment of HDACs and both HDAC1 and HDAC2 can regulate oligodendrocyte formation by disrupting the ß-catenin/TCF interaction {Ye, 2009 #5}. We have shown that Pitx2 acts downstream of the Wnt1/ß-catenin pathway, binding to TCF, releasing HDAC1 and thereby activate cell cycle control genes in myoblasts {Kioussi, 2002 #154}. This present study allows us to suggest a similar Pitx2 –dependent transcriptional mechanism present in the NSCs regulating maintenance and renewal.

NSCs in the adult subventricular zone give rise to highly proliferating transit-amplifying progenitor cells, which then differentiate into neuroblasts, astrocytes, and oligodendrocytes. Interestingly, many brain tumors are often characterized by altered CpG island methylation affecting the expression of genes functioning in cell cycle, apoptosis, DNA repair, and developmental transcription factors {Jones, 2007 #220} which may contribute to tumor initiation and progression. Overexpression of $p21^{Cip1}$ results in G1 arrest that suppresses tumor growth and knowing that Pitx2 regulate $p21^{Cip1}$ as shown in

this study and since Pitx2 exhibits consecutive hypermethylated CpG islands in human astrocytomas {Wu, 2010 #221} it is reasonable to speculate that Pitx2 might be involved in the NSC-derived tumor cell suppression via a dual mechanism in transcriptional and epigenetic level.

In summary, increased levels of Pitx2 can block the transition of the NSCs from G1 to S phase progression. This arrest in combination with an increased level of $p21^{Cip1}$, suggest that Pitx2 regulate mammalian neural progenitors cell cycle by occupying $p21^{Cip1}$ promoter region, activate its expression, and induces cell cycle exit in G1. Surprisingly, Pitx2 expression was not associated with an increase in differentiation markers, but instead the expression of nestin, associated with undifferentiated NSCs, was maintained. Further understanding of the involvement of sequence specific transcription factors in NSC fate in adult neurogenesis will be essential for developing therapeutic applications to intervene in progression of brain diseases.

ACKNOWLEDGMENTS

We thank the Hermanson and Joseph lab members for experimental guidance. This work was supported in part by grants from XXXX to BJ, the Swedish Childhood Cancer Society (BCF), the Swedish Research Council (VR-MH, DBRM), Karolinska Institutet, and the Swedish Cancer Society (CF) to OH, and NIH-NIAMS AR054406 to CK.

REFERENCES

FIGURE LEGENDS

Figure 1. Pitx2 Promotes Nestin Expression in NSCs

(A-F) Double labeling immunocytochemistry for EGFP(green)/Nestin(red). NSCs were nucleofected with control (A, C, E) and Pitx2-overexpressing plasmid (B, D, F) and cultured for 1 (A, B), 2 (C, D) and 3 (E, F) days. DAPI (blue) was used as counterstain. Nestin was colocalized with EGFP in almost all cells. (G-H) Quantitative RT-qPCR analysis for Nestin (G) and Acta2 (H) indicated increased expression levels for Nestin in the Pitx2-overexpressed NSCs at day one compared to control cells. No differences were observed for any other time points for Acta2.

Figure 2. Exogenous Pitx2 expression inhibits NSC proliferation

(A-D) EdU staining after 2 hrs pulse in NSC nucleofected ($EGFP^+$, green) cells. Decreased number of EdU^+ cells (red) in the presence of Pitx2. DAPI (blue) was used as counterstain. (E) NSC cells were counted for day 1 and day 3. Reduced number of proliferating cells in Pitx2-overexpressing NSC was detected in day 1 comparing to control cells.

Figure 3. Pitx2 expression leads to an accumulation of NSCs in G1 phase

FACS analysis of control (A, green) and Pitx2-overexperssed (B, red) NSCs stained with propidium iodine. (C) Cells were counted to 62% control and 79% Pitx2-overexpresed during the G1 phase respectively. Pitx2-overexpressed NSCs were arrested in G1 and failed to progress to S. Only 5% entered the S comparing to 25% of the control cells.

Figure 4. Exogenous Pitx2 expression promotes a dramatic increase in $p21^{Cip1}$ expression

RT-qPCR analysis in NSC control and Pitx2-overexpressing cells for $p21^{Cip1}$ (A), $p27^{Kip1}$ (B) and $p57^{Kip2}$ (C). Pitx2 dependent induction was specific to $p21^{Cip1}$ on day 1.

Figure 5. Pitx2 occupies the $p21^{Cip1}$ promoter

ChIP assays of control and Pitx2-overexpressed NSCs. Pitx2 occupancy was detected in the **$p21^{Cip1}$ promoter** region (A) in NSCs while no signal was detected in a randomly selected negative control region in chromosome 10 (B). Data are presented as fold over control antibody and error bars are based on data from three biological replicas.