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Dynamic expression of synemin isoforms in mouse embryonic stem cells and neural derivatives

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Abstract

Background: Intermediate filaments (IFs) are major components of the mammalian cytoskeleton and expressed in cell-type-specific patterns. Morphological changes during cell differentiation are linked to IF network remodeling. However, little is known concerning the presence and the role of IFs in embryonic stem (ES) cells and during their differentiation.

Results: We have examined the expression profile of synemin isoforms in mouse pluripotent ES cells and during their neural differentiation induced by retinoic acid. Using RT-PCR, Western blotting and immunostaining, we show that synemin M is present at both mRNA and protein levels in undifferentiated ES cells as early as pluripotency factor Oct-3/4 and IF keratin 8. Synemin H was produced only in neural precursors when neural differentiation started, concurrently with synemin M, nestin and glial fibrillary acidic protein. However, both synemin H and M were restricted to the progenitor line during the neural differentiation program. Our *in vivo* analysis also confirmed the expression of synemins H/M in multipotent neural stem cells in the subventricular zone of the adult brain, a neurogenic germinal niche of the mice. Knocking down synemin in ES cells by shRNA lentiviral particles transduction has no influence on expression of Oct4, Nanog and SOX2, but decreased keratin 8 expression.

Conclusions: Our study shows a developmental stage specific regulation of synemin isoforms in ES cells and its neural derivatives. These findings represent the first evidence that synemins could potentially be useful markers for distinguishing multipotent ES cells from undifferentiated neural stem cells and more committed progenitor cells.

Background

Synemin belongs to the intermediate filament (IF) protein family [1,2]. In mammals, the synemin gene is one of the rare IF genes encoding three isoforms (180, 150 and 41 kDa) achieved by alternative mRNA splicing, exon skipping and a shift in the open reading frame [3,4]. The two larger isoforms of synemin (H and M) harbor extended C-terminal tails that project from the surface of the filament and provide connecting arms that associate with neighboring proteins [5-7]. In contrast, the small isoform (L) lacks this tail domain [3,8].

Synemin forms obligatory heteropolymers in order to incorporate into filamentous networks and is associated with desmin and vimentin in muscle and endothelial cells [1-3,9]. It has been suggested that synemin could function as a linker between different cytoskeletal components based on the fact that it interacts with several proteins involved in the organization of the costameres, neuromuscular and myotendinous junctions within striated muscle cells. These proteins include α-actinin, vinculin, dystrophin, α-dystrobrevin, utrophin, zyxin and talin [1,3-7,10-13]. In addition, it has been reported that synemin is an A-kinase anchoring protein (AKAP), containing a binding region for protein kinase A (PKA) in its C-terminal domain which provides temporal and spatial targeting of PKA in cardiomyocytes [14]. In the nervous system, synemin was found to associate with ruffled membranes of reactive astrocytes and to also co-

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localize with α-actinin, suggesting a role in cell motility [15]. We have shown that synemins H and M were present with vimentin, nestin and glial fibrillary acidic protein (GFAP) in glial progenitors, whereas the small isoform appeared in neurons linked to the NF proteins associated with the membrane compartment [9,16].

The different expression of isoforms H, M and L of synemin in the nervous system raises several questions about the regulation of synemin gene expression during the determination of glial and neuronal cell lineages in the central and the peripheral nervous system (CNS and PNS). First, an unexpected finding is the selective synthesis of two high molecular weight synemin isoforms (H and M) in CNS astrocytes, while the smallest synemin isoform (L) is present only in neurons [16]. This selectivity suggests that the commitment of CNS precursor cells to form glia or neuron involves the direct regulation of the single synemin gene. Our analysis of mouse development from embryonic day 5 to 15 (E5 to E15) has demonstrated that synemin M mRNA is produced at E5 as early as nestin and vimentin mRNA, prior to the appearance of the H isoform. *In toto* hybridization at E7.5 showed synemin M labeling in the embryonic mesoderm and the neuroectoderm [9]. We asked if synemin is also expressed in mouse embryonic stem (ES) cells, which can differentiate into a variety of somatic cell types including lineages from three embryonic germ layers? The presence and function of IF proteins in ES cells are not yet fully understood, only a few members of this family have been studied [17-20]. The mRNAs coding for keratins 7, 8, 18 and 19 are present in the 2-cell stage embryo, but only K7 and K8 (type II) are translated into protein in 4- to 8-cell stage embryos at a low level, which is deposited in aggregates [18,19,21-23]. The K18 and K19 proteins were identified from E3.5 mouse embryo [19,24]. After differentiation of ES cells into neuronal progenitors, K8 and K18 protein expression decreased [24]. The nuclear IF protein lamin B1 and B2 were identified as markers of ES cells; however the lamins A/C were activated during ES cell differentiation [25].

In this report, we examined the expression profile of synemin isoforms in mouse pluripotent ES cells and during their early neural differentiation induced by retinoic acid to answer the following questions: 1) Are synemin isoforms expressed in pluripotent ES cells? 2) How are synemin isoforms expressed during neural differentiation of ES cells? We detected the expression of synemin M at both mRNA and protein levels in pluripotent ES cells, as early as Oct-3/4 and keratin 8. Synemin H was produced only in neural precursors when the neural differentiation started, along with synemin M, nestin and GFAP.

Results

Synemin M expression in mouse pluripotent ES cells

To investigate which synemin isoforms are expressed in ES cells, two mouse ES cell lines were used. We first analyzed the pluripotent state of the cells by PCR-Array. We identified the expression of transcription factors maintaining "stem-ness" as Nanog, Nr6a1, Utf1, Pou5f1 (Oct-3/4), and signaling molecules required for pluripotency and self-renewal genes such as Ifitm1 and Pten. No expression of embryonic stem cell differentiation genes was detected in the ES cells (Data not shown). Expression of synemin mRNA in ES cells was analyzed by RT-PCR. As shown in Figure 1A, we detected the synemin M mRNA in ES cells. In contrast, neither synemin H nor nestin mRNA was found. The E9.5 mouse embryos containing synemin M and H were used as a positive control (Figure 1A).

The presence of synemin M protein, but not synemin H, was confirmed by Western blot analysis in ES cells (Figure 1B). In the positive control, both synemin M and H isoforms were identified in the adult mouse bladder. The presence of Oct-3/4, a marker of the pluripotency of ES cells, was also confirmed by Western blotting (Figure 1B). Two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) followed by Western blotting was used to further analyze the synemin profile from ES cells extracts. This experiment showed two spot trains (a and b) of synemin M with an apparent molecular weight of 148-150 kDa and isoelectric point (pI) 5.1-5.2 in each ES cell line (Figure 1C), corresponding to its theoretical pI (5.14), in agreement with its high content in glutamic acid and glutamine (18%, 228 residues/molecule), aspartic acid (6%, 70 residues/molecule), and rich in serine (9%, 111 residues/molecule) and poor in cysteine (0.1%, 2 residues/molecule). We could observe that the quantity of spot train-a of synemin M was more abundant than spot train-b. In order to investigate the possible post-translational modifications of synemin, we analyzed the phosphorylation of synemin M in ES cells with anti-phosphoserine antibody. This result revealed that synemin M was phosphorylated in serine with isoelectric variants (Figure 1C).

To determine the synemin localization in ES cells, we performed double immunofluorescence staining utilizing synemin, Oct-3/4, cytokeratin 8 (K8), and SSEA-1 antibodies. In order to study specifically the expression of the synemin H isoform, we produced a polyclonal antibody with a polypeptide corresponding to the exon 4a of human synemin, a specific fragment for synemin H [4]. Western blotting analysis with proteins extracted from the mouse bladder showed that this antibody recognized specifically one band of 180 kDa, corresponding to the synemin H isoform (Figure 2A). The
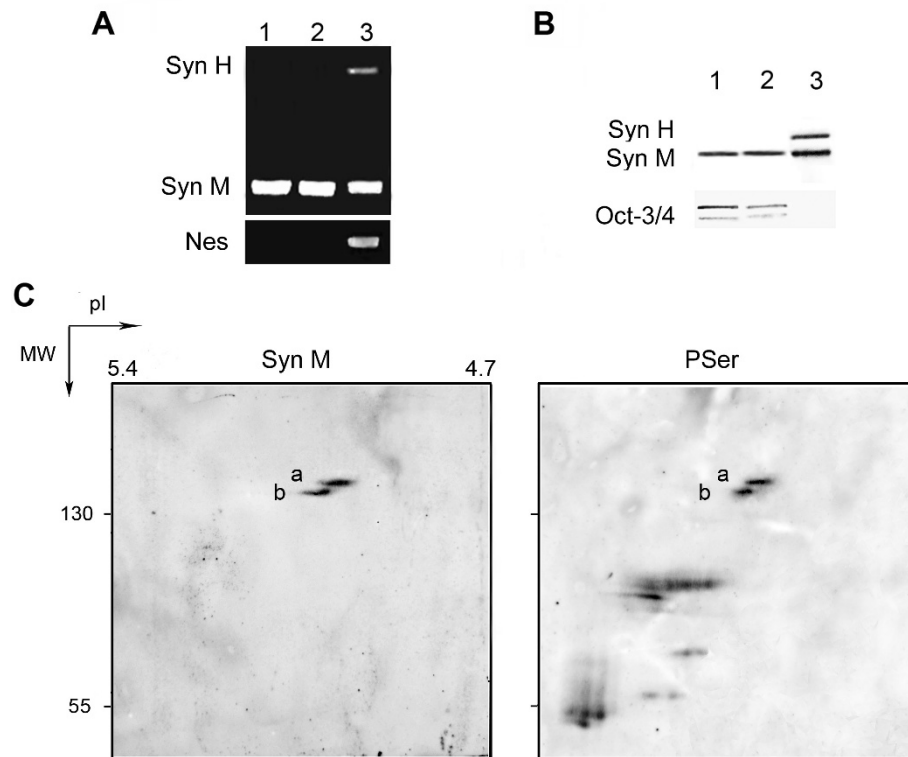
Figure 1 Characterization of synemin M in mouse ES cells. A, Analysis by RT-PCR of mRNA encoding synemins H and M isoforms and nestin in USP-1 (lane 1), CGR8 (lane 2) and mouse embryo at E9.5 (lane 3). Synemin M mRNA was found in pluripotent ES cells, while synemin H and nestin mRNAs were absent (lanes 1 and 2). Both synemin isoforms were detected in the 9.5-day-old embryo (lane 3). B, Characterization of synemins H, M and Oct-3/4 proteins by Western blot. USP-1 (lane 1), CGR8 (lane 2) ES cells and the adult mouse bladder (lane 3) were used for this study. Only the synemin M and Oct-3/4 were found in pluripotent ES cells (lanes 1 and 2). The synemin H was absent from undifferentiated ES cells (lanes 1 and 2). The adult mouse bladder was used for a control (lane 3). C, Analysis of synemin in mouse ES cells by 2D-PAGE and immunoblotting. Total protein extract of ES cells was separated by using pH 4-6 gradients IPG strips and 8% SDS/PAGE gels. Two spot trains of synemin M with isoelectric points (pI) 5.1-5.2 and apparent 148-150 kDa molecular weights (MW) were characterized. Analysis of the phosphorylation of synemin M revealed that two spot trains of synemin M contained phosphoserines (PSer) with isoelectric variants. Syn: synemin.

immunocytochemistry study clearly showed the presence of the synemin M protein in pluripotent ES cells expressing Oct-3/4 (Figures 2C-E) and K8 (Figures 2F-H). However, synemin H was never detected in undifferentiated ES cells (Figure 2J) expressing the pluripotent ES marker such as SSEA-1 (Figures 2I-K). It is remarkable that neither synemin nor keratin was organized in filamentous IF networks in pluripotent ES cells due to the absence of their partners.

Change of synemin expression profile during neural differentiation of ES cells

Since synemin isoform expression during the mouse embryo development is complex [9], we decided to study the possible change of synemin isoform expression profile during ES cell neural differentiation. To achieve this, cultured embryoid bodies (EBs) were treated with retinoic acid (RA), an inducer of neural differentiation

[26-28]. The characteristics of differentiated ES cells were then analyzed by RT-PCR, Western blotting and immunochemistry. The expression of synemin H mRNA as well as synemin L mRNA was detected in differentiated ES cells along with synemin M, nestin, a specific marker of neural precursor cells [29,30], and GFAP, a specific marker of the glial cell lineage [31-34] (Figure 3A). However, the accumulation of synemin H mRNA was less abundant than synemin M (the ratio of two isoforms is about 5). The appearance of these neural markers (nestin, GFAP, and β-III tubulin) in RA treated EBs was also confirmed by Western blotting (Figure 3B). As shown in Figure 3B, the induction of synemin H and synemin L was observed in differentiated ES cells after RA treatment as well as an increase of synemin M.

When RA was added to EBs cells, the distribution of synemin H and M was limited to the inner cells of the EB and to a subpopulation of cells that migrated from
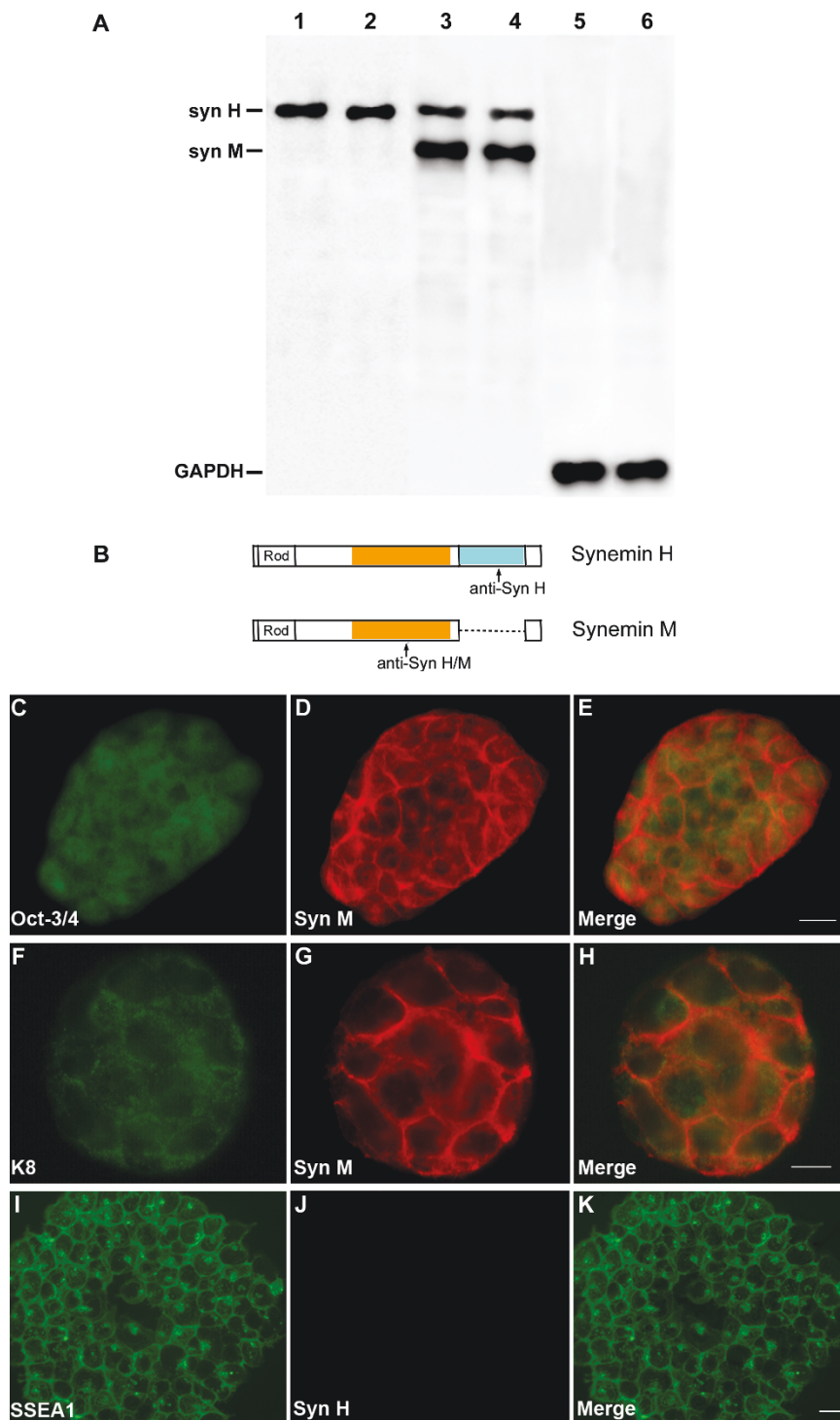
Figure 2 Immunofluorescence detection of synemin in mouse ES cells. A, Western blotting showing the specificity of synemin antibodies with proteins extracted from mouse bladder. The antibody anti-synemin H detected one band of 180 kDa, corresponding to synemin H isoform (lanes 1-2), and the anti-synemin H/M detected two bands 150 kDa and 180 kDa that correspond to H and M isoforms (lanes 3-4). GAPDH was used for the loading control (lanes 5-6). B, Diagram illustrating the regions recognized by antibodies anti-synemin H and anti-synemins H/M. C-K, Immunochemistry analysis demonstrating ES cells colonies labeled for Oct-3/4 (C), keratin 8 (F), SSEA-1 (I), synemin M (D, G) and synemin H (J). Synemin M was present with Oct-3/4 (C-E) and keratin 8 (F-H) in pluripotent ES cells. No immunolabelling for synemin H was observed in undifferentiated ES cells expressing SSEA-1 (I-K). Syn: synemin; K8: keratin 8. Bar = 20 μm.
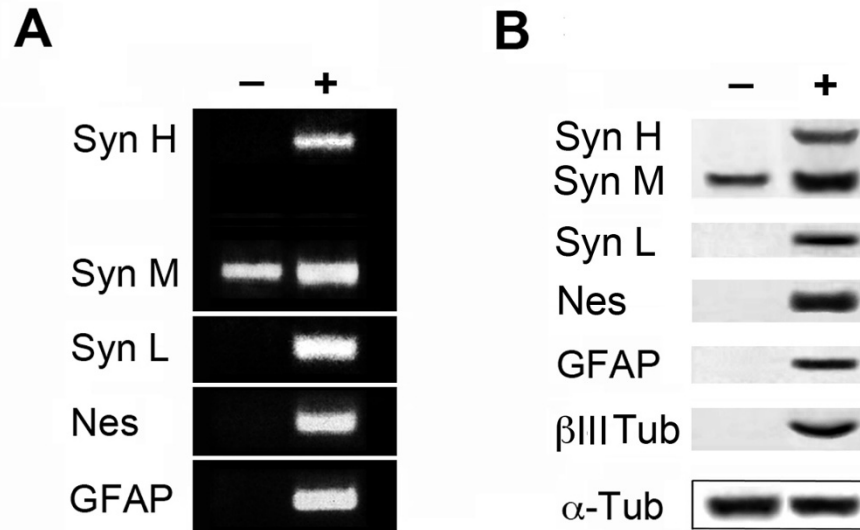
Figure 3 Analysis of synemin isoforms after neural differentiation of mouse ES cells. A, RT-PCR analysis of mRNA encoding synemins H, M and L isoforms, nestin, and GFAP in retinoic acid (RA) treated (+) and control (-) EBs. Synemin M mRNA was found in both control and differentiated ES cells, while synemin H, synemin L, nestin and GFAP mRNA were found only in differentiated ES cells (+) and absent in control ES cells (-). B, Western blotting analysis of synemins (H, M and L), nestin, GFAP and β-III tubulin proteins extracted from RA treated (+) and control (-) EBs. Synemins H and L, nestin, GFAP, and β-III tubulin proteins were detected in retinoic acid treated (+) EBs and absent in control (-) EBs. An increase of synemin M protein was observed in EBs after retinoic acid treated (+). α-tubulin was used for the loading control. Syn: synemin; Nes: nestin; βIII Tub: β-III tubulin; α-Tub: α-tubulin.

it, presumably radial glia cells. Differentiated ES cells presented an evident heterogeneity. We observed that almost all of the neural precursors (90%) expressing synemin H and synemin M are nestin positive (Figures 4A-C, G-I). The expression of synemin H in differentiated ES cells was always associated with synemin M. During glial cell differentiation, synemin H and M were expressed in the immature astrocytes cells harboring low level of GFAP (Figures 4D-F and data not shown). In contrast, synemin H/M expression became extinct when astrocytes express high level of GFAP (Figures 4J-L).

The neuronal differentiation in EBs was also observed after RA treatment. These neuronal cells positive to β-III tubulin did not contain the synemin H/M (Figures 4M-O). However, the antibody directed against synemin L reacted with the neurons expressing β-III tubulin (Figures 4P-R).

Our *in vivo* analysis in the adult mouse brain also confirmed the expression of synemins H and M in multipotent neural stem cells. These synemin isoforms were detected only in multipotent neural stem cells, particularly in the subventricular zone of the lateral ventricle (Figure 5A), a neurogenic germinal site in adult mice [33,35]. However, GFAP was present in these neural stem cells as well as in mature cortical astrocytes (Figure 5B).

Synemin knockdown in mouse ES Cells by shRNA lentiviral particles

To test whether synemin expression is functionally required in ES cells, we carried out a synemin knockdown experiment in mouse ES cells by shRNA lentiviral particles transduction. The transduced mouse ES cell clumps were selected via Puromycin dihydrochloride selection, and then analyzed by qRT-PCR, Western blot and immunocytochemistry. This study showed that knocking down synemin (by 61.5%) decreased the expression of keratin 8 by 42.6% at mRNA level (Figure 6A). A similar decrease was also detected by Western blotting (Figure 6B) and immunocytochemistry analyses (Figures 6I-N). Blast program (NCBI) analysis indicates that three synemin siRNA sequences used in this experiment do not share any homology with other genes, so K8 decrease in this study is unlikely due to the direct inhibition of synemin siRNA. The relationship between synemin and K8 in ES cells remains to be studied. Synemin knockdown in mouse ES cells did not influence the expression of Oct4, Nanog and SOX2 (Figures 6A-B). This result was also confirmed by immunocytochemistry study (Figures 6C-H). In order to determine whether synemin knockdown can affect the growth ability of mouse ES cells, MTT assay was used to test the changes of cell growth ability. As seen in Figure 7, there is no
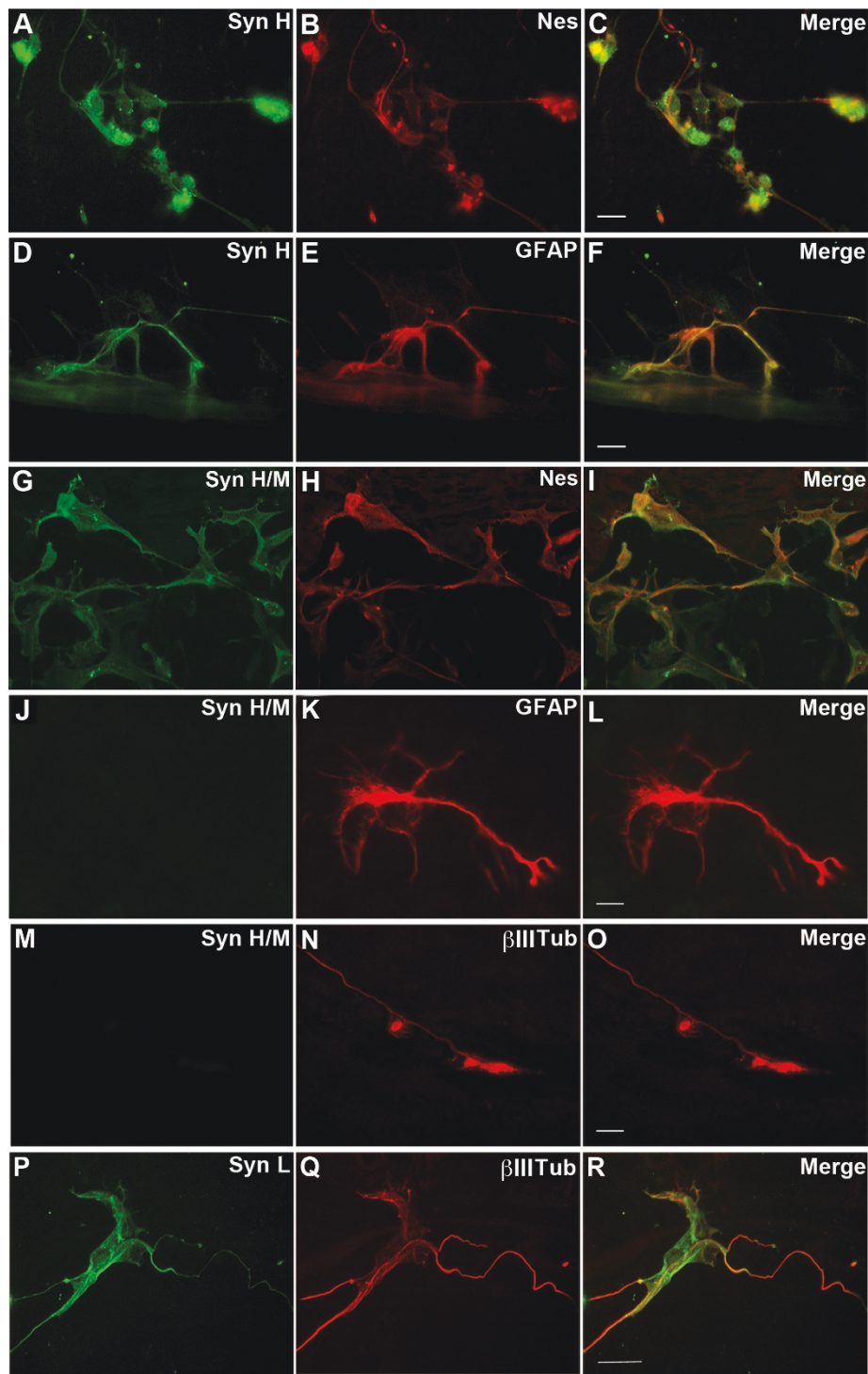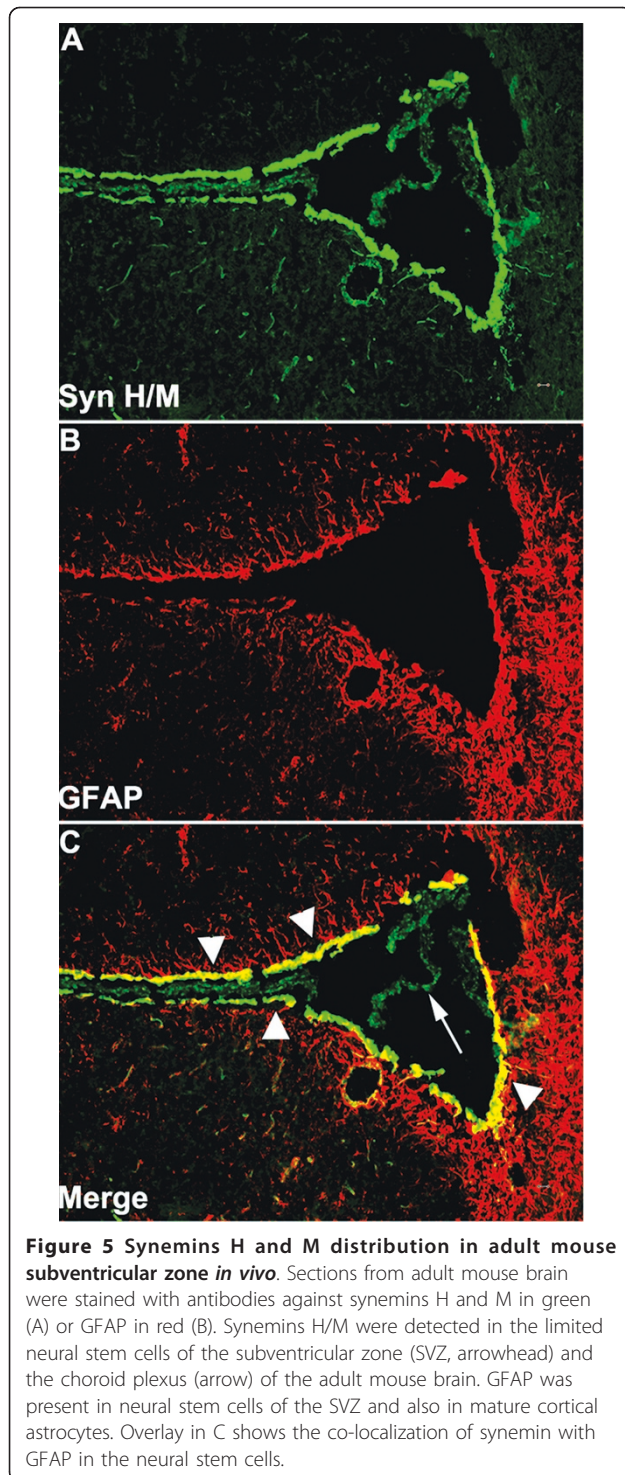
Figure 4 Synemins H and M distribution during neural differentiation of mouse ES cells. Retinoic acid-treated EBs were immunolabelled for synemin H (A, D), synemin H/M (G, J, M), synemin L (P), nestin (B, H), GFAP (E, K) and β-III tubulin (N, Q). The heterogeneity of differentiated ES cells was observed. Synemin H and M were co-expressed with nestin in a subpopulation of neural precursors (A-C, G-I), also with GFAP in a subpopulation of glia-astrocyte precursors harboring low level of GFAP (D-F). In contrast, synemin expression became extinct when astrocytes expressing high level of GFAP (J-L). During neuronal differentiation, no synemin H or M was detected in the neuronal cell expressing β-III tubulin (M-O). However, synemin L was co-expressed with β-III tubulin in neurons (P-R). Syn: synemin; Nes: nestin; βIII Tub: β-III tubulin. Bar = 20 μm.
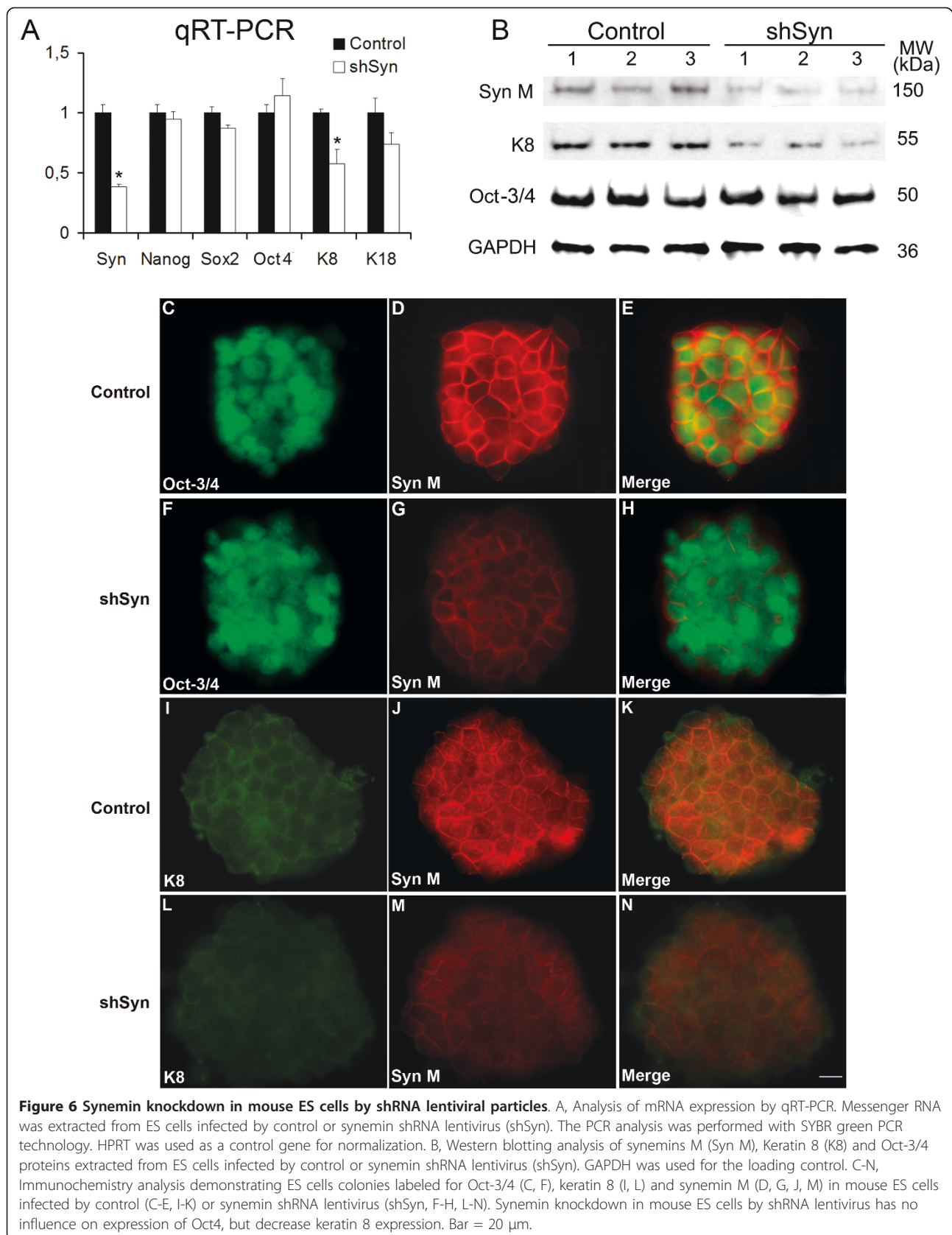
Figure 5 Synemins H and M distribution in adult mouse subventricular zone *in vivo*. Sections from adult mouse brain were stained with antibodies against synemins H and M in green (A) or GFAP in red (B). Synemins H/M were detected in the limited neural stem cells of the subventricular zone (SVZ, arrowhead) and the choroid plexus (arrow) of the adult mouse brain. GFAP was present in neural stem cells of the SVZ and also in mature cortical astrocytes. Overlay in C shows the co-localization of synemin with GFAP in the neural stem cells.

significant modification of cell proliferation in the shSyn group when compared to the control group.

Discussion

The exact mechanisms underlying the developmental changes in stem cell and precursor population are not understood. The adult vertebrate CNS consists of four major differentiated cell types: neurons, astrocytes, oligodendrocytes and ependymal cells. How these differentiated cell types are generated during early embryogenesis remains mostly unknown due to the complexity of cell structure in this system, the lack of specific cell surface markers to identify distinct cell types and the presence of numerous amplifying cell population in transit that rapidly generate early progenitors. We demonstrated here that synemin expression is characteristic of mouse ES cell lines in the undifferentiated state and the neural stem cells at both mRNA and protein levels. Our experiment showed that mouse pluripotent ES cells expressed synemin M, prior to nestin, which is considered as a marker of neural stem cells [20,29,30]. Moreover, this expression was associated with a common transcriptional signature that is shared by stem cells. This signature includes the expression of stem cell markers, such as homeobox protein Nanog, a transcription factor that functions in concert with other factors such as Oct4 and SOX2 to establish the self-renewal of undifferentiated ES cells [36-38], Oct4, a transcription factor restricted to totipotent and pluripotent cells [39-41], and SSEA-1, an ES cell surface biomarker [42]. It has been reported that there is a strong correlation between Oct4 gene expression and synemin regulation. Knocking down Oct4 by RNAi resulted in a dramatic decrease of synemin M [40]. Synemin knockdown in mouse ES cells by shRNA lentiviral particles did not modify the expression of Oct4, Nanog and Sox2 (Figures 6A-B) showing that synemin can not influence these transcriptional factors. As Oct4 is required to maintain the pluripotentiality and self-renewal of ES cells and since synemin M expression is under its regulation, this suggests that synemin M could be implicated in ES cell maintenance. Synemin knockdown did not influence the ES cell growth. The exact nature of how synemin is implicated in the maintenance of mouse ES cells remains to be studied.

Cytoskeletal proteins play important regulatory roles in a variety of cellular processes including proliferation, migration and differentiation. Among the three types of cytoskeletal elements, two are clearly present in mouse oocytes: microtubules and microfilaments, the presence of IFs being more controversial. The organization and role of the cytoskeletal networks (mainly microtubules and microfilaments) during oogenesis, fertilization and pre-implantation development of the mouse are described given the importance of cell-cell interactions and of the subcellular organization in events leading to the formation of the first two lineages of the mouse embryo [43]. In recent years, IFs have attracted much interest, largely because their constitutive polypeptide units are specifically expressed in various cell types and
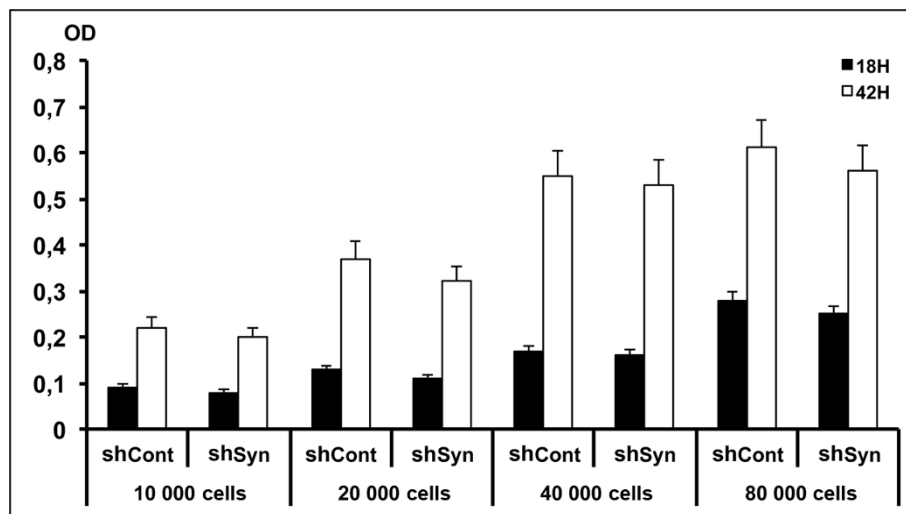
Figure 6 Synemin knockdown in mouse ES cells by shRNA lentiviral particles. A, Analysis of mRNA expression by qRT-PCR. Messenger RNA was extracted from ES cells infected by control or synemin shRNA lentivirus (shSyn). The PCR analysis was performed with SYBR green PCR technology. HPRT was used as a control gene for normalization. B, Western blotting analysis of synemins M (Syn M), Keratin 8 (K8) and Oct-3/4 proteins extracted from ES cells infected by control or synemin shRNA lentivirus (shSyn). GAPDH was used for the loading control. C-N, Immunochemistry analysis demonstrating ES cells colonies labeled for Oct-3/4 (C, F), keratin 8 (I, L) and synemin M (D, G, J, M) in mouse ES cells infected by control (C-E, I-K) or synemin shRNA lentivirus (shSyn, F-H, L-N). Synemin knockdown in mouse ES cells by shRNA lentivirus has no influence on expression of Oct4, but decrease keratin 8 expression. Bar = 20 μm.

Figure 7 MTT assay was used to determine the effect of synemin knockdown on mouse ES cell proliferation activity at 18 and 42 hours culture. No significant modification of cell proliferation in the shSyn group when compared to the control group. OD: optical density.

thus represent excellent differentiation markers [17,44]. However, the presence of IFs in ES cells is not clear, and there is no evidence supporting a role for IF proteins in ES cell. Cytokeratins seem to be the first IFs expressed in embryogenesis [18,19]. Our findings represent the first evidence that synemin M could be a novel characteristic of stem cells. Our functional study demonstrated that synemin knockdown in ES cells by shRNA lentiviral particle transduction also decreased keratin 8 expression. However, their correlation in ES cells is still not clear. In addition, our protein analysis showed that pluripotent ES cells contain two spot groups with isoelectric variants of synemin M (Figure 1C). These isoelectric variations of synemin M may reflect differences on its phosphorylation level. In fact, we have observed that two spot trains of synemin M were phosphorylated (Figure 1C). It is known that IFs are among the most abundant cellular phosphoproteins, their physiologic phosphorylation is typically heterogeneous and implicated in the structural organization of IFs [2,45]. Early studies indicated that synemin provides temporal and spatial targeting of protein kinase A (PKA) in adult and neonatal cardiac myocytes [14]. PKA targeted to IFs could phosphorylate its substrates and it was previously reported that the synemin avian ortholog is phosphorylated by PKA [46]. Moreover, synemin itself may be regulated through phosphorylation by the catalytic subunit of PKA [14]. It is also noteworthy that the cAMP/PKA pathway plays an important role in murine ES cell self-renewal pathways. Directly inhibiting PKA activity in the presence of LIF causes a decrease in Oct4 expression; however, in the absence of LIF, there is increased Oct4 expression [47]. The further

investigation may be particularly attractive to identify the mechanism by which the post-translational modification of synemin protein is taken place during stem cell differentiate.

During neural differentiation of ES cells evoked by RA-treatment, the expression of synemin H was induced in neural stem cells along with synemin M, nestin and GFAP expression, which characterizes synemin H as a potential marker of the neural stem cell differentiation. Our *in vivo* analysis also confirmed the expression of synemins H and M in multipotent neural stem cells in the subventricular zone of the adult mouse brain, a niche of the neural stem cells [33,35]. Furthermore, in agreement with our previous data, synemin L is expressed only in mature neurons [9,16]. This selectivity suggests that the commitment of neural precursors to form astroglia or neuron as early as stem cell differentiation involves the direct regulation of the single synemin gene. We therefore believe that the production of synemin proteins is closely associated with the early specialization of ES cells.

Conclusions

The work presented here demonstrates a complex regulation of synemin gene expression and a developmental stage specific production of each isoform in ES cells and during their neural differentiation. The expression of synemins is characteristic of mouse ES cell lines in the undifferentiated state and the neural stem cells. Synemin M was present in pluripotent ES cells, together with Oct-3/4 and SSEA-1, prior to nestin, a known marker of neural stem cells. Synemin H is up regulated during retinoic acid induced neural differentiation and allows a

distinction between an ES cell state and a neural committed stage. During neural differentiation there was also a distinct expression of synemin H, M and L. Synemin H and M are specific for the glial lineage during neural differentiation, whereas synemin L characterized only neuronal cells. The synemin knockdown in ES cells has no influence on expression of Oct4, Nanog and SOX2, but decreased keratin 8 expression. Taken together, the findings herein represent the first evidence that synemins might offer potentially novel and useful markers for distinguishing multipotent ES cells from undifferentiated neural stem cells and more committed progenitor cells.

Methods
Cell culture
Two mouse ES cell lines were used during our work: USP1 [48] and CGR8 [22]. USP1 ES cells were maintained in an undifferentiated state on mouse embryonic fibroblast (MEF) feeder layer, mitotic inactivated with mitomycin C (Sigma-Aldrich), in DMEM/F12 with 15% (v/v) knockout serum replacement, 2 mM L-glutamine, 1% (v/v) nonessential amino acids, 0.1 mM β-mercaptoethanol (Invitrogen), 40 μg/mL gentamicin sulfate (Scheing-Plough) and 1000 U/mL of leukemia inhibitory factor (LIF, Invitrogen). CGR8 murine ES cells were cultured on 0,2% (w/v) gelatin (Sigma) coated tissue culture plates in Glasgow's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum, 2 mM L-glutamine, 1% (v/v) nonessential amino acids, 0.1 mM β-mercaptoethanol, 1 mM sodium pyruvate, 50 U/mL penicillin, 50 mg/mL streptomycin (Invitrogen). 1000 U/mL of LIF were added prior to use. All cultures were incubated at 37°C in a humidified 5% CO_2/95% air.

Formation and treatment of embryoid bodies (EBs)
A standard protocol for EB formation was used. Briefly, USP1 ES cells and MEF cell monolayers were dissociated with TrypLE™ Express (Invitrogen) and were plated on gelatin-treated dishes for 30 min to remove the feeder cells. This procedure was repeated twice. CGR8 ES cells were used directly. For EBs formation, ES cells (4×10^5/mL) were passed to a 6-well plate covered with 2% (w/v) gelatin (Sigma) and cultured for 48 h (d0-d2) in ES medium as described above. Colonies were then treated with TrypLE™ Express for 5 minutes at 37°C, transferred to non-adherent plate dishes (2.5×10^5/mL) with EB medium (DMEM/F12 medium with 15% (v/v) fetal bovine serum (Invitrogen), 2 mM glutamine, 1% (v/v) nonessential amino acids, 0.1 mM β-mercaptoethanol, 50 U/mL penicillin, 50 mg/mL streptomycin without LIF). ES cells were plated as hanging drops on a lid of 10-cm non-adherent Petri dish. The lid was inverted over the bottom of the dish filled with culture medium.

Spherical cell aggregates (EBs) were formed during 4 days (d3-d6). The resulting EBs were transferred into Petri dishes (10-15 EBs per 6-cm dish) in 4 mL of the EB medium, and treated with all-trans-retinoic acid (RA, Sigma-Aldrich) during additional 4 days (from d7 to d10) to induce neural differentiation. At d7, EBs were divided in 2 groups: a) 0.02% (v/v) vehicle (DMSO) and b) 2 μM RA. The medium was changed every two days.

For studying the neuronal differentiation, EBs were plated onto 1 μg/mL laminin (Invitrogen) and 1 μg/mL fibronectin (Invitrogen) coated dishes at day 10 and cultured in DMEM/F12, with 1% (v/v) N2 supplement and 20 ng/mL of FGF-2 (Invitrogen) for additional 4 days.

Transduction of mouse ES cells by lentivirus
Mouse ES cells were cultured described above. The shRNA lentiviral particles transduction was performed according to the manufacturer's protocol (Santa Cruz). Synemin shRNA lentiviral particles (sc-60526-V, Santa Cruz) and control shRNA lentiviral particles (sc-108080, Santa Cruz) were used for this study. Synemin shRNA (m) Lentiviral Particles (sc-60526, Santa Cruz) is a pool of 3 different shRNA plasmids. Corresponding siRNA sequences are following:

A: Sense GCAAGACUAUGUUUGGAAAtt/Antisense UUUCCAAACAUAGUCUUGCtt

B: Sense GCAAGGCAUUGCCUAUGAAtt/Antisense UUCAUAGGCAAUGCCUUGCtt

C: Sense CAGUCACUCUGGAGUCAAAtt/Antisense UUUGACUCCAGAGUGACUGtt

The transduced mouse ES cell clumps were selected via Puromycin dihydrochloride selection (2 μg/ml), and then analyzed by qRT-PCR, Western blot and immunocytochemistry.

MTT assay
Different quantities (1×10^4, 2×10^4, 4×10^4 and 8×10^4 cells) of three selected synemin knockdown and control clones of mouse ES cells were cultured in 96-well flat-bottomed plates in a triplicate pattern. After eighteen or Forty-two hours, 20 μl 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg/ml) was added to each well and incubated for 3.5 h at 37°C. Then media were carefully removed, 200 μl of DMSO was added to each well and the plate was agitated for 10 min at 37°C. Finally, Absorbance of the converted dye of each well is measured at a wavelength of 570 nm.

RT-PCR analysis
Total RNA from ES cells and mouse embryos was isolated with Trizol Reagent (Invitrogen) and then treated with RNase-free DNase (Qiagen). Aliquots (1 mg) of total RNA were reverse-transcribed into cDNA with

first-strand DNA synthesis kit (Roche Diagnostic) following the manufacturer's instructions, and RT-PCR products were amplified as previously described [9]. For quantitative RT-PCR, the PCR analysis was performed with SYBR green PCR technology (Light Cycler 480 system, Roche Diagnostics). Primers were selected with the Primer3 program http://biotools.umassmed.edu. Relative quantification was achieved with the following equation: $R = 2^{\Delta Ct}$target$^{(control-sample)-\Delta Ct}ref^{(control-sample)}$, Ct = cycle threshold http://www.gene-quantification.de. HPRT was used as the reference transcript. Results from 3 independent RT-PCR analyses were expressed as the ratio between mutant and control samples.

Primers for RT-PCR:

Synemin H or M: SynF (5'-AGTCAGGGAGCGT TTCTGTGGACG-3') and SynR (5'-ATCGCTTCTCGT GTCGCTCAAATCC-3');

Synemin L: SynLF (5'-AGAGTGATTGACAGCCTG-GAGGAT-3') and SynLR (5'-ACTGTGTGCAATTCTC-CAGCCACC-3');

Nestin: NesF (5'-AGGCTTCTCTTGGCTTTCCT-3') and NesR (5'-TGGATCATCAGGGAAGTGGT-3');

GFAP: gfapF (5'-TGGATTTGGAGAGAAAGGTT GAAT-3') and gfapR (5'-CGATAGTGGTTAGCTTCGT GTTTG-3').

Primers for qRT-PCR:

Synemin: F (5'-GGTGGCCTCAGATGAGAAGA-3') and R (5'-GGCTTGCATGTCGGTATTTT-3');

Keratin 8: K8F (5'-TCATCCTATGGGGGACTCAC-3') and K8R (5'-TCTTCACAACCACAGCCTTG-3');

Keratin 18: K18F (5'-CTTGCTGGAGGATGGA GAAG-3') and K18R (5'-GCCTCAGTGCCTCAGAA CTC-3')

Oct4: Oct4F (5'-GGGGCTGTATCCTTTCCTCT-3') and Oct4R (5'-GCTGGTGCCTCAGTTTGAAT-3');

Nanog: NanogF (5'-GGACTTTCTGCAGCCTTACG-3') and NanogR (5'-TTTCACCTGGTGGAGTCACA-3');

SOX2: Sox2F (5'-TACCTCTTCCTCCCACTCCA-3') and Sox2R (5'-CTGGGCCATGTGCAGTCTAC-3').

Production of the antibody against synemin H
A cDNA fragment encoding the exon 4a (formerly named intron IV) of human synemin [4], a specific fragment for synemin H (amino acid residues 1151-1462) was cloned into a pQE32 plasmid (Qiagen). The antibody anti-synemin H was produced as described previously [4]. The resulting rabbit antiserum was characterized by Western blotting analysis. This antibody recognizes both human and mouse synemin H isoforms.

Immunocytochemistry
ES cells were fixed with cold methanol during 5 minutes, washed with PBS, and then with PBS-Triton X-100

(0.1% v/v). Nonspecific sites were blocked with PBS + 5% (w/v) bovine serum albumin (BSA) during 45 minutes and incubated with primary antibodies for 90 minutes at room temperature (RT). The primary antibodies were polyclonal anti-synemins H/M (1:400), anti-synemin H (1:400) and anti-synemin L (1:100) produced in our laboratory [3], mouse monoclonal antibodies anti-Oct-3/4 (1:100, Santa Cruz), anti-stage-specific embryonic antigen-1 (SSEA-1, 1:100, Chemicon), anti-glial fibrillary acidic protein (GFAP, 1:1000, Sigma), anti-nestin (1:200, Chemicon), anti-β-III tubulin (1:500, Sigma) or rat anti-keratin 8 (TROMA 1, 1:100) [48]. The secondary antibodies used were goat anti-mouse, anti-rat or anti-rabbit immunoglobulins, coupled to the fluorochromes Alex 488 (1:400, Invitrogen) or Cy3 (1:500, Jackson) for 40 minutes at RT. Controls were performed without the primary antibody.

One- and two-dimensional PAGE and Western blotting
Protein separation by 1D- or 2D-PAGE was carried out as described previously [49,50]. For 2D-PAGE, total protein extract of ES cells was separated by using pH 4-6 gradients IPG strips and 8% SDS/PAGE gels. The proteins were transferred onto nitrocellulose Hybond-C$^+$ or PVDF (GE Healthcare) membranes and nonspecific protein-binding sites were blocked with TBS-T (20 mM Tris-HCl, pH 7.5, 136.8 mM NaCl and 0.1% (v/v) Tween 20) containing 5% (w/v) non-fat milk. The blots were reacted with primary antibodies against synemins H/M (1:3000), synemin L (1:1000), nestin (1:1000), GFAP (1:5000), β-III tubulin (1:3000), Oct-3/4 (1:500), GAPDH (1:5000, Sigma) or α-tubulin (1:5000, Dako) in TBS-T containing 2% (w/v) non-fat milk overnight at 4°C followed by corresponding secondary antibodies anti-rabbit or mouse IgG HRP-conjugated (1:5000; GE Healthcare). The specific binding was revealed by an enhanced chemiluminescence (ECL) detection system (GE Healthcare). For synemin phosphorylation study, the anti-phosphoserine CDKs substrate antibody (1:1000, Cell Signaling) in TBS-T containing 5% (w/v) BSA were used. This antibody detects phosphosérine in a $(K/R)(S^*)PX(K/R)$ cyclin-dependent kinase (CDK) motif.

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de Souza Martins *et al. BMC Cell Biology* 2011, **12**:51
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