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miR-125 potentiates early neural specification of human embryonic stem cells

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SUMMARY

The role of microRNAs (miRNAs) as coordinators of stem cell fate has emerged over the last decade. We have used human embryonic stem cells to identify miRNAs involved in neural lineage commitment induced by the inhibition of TGF β -like molecule-mediated pathways. Among several candidate miRNAs expressed in the fetal brain, the two isoforms of miR-125 alone were detected in a time window compatible with a role in neural commitment *in vitro*. Functional analysis indicated that miR-125 isoforms were actively involved in the promotion of pluripotent cell conversion into SOX1-positive neural precursors. miR-125 promotes neural conversion by avoiding the persistence of non-differentiated stem cells and repressing alternative fate choices. This was associated with the regulation by miR-125 of SMAD4, a key regulator of pluripotent stem cell lineage commitment. Activation of miR-125 was directly responsive to the levels of TGF β -like molecules, placing miR-125 at the core of mechanisms that lead to the irreversible neural lineage commitment of pluripotent stem cells in response to external stimuli.

KEY WORDS: Human pluripotent stem cells, Neural commitment, MicroRNA

INTRODUCTION

The formation of early embryonic tissues is the result of a tightly controlled sequence of events that associates environmental signals and intracellular molecular mechanisms. The cellular environment, or niche, plays an instrumental role, in particular via secreted molecules that coax progenitors or stem cells toward a specific lineage. In the neural system, the earliest precursor cells are found in the neuroectoderm, a tissue that derives from the central part of the primitive ectoderm just after the appearance during gastrulation of mesodermal structures called organizers, which include the notochord (Harland, 2000). Noggin, an antagonist of the bone morphogenetic protein (BMP) receptors, was one of the first instructive secreted proteins identified as being crucial for the formation of neuroectoderm. Noggin is secreted by the cells of the notochord and, by blocking BMP-dependent pathways, induces the surrounding ectodermal cells to adopt a more specialized neural fate, which culminates in the formation of the neural plate (Lamb et al., 1993; Zimmerman et al., 1996).

It has recently emerged that inhibition of BMP-dependent pathways by their endogenous inhibitors noggin, follistatin or chordin, is not sufficient to completely induce neuralization in vertebrates. A wider blockage of the pathways that activate the transcription factors of the Smad family is required. Recently, Chambers and collaborators (Chambers et al., 2009) have shown that dual inhibition of both the activin/nodal and BMP pathways, the two canonical Smad-dependant pathways, is necessary to promote the early neural commitment of human embryonic stem cells (hESCs). However, the pathways initiated by these secreted molecules that, next to classical transcription factors, can further synchronize neural lineage entry remain to be identified.

Small non-coding RNAs of the microRNA (miRNA) family have been identified in recent years as key regulators of embryonic development (Chua et al., 2009; Fineberg et al., 2009) and represent particularly attractive candidates for regulating neural commitment. One clear role that has been identified for miRNAs is that of facilitating the coordinated phenotypic transitions that govern stem cell differentiation by blocking alternative cell fate choices or by participating in negative-feedback loops (Rybak et al., 2008). Although miRNAs appear to be plausible players in the earliest steps of nervous system formation, to date a requirement for any particular miRNA during the neural commitment of human pluripotent cells has not been reported. *In vivo* experiments employing miRNA ablation or overexpression as neural commitment proceeds in the embryo are particularly difficult to conduct and to interpret because they often lead to severe phenotypes and, in some cases, to early embryonic lethality. For evident ethical reasons, they are impossible to conduct in human embryos.

Thus, hESCs offer the unique possibility of modeling *in vitro* the first stages of neurulation (Chambers et al., 2009; Lowell et al., 2006) and assessing the involvement of miRNAs that have been described in rodent embryonic neural tissues and that constitute putative candidates for synchronizing human neural commitment. In the present study, we focused on a discrete set of miRNAs, namely miR-9, miR-124 and the two isoforms miR-125a and miR-125b (Cao et al., 2007; Sempere et al., 2004; Smirnova et al., 2005; Wulczyn et al., 2007), that were candidates for linking the extracellular signals required for neural commitment with the triggering of a genetic program of neuralization owing to their high level of expression in the neural tube of early rodent embryos.

MATERIALS AND METHODS

hESC culture

hESCs used in this study were from the H9 line (WA09, Wicell, Madison, WI, USA). hESCs were maintained on a layer of mitotically inactivated murine embryonic STO fibroblasts for a variable number of passages. Manual dissection, rather than enzymatic methods, was routinely used to passage the cells. The hESCs were cultured in DMEM/F12 glutamax

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supplemented with 20% knockout serum replacement, 1 mM non-essential amino acids, 1% penicillin/streptomycin, 0.55 mM 2-mercaptoethanol and 5 ng/ml recombinant human FGF2 (all from Invitrogen, Cergy Pontoise, France). Cultures were fed daily and passaged every 5–7 days. To study the effect of pre-miR complexes under pluripotency conditions, hESCs were plated on Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) in hESC medium preconditioned by feeders for 16 hours.

hESC neural differentiation and treatment with antagonists or inhibitors

Neural differentiation was performed as described (Lowell et al., 2006) with some modifications. hESC cultures were washed once in PBS then the hESC medium was changed for differentiation medium composed of N2B27 medium (Ying et al., 2003) supplemented with FGF2 (5 ng/ml). hESCs were manually detached from the feeder layer, collected in N2B27 and transferred to a low-attachment Petri dish in order to fully remove them from the influence of the feeders. Cells were finally seeded on tissue culture multi-well plates coated with poly-ornithine and laminin (Sigma, St Louis, MO, USA). The ROCK inhibitor Y27632 (Calbiochem, San Diego, CA, USA) was used at 10 μ M at the time of plating to optimize cell survival and seeding. The differentiation medium was changed after 24 hours, then every other day. Where indicated, human recombinant noggin (300 ng/ml; Peprotech, London, UK) and SB431542 (20 μ M; Tocris Biosciences, Ellisville, MO, USA) were added from day 0, then in every medium change. Activin A and BMP4 were both from R&D Systems and used at 10 ng/ml.

Immunostaining

Cells were fixed in 4% paraformaldehyde (PFA) and incubated for 10 minutes in blocking buffer (PBS containing 3% goat serum and 0.1% Triton X-100). Primary antibodies (supplementary material Table S2) were diluted in blocking buffer and applied overnight at 4°C. After three washes in PBS, secondary antibodies conjugated to Alexa fluorophores (Molecular Probes, Eugene, OR, USA) were diluted 1:1000 in blocking buffer and applied for 2 hours at room temperature. The cells were washed at least three times in PBS and visualized on an Axiovert inverted fluorescence microscope (Carl Zeiss, Le Pecq, France). Image acquisition was performed using AxioVision LE software (Carl Zeiss). For nuclear counterstaining, cells were incubated in 10 μ g/ml DAPI (Sigma) for 10 minutes after immunostaining.

Flow cytometry (FACS)

Cells were trypsinized for 5 minutes and 10% fetal bovine serum added to stop the reaction. The cells were collected and washed by resuspension in PBS and centrifugation at 1500 *g* for 10 minutes. Cells were fixed in 200 μ l freshly prepared 2% PFA for 10 minutes on ice, washed by centrifugation and permeabilized with 0.1% saponin. After centrifugation, cells were incubated for 1 hour at room temperature in 200 μ l primary antibody as described in supplementary material Table S2. Cells were washed and incubated for 30 minutes at room temperature with Alexa Fluor 488-coupled anti-isotype antibodies. FACS-based counting was performed on a FACScalibur analyzer using CellQuest software (BD Bioscience). Proper gating, compensations and exclusion of autofluorescent cells were performed using several control conditions, which included cells probed with Alexa Fluor 488-coupled secondary antibodies only and isotype-related non-fluorescent antibodies.

Quantitative RT-PCR (qPCR)

Total RNA was isolated using an RNeasy Mini extraction kit (Qiagen, Courtabouef, France) according to the manufacturer's protocol, and 500 ng used for reverse transcription using Superscript III (Invitrogen). PCR primers are listed in supplementary material Table S1. qPCR assays included 1 μ M each primer and 12.5 ng cDNA in SYBR Green PCR Master Mix (Roche Diagnostics, Basel, Switzerland). PCR reactions were performed on an ABI 7900 light cycler (Applied Biosystems, Foster City, CA, USA). Quantification of gene expression was based on the Δ Ct method and normalized to 18S rRNA (*RN18S1*) housekeeping gene levels. RNAs isolated from pooled human fetal brains, aged from 20 to 33 weeks, were obtained from Clontech (Clontech Laboratories,

Mountain view, CA, USA). Melting curve and electrophoresis analysis were performed to control PCR product specificities and exclude non-specific amplification.

miRNA extraction and TaqMan assay

miRNAs were extracted using the miRvana extraction kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions after a phenol-chloroform purification step. They were reverse transcribed and prepared for qPCR using the TaqMan miRNA reverse transcriptase kit and miRNA-specific stem-loop primers (Applied Biosystems). An individual TaqMan miRNA assay was performed on the ABI 7900 with a no-Amperase UNG Master Mix. Data were analyzed with SDS relative quantification software (Applied Biosystems) with an automatic Ct setting for assigning threshold and baseline for Ct determination. Results were normalized against *RNU48* (*SNORD48* – Human Gene Nomenclature Committee) small nucleolar RNA.

Transfection of hESCs with antago-miR or pre-miR-125 complexes

Neural induction was initiated as described above. After 24 hours, cells were transfected with 20 nM antago-miR or pre-miR-125 complexes using Lipofectamine RNAiMAX (Invitrogen). Cells were exposed to the transfection complexes for 16 hours before the first medium change. Western blot analyses of Smad protein levels were performed 48 hours after this medium change, and immunocytochemistry and qPCR analyses were performed at day 7. Non-targeting antago-miR or pre-miR complexes were used as controls. In addition, a non-targeting antago-miR linked to Cy3 was used to evaluate the efficiency of transfection. All antago-miR and pre-miR complexes were from Ambion.

Co-transfection of antago-miR complexes with siRNA was as described previously (Ma et al., 2010). Clusters of hESCs were co-transfected with antago-miR complexes and 15 μ M *SMAD4* siRNA or non-targeting siRNA (Ambion) using Lipofectamine RNAiMAX, then seeded on laminin-coated culture dishes with or without SB431542 and noggin.

Luciferase reporter assay

Plasmids containing the 3'UTR of human *SMAD4* or of human beta-actin (*ACTB*) downstream of the gene encoding the *Renilla* luciferase were obtained from Switchgear Genomics (Menlo Park, CA, USA). HEK cells were seeded in 96-well plates at a density of 25,000 cells per cm². Twenty-four hours post-plating, 80 ng plasmid was co-transfected in each well with 10 pmoles pre-miR complexes using DharmaFECT Duo reagent (Dharmacon, Lafayette, CO, USA) following the manufacturer's instructions and with 20 ng of a plasmid lacking any 3'UTR but expressing the firefly luciferase. Forty-eight hours post-transfection, the EnduRen kit from Promega was used to detect *Renilla* luciferase activity without lysing the cells. After this first reading on an Analyst GT luminometer (Molecular Devices, Sunnyvale, CA, USA), the cells were washed, lysed and the activity of the firefly luciferase was detected using Steady-Glo luciferase assay reagent (Promega). The signal obtained from the firefly luciferase activity of the plasmid lacking any 3'UTR was used to normalize the signals obtained with the 3'UTR-containing constructs in order to track equal transfection efficiency in wells. Each 3'UTR/miR combination was analyzed in quadruplicate.

Western blot analysis

Cells were trypsinized and collected by centrifugation (1000 *g*, 10 minutes). The pellets were incubated on ice in lysis buffer [150 mM NaCl, 50 mM Tris pH 8.0, 5 mM EDTA, 0.5% Triton X-100, 0.5% protease inhibitor cocktail (Sigma)]. Homogenates were centrifuged, supernatants were collected and stored at –80°C. Protein concentrations were determined with the BCA protein assay (Pierce, Rockford, IL, USA). Proteins (10 μ g) were resolved on a 10% NuPAGE SDS-PAGE gel (Invitrogen) and transferred onto polyvinylidene difluoride membranes. The membranes were incubated with anti-Smad antibodies (Cell Signaling Technology, Boston, MA, USA) at 1/1000 in 5% non-fat dry milk in TBS (100 mM NaCl, 10 mM Tris base pH 7.5) containing 0.1% Tween 20 (T-TBS) overnight at 4°C. After 1 hour incubation with anti-rabbit IgG HRP-coupled secondary antibodies, antigens were revealed using ECL reagents (Amersham Pharmacia Biotechnology, Les Ulis, France). Blots were

routinely stripped in a denaturing solution (0.5 M Tris-HCl pH 6.8, 10% SDS, 0.8% beta-mercaptoethanol), and reprobed with anti-beta-actin uncoupled to HRP (Sigma) in order to confirm equal loading.

Statistics

All experiments were carried out independently at least three times with triplicates included in each experiment. Error bars represent s.d. Statistical significance was estimated with an ANOVA test and *P*-values were calculated using Student's *t*-test.

RESULTS

Timecourse of hESC neural commitment following treatment by noggin and SB431542

The combined effects of noggin and SB431542, which are both inhibitors of TGF β -like molecules, as shown by Chambers et al. (Chambers et al., 2009) were confirmed under conditions in which the levels of TGF β -like molecules were optimally lowered in the culture medium (Lowell et al., 2006), using N2B27 medium in combination with laminin instead of knockout serum replacement-supplemented medium and Matrigel, and avoiding the first step of

culture in feeder cell-conditioned medium. Cultures treated with the two inhibitors homogeneously expressed the early neural marker PAX6 as well as SOX2. By contrast, when the inhibitors were omitted, expression of the ESC marker OCT4 (POU5F1 – Human Gene Nomenclature Committee) persisted and numerous non-neural cells (PAX6[−] SOX2[−]) appeared (Fig. 1A). FACS analyses of nestin and SOX1 were performed (Fig. 1B,C), in addition to OCT4 and SOX2 (supplementary material Fig. S1), to allow hESCs (OCT4⁺ SOX2⁺) to be differentiated from early neural precursor cells (OCT4[−] SOX2⁺) and other lineages (OCT4[−] SOX2[−]) (Lowell et al., 2006). This indicated that efficient neural induction (over 80%) was obtained over 8 days using noggin and SB431542, whereas differentiation in N2B27 alone remained suboptimal.

A timecourse analysis using qPCR showed that pluripotency markers decreased rapidly between days 2 and 5 of the treatment with noggin and SB431542 (Fig. 1D). Conversely, expression of the neural markers *PAX6* and *SOX1* was initiated between day 2 and day 5. Immunocytochemistry (Fig. 1E) and FACS analyses (Fig. 2D) confirmed this sequence of phenotypic changes at the cellular level. Altogether, these results confirmed an efficient and

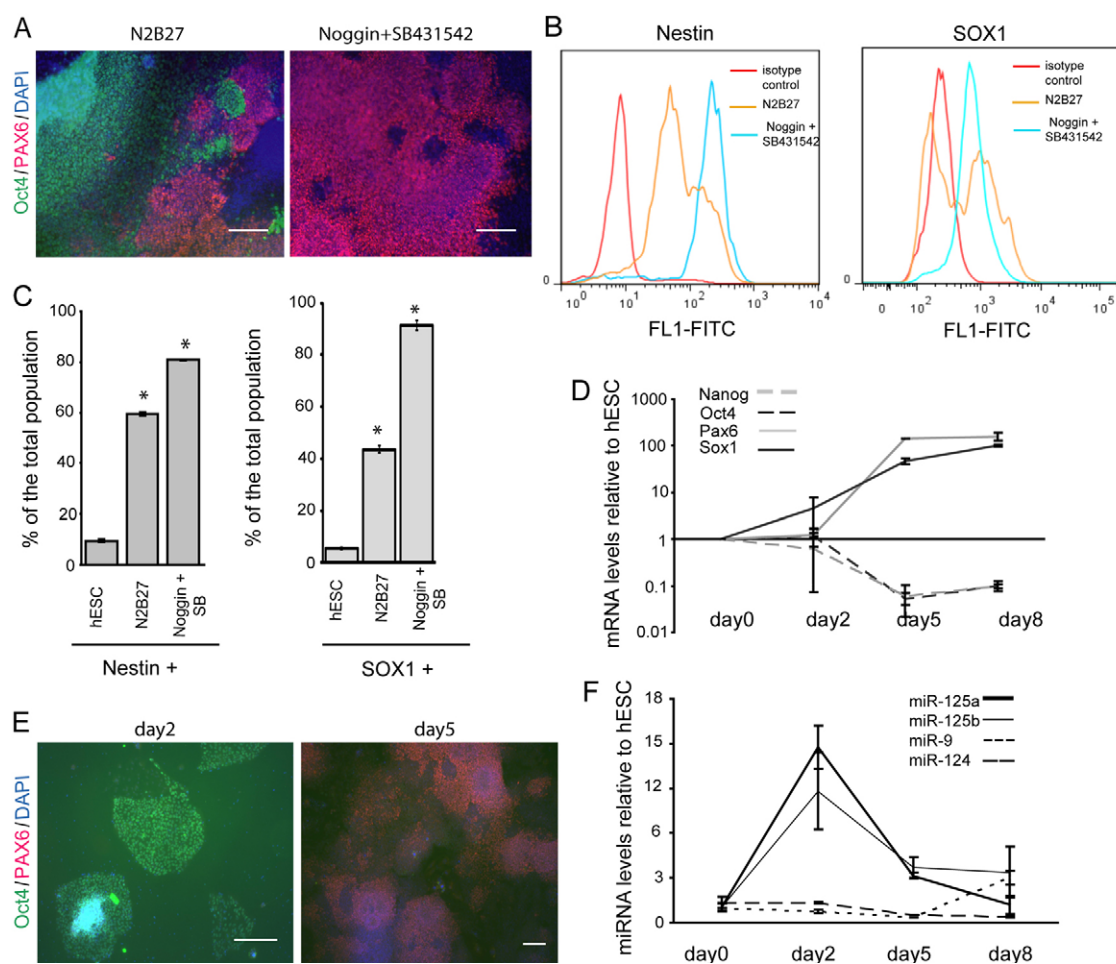


Fig. 1. Timecourse of the neural conversion of hESCs after treatment with noggin and SB431542. (A) Qualitative analysis of the effect of noggin and SB431542 by immunocytochemistry using OCT4 to label hESCs (green) combined with labeling for the pro-neural gene PAX6 (red). (B,C) The efficiency of neural conversion of hESCs into neuroepithelial precursors following 8 days of treatment with noggin and SB431542 was monitored by FACS. SOX1 and nestin were chosen as markers of neural lineage-committed cells. (B) Representative FACS profiles. (C) Quantitative analyses of the percentage of cells expressing each marker. **P* < 0.01, two-tailed Student's *t*-test. Error bars indicate s.d. (D) Expression of genes representative of the neural lineage (*PAX6* and *SOX1*) or of pluripotent and undifferentiated cells (*OCT4* and *NANOG*) was monitored over time by qPCR and indicated that the neural commitment decision occurred between day 2 and day 5 post-treatment. (E) This was confirmed by immunocytochemistry for PAX6 and OCT4. (F) The timecourse expression of selected miRNAs was analyzed using TaqMan assays following the treatment of hESCs with noggin and SB431542 and normalized to the expression measured in pluripotent hESCs. Scale bars: 100 μ m.

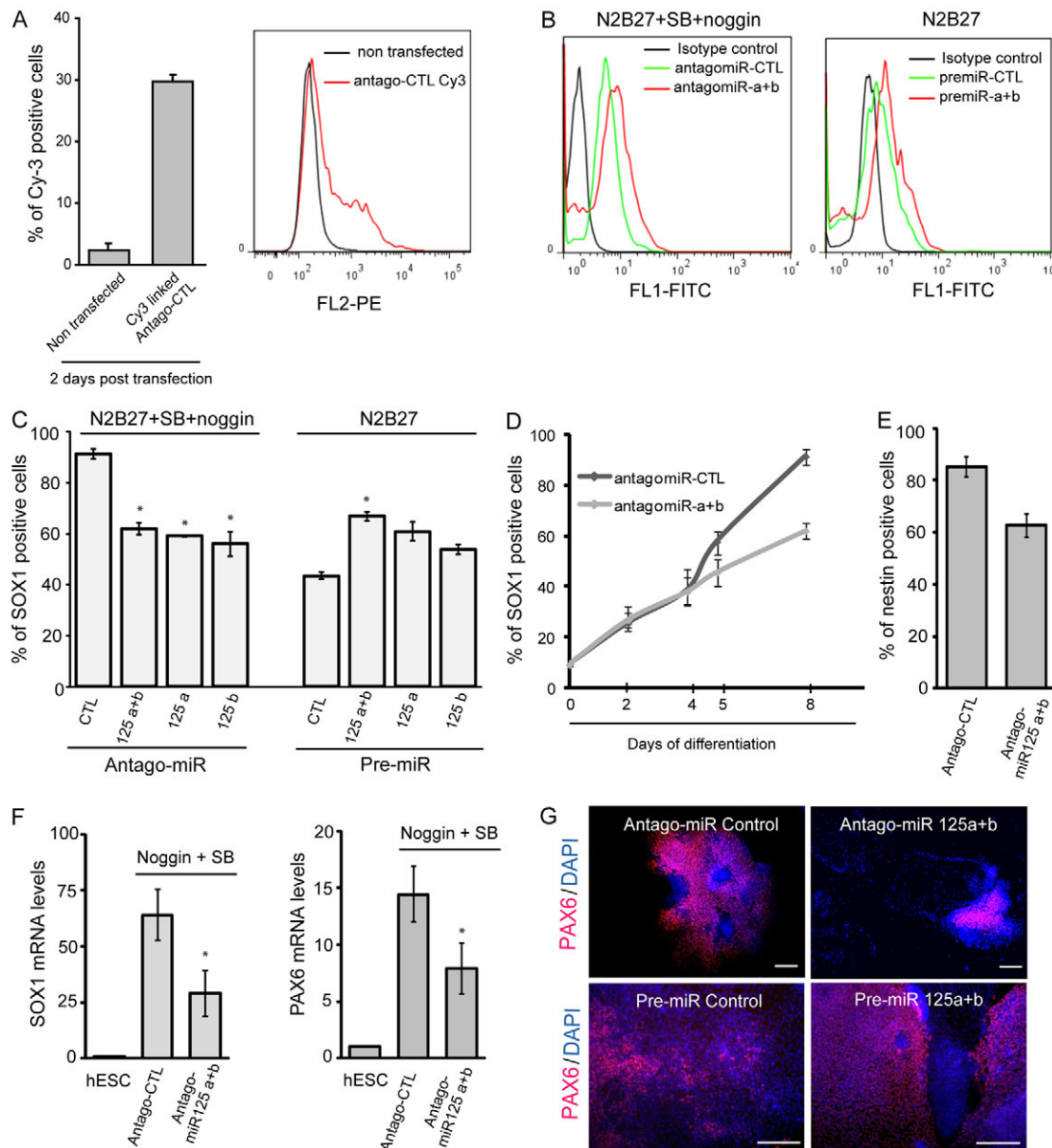


Fig. 2. miR-125 isoforms promote neural conversion of hESCs. The functional involvement of miR-125 in the promotion of the neural conversion of hESCs was investigated using antago-miR or pre-miR complexes. (A) The efficiency of transfection of hESCs with antago-miR complexes was quantified 2 days after transfection using a non-targeting antago-miR linked to Cy3. (B–D) Functional consequences of antago-miR and pre-miR transfections were analyzed by FACS using SOX1 as a marker of neural commitment. (B) Representative FACS profiles of the SOX1-labeled population in hESCs transfected with antago-miR or pre-miR control (CTL) or with antago-miR or pre-miR complexes targeting miR-125a and miR-125b (a+b). (C) Quantification of the functional effect of each miR-125 isoform manipulation after 7 days of differentiation. (D) FACS quantification of the functional effect of the inactivation of both miR-125 isoforms as neural commitment proceeds indicated that miR-125 expression was necessary to fully achieve neural commitment. (E) Quantification of the number of nestin-positive cells after antago-miR treatment. (F) SOX1 and PAX6 mRNA quantified by qPCR 7 days after antago-miR treatment. (G) PAX6 protein expression visualized by immunocytochemistry 7 days after antago-miR or pre-miR treatment. * $P < 0.05$, one-tailed Student's *t*-test compared with CTL miR complexes. Error bars indicate s.d. Scale bars: 100 μ m.

homogeneous conversion of hESCs into neural precursors upon treatment with the two inhibitors and pointed to the day 2–5 time window as the crucial period for cell fate decision.

Activation of miRNAs following noggin and SB431542 treatment

This induction paradigm, and particularly its precise schedule, was used to examine the selective involvement of miRNAs in early neural commitment. Three miRNAs, miR-9, miR-124 and miR-

125, were selected because of their high expression in the developing nervous system of the mouse (Sempere et al., 2004). Because no relevant information was available for human, high and selective expression of miR-9, miR-124 and the two miR-125 isoforms a and b was first confirmed in the human fetal brain (supplementary material Fig. S2). As hESC neural commitment proceeded, these miRNAs displayed very different timecourses of appearance (Fig. 1F). There was no expression of miR-9 or miR-124 until day 8, pointing to a potential role beyond the time

window of cell fate decision triggered by SB431542 and noggin. Only miR-125a and miR-125b were activated during the time window of hESC commitment to the neural lineage, with the strongest expression at day 2.

miR-125 isoforms promote neural conversion of hESCs

As these experiments pointed to miR-125a and miR-125b as potential candidates for a role in early neural commitment, their functional roles were independently analyzed using antago-miR and pre-miR complexes. Transfection of hESCs with a Cy3-labeled non-targeting control antago-miR indicated that the efficiency of transfection was ~30% after 2 days (Fig. 2A). The efficiency with which antago-miRs silence their respective targets was checked using a TaqMan-based miRNA detection system (supplementary material Fig. S3A). The efficiency with which pre-miRs induce an increase in their respective miRNA expression was also tracked using the same system (supplementary material Fig. S3B).

Antagonizing the action of either miR-125a or miR-125b independently, or both in combination, compromised to the same extent the efficiency of neural induction as quantified by SOX1-expressing cells 8 days after transfection (Fig. 2B,C), indicating that both isoforms were actively involved. Timecourse experiment showed that the reduction in the efficiency of neural differentiation induced by inactivation of both miR-125 isoforms was not detectable before day 5, with the strongest effect at day 8 (Fig. 2D) when the cells normally reach the full neural phenotype (Fig. 1A,B). This reduction of neural induction efficiency was confirmed using nestin as a neural marker (Fig. 2E). Accordingly, the mRNA levels of *SOX1* and *PAX6* were strongly reduced following treatment with antago-miR (Fig. 2F) and immunocytochemistry confirmed the presence in the cell cultures of areas that were negative for the neural marker PAX6 (Fig. 2G).

Mirror experiments analyzing neural conversion under conditions of forced expression of miR-125 isoforms in suboptimal conditions (i.e. without noggin and SB 431542 in the medium) provided exactly the opposite results. SOX1-positive cells were more numerous than in untreated controls when the expression of each isoform was induced independently, and maximal neural induction efficiency was obtained when the two pre-miR complexes were transfected together (Fig. 2B,C). Promotion of neural induction under those conditions was confirmed by PAX6 immunocytochemistry (Fig. 2G).

In order to determine whether overexpression of miR-125 was sufficient to initiate neural differentiation in cells maintained under pluripotency-promoting conditions, hESCs were transfected with both pre-miR-125 isoforms and grown on Matrigel in hESC medium preconditioned by feeder cells for at least 16 hours (Fig. 3). After 8 days of culture, most cells remained undifferentiated regardless of whether they had been transfected with pre-miR-125, indicating that the forced expression of miR-125 was not sufficient to bypass the pluripotency signals.

miR-125 activation is modulated by extracellular levels of activin and BMP4

The activation of each miR-125 isoform was measured after 2 days of treatment with either noggin or SB431542, or both in combination, in order to investigate their roles more specifically (Fig. 4A). Strikingly, the expression of miR-125a and miR-125b was only strongly induced when the two inhibitors were used together, indicating that inhibition of both the activin-dependent and BMP-dependent signaling pathways was required to efficiently

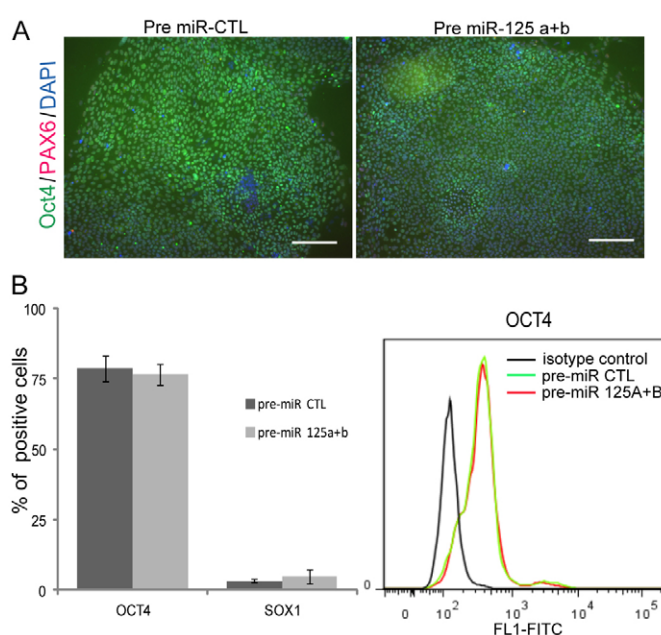


Fig. 3. Forced expression of miR-125 is not sufficient to bypass pluripotency signals. hESCs were transfected using non-targeting pre-miR complexes (pre-miR CTL) or pre-miR complexes targeting both isoforms of miR-125 (pre-miR a+b) and cultivated under culture conditions supporting self-renewal. The resulting phenotype was analyzed after 8 days of culture by (A) immunocytochemistry for OCT4 and PAX6 and (B) FACS using OCT4 and SOX1. Error bars indicate s.d. Scale bars: 100 μ m.

activate miR-125. This strongly suggested a physiological role for the TGF β -like molecules in the process. This was confirmed by measuring early modulations of the activation of miR-125 isoforms after 2 days of treatment with activin, BMP4, or both at days 0 and 1 after plating. Levels of miR-125 isoforms remained unchanged when either 20 ng/ml activin or 20 ng/ml BMP4 was added. By contrast, the two molecules strongly inhibited miR-125 activation when used in combination (Fig. 4B). This mirrored the effects of the two inhibitors noggin and SB431542 and pointed to miR-125 as a direct sensor of extracellular levels of TGF β -like factors. This prompted us to analyze the cell fates induced differentially by noggin or SB431542 treatment.

An 8-day treatment with SB431542 triggered the complete disappearance of OCT4⁺ pluripotent hESCs as monitored by FACS (supplementary material Fig. S4B). However, this was not accompanied by a general commitment to the neural lineage but instead by the appearance of a large number of OCT4⁻ SOX2⁻ non-neural cells. qPCR revealed high levels of the two neural crest markers *SOX10* and *SLUG* (*SNAIL2* – Human Gene Nomenclature Committee) and of the two early epidermal markers *CK18* and *P63* (*KRT18* and *TP63*, respectively – Human Gene Nomenclature Committee) (supplementary material Fig. S4B). The presence of neural crest and epidermal derivatives was confirmed by immunocytochemistry for PAX3 and CK18, respectively (supplementary material Fig. S4C). Therefore, SB431542 promoted differentiation to all ectodermal cell fates and not simply to those related to the neural lineage. Conversely, the same analyses performed after noggin treatment resulted in a large number of hESCs that were resistant to differentiation, with most differentiated cells exhibiting a neural phenotype, indicating that the BMP inhibitor was not sufficient by itself to

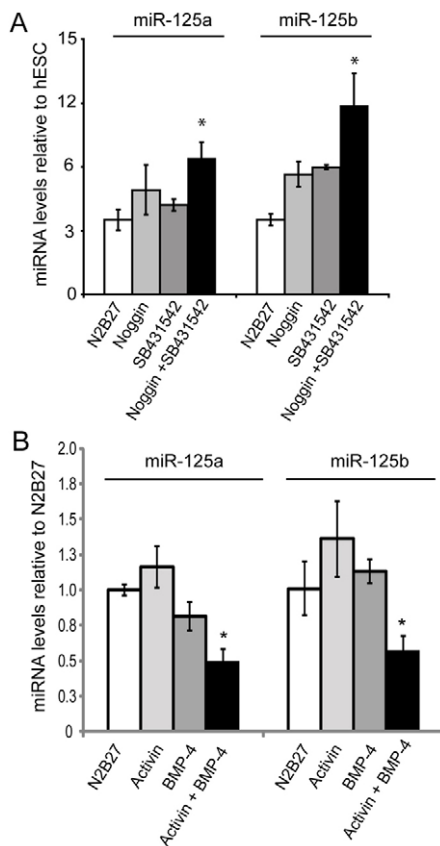


Fig. 4. The expression of miR-125 isoforms is regulated by extracellular levels of activin and BMPs. (A) The activation of miR-125 isoforms a and b was quantified using TaqMan assays following 2 days of treatment of hESCs with noggin, SB431542, or both. (B) The same experiments were performed after activin and BMP treatments. The results represent the mean \pm s.d. of three experiments. * $P < 0.01$, one-tailed Student's t -test.

induce mass differentiation, but rather blocked the emergence of non-neural ectodermal derivatives when used together with SB431542.

These results indicated that miR-125 was selectively activated in culture conditions that promote the neural fate.

The miR-125a and miR-125b isoforms promote hESC entry into the neural lineage by inhibiting alternative cell fate choices

In order to investigate how miR-125 isoforms promote neural lineage specification, neural induction was initiated with noggin and SB431542 and cells were transfected with 20 nM antago-miR-125 or pre-miR-125 complexes 24 hours later (Fig. 5A). qPCR analyses performed after 8 days of differentiation indicated that, when both isoforms were antagonized simultaneously, expression of all non-neural markers was significantly induced. This included the pluripotency markers *OCT4* and *NANOG*, the epithelial markers *CK8* (*KRT8* – Human Gene Nomenclature Committee) and *CK18*, and the neural crest markers *SOX10* and *P75* (*NGFR* – Human Gene Nomenclature Committee). These results were confirmed by immunocytochemistry, which revealed patches of *OCT4*⁺ and *CK18*⁺ cells in cultures transfected with the two antago-miRs (Fig. 5B). Altogether, these results indicated that the complementary action of the two miR-125 isoforms may promote

the exit of hESCs from pluripotency and then promote their neural lineage commitment by blocking the emergence of non-neural ectoderm derivatives.

SMAD4 is a direct target of miR-125

A target of miR-125 isoforms that could support this pro-neural role was then sought, focusing on factors commonly involved in both the BMP and TGF β signaling pathways. On the basis of the miRBase prediction algorithm (Griffiths-Jones et al., 2008), *SMAD4*, a common co-factor of the two Smad-dependent pathways activated both by TGF β and BMPs, was identified as a candidate (Fig. 6A). The functional interaction of miR-125 isoforms with the 3'UTR of *SMAD4* was evaluated using a luciferase reporter assay. HEK cells were co-transfected with a construct containing the entire 3'UTR of *SMAD4* cloned downstream of the *Renilla* luciferase gene and with pre-miR-125a, pre-miR-125b or a non-targeting pre-miR control. Transfection of the *SMAD4* 3'UTR construct with either pre-miR-125a or pre-miR-125b led to the same, significant reduction in luciferase activity, in contrast to the pre-miR control (Fig. 6B). This effect was not observed when the 3'UTR of *SMAD4* was replaced by the 3'UTR of two genes that did not contain the predicted miR-125 binding sequence: alpha-actin (*ACTA1*) and *CUGBP* (*CELF1* – Human Gene Nomenclature Committee).

To evaluate whether *SMAD4* mRNA and protein levels are directly affected by the activation of miR-125 isoforms, hESCs were transfected with either pre-miR-125a, pre-miR-125b or a non-targeting pre-miR control. Two days post-transfection, the level of *SMAD4* mRNA was analyzed by qPCR (Fig. 6C) and *SMAD4* protein by western blotting (Fig. 6D,E). Activation of each isoform alone led to a significant decrease in *SMAD4* protein levels, whereas mRNAs remained unaffected, consistent with the post-transcriptional regulation of a miRNA target. The specific targeting of *SMAD4* was further suggested by the fact that the levels of *SMAD2* and *SMAD5* were unaffected (Fig. 6E). A reverse experiment was performed using antago-miRs (Fig. 6F). Inactivation of each miR-125 isoform alone restored *SMAD4* protein levels.

Taken together, these experiments support the hypothesis that *SMAD4* is a direct target of miR-125 and that miR-125 activation in hESCs elicits a depletion of *SMAD4* protein.

The relationship between decreased expression of *SMAD4* and the progression of neural induction was then investigated. *SMAD4* protein levels were shown by western blot analysis to decrease quickly and strongly as neural commitment induced by noggin and SB431542 proceeded (Fig. 7A). *SMAD4* was then knocked down using siRNAs in hESCs. Treated cells were then placed in suboptimal conditions of neural differentiation, i.e. in N2B27 without noggin and SB431542, and their ability to exit the pluripotent compartment and to enter the neural lineage was evaluated 5 days post-transfection. In keeping with the working hypothesis, *SMAD4* knockdown cells expressed lower levels of pluripotency markers *OCT4* and *NANOG* and higher levels of the early neural markers *PAX6* and *SOX1* than untreated controls (Fig. 7B). FACS experiments using *SOX1* protein as a neural marker confirmed these results (Fig. 7C). Finally, neural induction was induced in *SMAD4*-inactivated hESCs using SB431542 plus noggin, and the effects of antago-miR-125 isoforms tested by FACS using *SOX1* and nestin proteins as neural markers (Fig. 7D). In agreement with the working hypothesis that miR-125 induces neural conversion by affecting the expression of *SMAD4*, knockdown of miR-125 did not affect

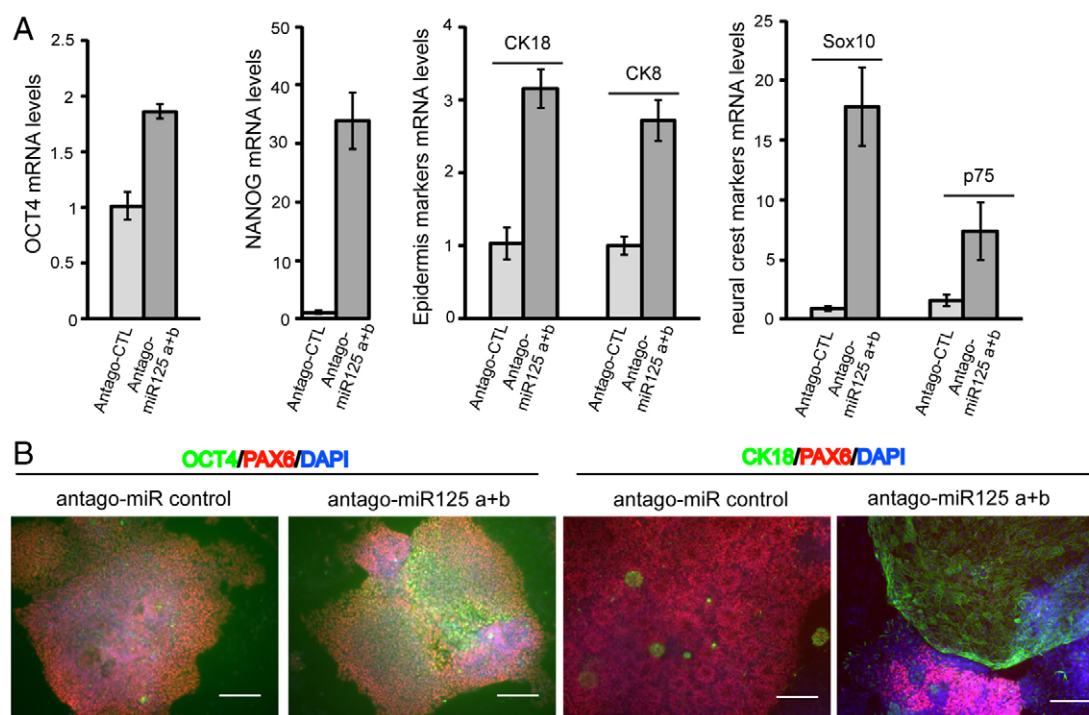


Fig. 5. miR-125 isoforms promote hESC entry into the neural lineage by the persistence of undifferentiated and inhibiting alternative cell fate choices. The effect of blockage of miR-125 isoform function was evaluated by transfecting hESCs with antago-miRs 24 hours after initiating the neural induction process with noggin and SB431542. **(A)** Levels of expression of the pluripotency markers *OCT4* and *NANOG*, the epidermal markers *CK8* and *CK18*, and the neural crest cell markers *SOX10* and *P75* were monitored by qPCR and normalized to the levels measured in cells treated with a non-targeting antago-miR (antago-CTL). The results represent the mean \pm s.d. of three experiments. **(B)** Immunocytochemistry for PAX6 (red) was performed in combination with *OCT4* or *CK18* (both in green) in cells treated with antago-miR-125a and antago-miR-125b or a non-targeting antago-miR control in the presence of noggin and SB431542. Scale bars: 100 μ m.

the neural conversion observed in SMAD4 knockdown hESCs, whereas it did decrease it significantly in SMAD4-expressing control cells.

DISCUSSION

The main result of this study is the identification of miR-125 as a key player in the molecular cascade that contributes to the irreversible commitment of pluripotent human stem cells to the neural lineage. In response to a complete inhibition of both the activin- and BMP-dependent pathways, the activity of the two miR-125 isoforms is released, promoting and securing the commitment of hESCs toward the neural lineage, at least in part by blocking the expression of SMAD4, a central regulator of ESC fate.

The role of miRNAs in the neurogenesis of early mammals has been assessed *in vivo* using mouse models in which the *Dicer1* gene has been knocked down in specific cell types, resulting in a global loss of miRNAs. These experiments have highlighted the role of miRNAs in the maintenance of a pool of neural progenitors and in the survival and homeostasis of differentiated neurons (Davis et al., 2008; Schaefer et al., 2007). However, it was difficult to investigate *in vivo* whether miRNAs were involved at an earlier developmental stage, such as in neural commitment, because inactivation of the *Dicer1* gene at an early time point leads to dramatic and lethal phenotypes.

We took the opportunity to homogeneously convert pluripotent hESCs into neuroepithelial cells using noggin and SB431542 treatment (Chambers et al., 2009) in order to identify the miRNAs

involved in the earliest stages of human neural differentiation. Timecourse analysis of representative lineage markers allowed us to define a time window of commitment between day 2 and day 5. It was assumed that the miRNAs involved in the entry of pluripotent cells into the neural lineage would be activated before or during this time window, whereas any miRNA activated later would serve to stabilize the neural phenotype or to induce terminal differentiation in neurons. Particular focus was placed on miR-9, miR-124 and miR-125 isoforms a and b because they are all expressed throughout the neural tube of the mouse, suggesting that they play a role during the early steps of nervous system formation (Smirnova et al., 2005). In fact, miR-9 and miR-124 were activated only after the time window of cell fate decision. This is in line with the well-documented roles of these two miRNAs in the homeostasis of neural stem cells and in control of the balance between self-renewal and neuronal differentiation (Delaloy et al., 2010; Packer et al., 2008), as well as in neuronal maintenance and plasticity (Makeyev et al., 2007; Visvanathan et al., 2007; Yu et al., 2008). Less has been known, to date, about the role of miR-125 during early neurogenesis, apart from its expression in the nervous system of the mouse from embryonic day 12 (Smirnova et al., 2005). We observed that the activation timecourse of the two human isoforms of miR-125 was compatible with a role in the very first stage of neural commitment of pluripotent stem cells. Functional inactivation of miR-125a and miR-125b in hESCs using specific antagonists indicated that miR-125 activation is instrumental in promoting the homogeneous and efficient entry of these cells into the neural lineage. These results were strengthened

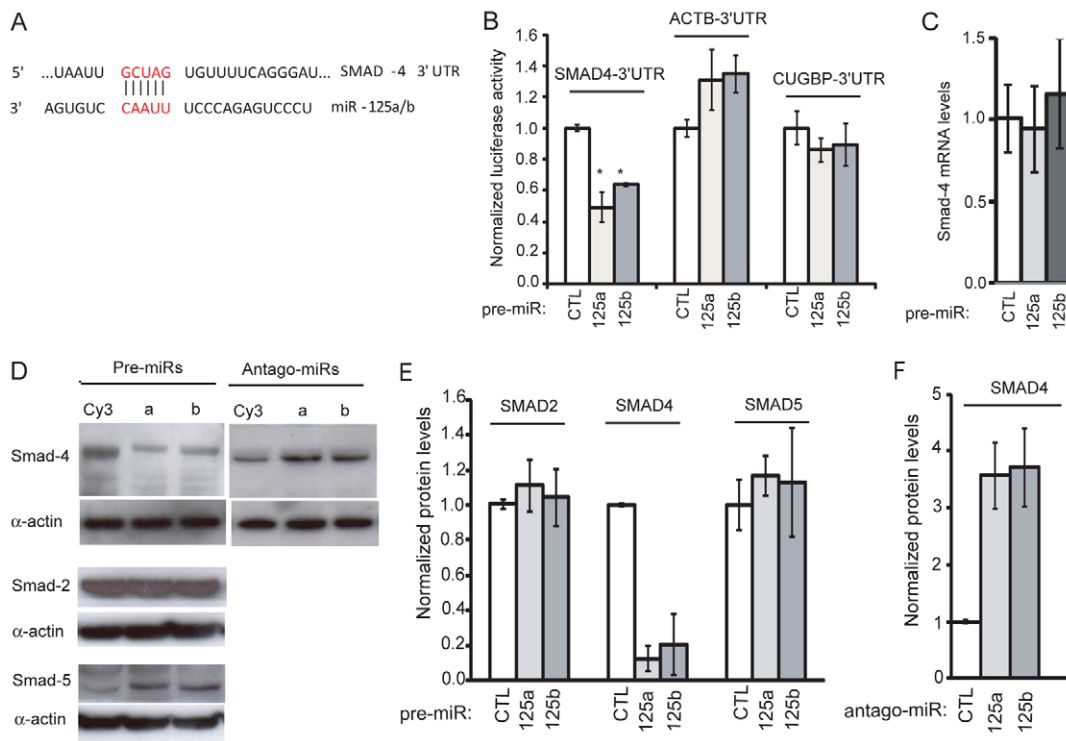


Fig. 6. miR-125 isoforms interact directly with *SMAD4* mRNA and control *SMAD4* protein expression. (A) The predicted consensus sequences (red) for miR-125a and miR-125b binding located on the 3'UTR of human *SMAD4* mRNA. (B) Pre-miR-125 complexes were co-transfected into HEK cells with luciferase reporter constructs containing the 3'UTR sequence of *SMAD4* or of two genes predicted not to be targeted by miR-125: beta-actin (*ACTB*) and the splicing factor *CUGBP*. A non-targeting pre-miR (pre-miR CTL) was used as a control. The luciferase activity was quantified 48 hours after transfection. (C) *SMAD4* mRNA levels were quantified by qPCR 48 hours after treatment of hESCs with a non-targeting pre-miR (CTL), pre-miR-125a or pre-miR-125b. (D–F) Pre-miR-125 or antago-miR-125 was transfected into hESCs and the protein levels of Smad factors quantified by western blot analysis 48 hours later. Non-targeting antago-miR or pre-miR complexes were used as controls. (D) Representative immunoblots. Quantification of protein content after pre-miR (E) or antago-miR (F) complexes was normalized to actin content. The results represent the mean \pm s.d. of three experiments. * $P < 0.01$, one-tailed Student's *t*-test.

by the fact that forced expression of these isoforms using pre-miR complexes increased the efficiency of neural conversion when the hESCs were placed in a suboptimal system lacking noggin and SB431542.

The promotion of neural conversion by miR-125 involved both the facilitation of differentiation and the repression of alternative fate choices, including non-neural ectodermal lineages. miR-125 has previously been reported, along with let-7, as being involved in mechanisms governing the repression of self-renewal in embryonal carcinoma cells (Rybak et al., 2008; Viswanathan et al., 2008). In these cells, miR-125 and let-7 are targeted by the RNA-binding protein LIN28, which actively represses their maturation and activation in conditions promoting self-renewal, a mechanism that plays a role in the maintenance of the undifferentiated state. Conversely, miR-125 and let-7 were overactivated and contributed to the downregulation of LIN28 when embryonal carcinoma cells were placed in pro-differentiating conditions, suggesting a direct role of miR-125 in repressing self-renewal and pluripotency. These mechanisms might be transposable to the hESC system, as indicated by the persistence of undifferentiated hESCs upon repression of miR-125 activity, but would not account for the additional suppressive action of miR-125 on non-neural lineages.

Smad factors were candidate targets of the effects of miR-125 because of their central role in the control of the balance between the self-renewal and differentiation of hESCs (Xu et al., 2008).

Activin-like molecules support the self-renewal of hESCs by activating the kinase domain of type-I TGF β receptors and by inducing the phosphorylation of receptor-associated SMAD2. Once phosphorylated, SMAD2 binds to SMAD4 and translocates to the nucleus to exert a pro-transcriptional activity (Shi and Massague, 2003). Conversely, members of the BMP family, such as BMP4, induce rapid differentiation of hESCs toward extra-embryonic tissues (Xu et al., 2002) and, in a dose-dependent manner, epidermis and neural crest cells (Guenou et al., 2009; Mizuseki et al., 2003). BMPs signal through the phosphorylation of SMAD1/5 proteins, which, in turn, bind to SMAD4 to form a transcriptional core (Shi and Massague, 2003).

Our working hypothesis of an action of miR-125 on the Smad-related pathways was validated in a culture system that recapitulated the variety of phenotypes driven by Smad-dependent pathways, and *SMAD4* was identified as a target of the suppressive effects of miR-125. The central role of SMAD4 in balancing the self-renewal and fate decisions of hESCs has been demonstrated by knocking down its expression using an inducible shRNA system (Avery et al., 2010). Whereas SMAD4 did not prove to be essential for the maintenance of short-term self-renewal, SMAD4 knockdown hESCs were, however, less stable and more sensitive to pro-differentiating signals. Upon differentiation, SMAD4 knockdown hESCs were not able to respond to BMP4 and had a greater propensity to differentiate toward the neural lineage than

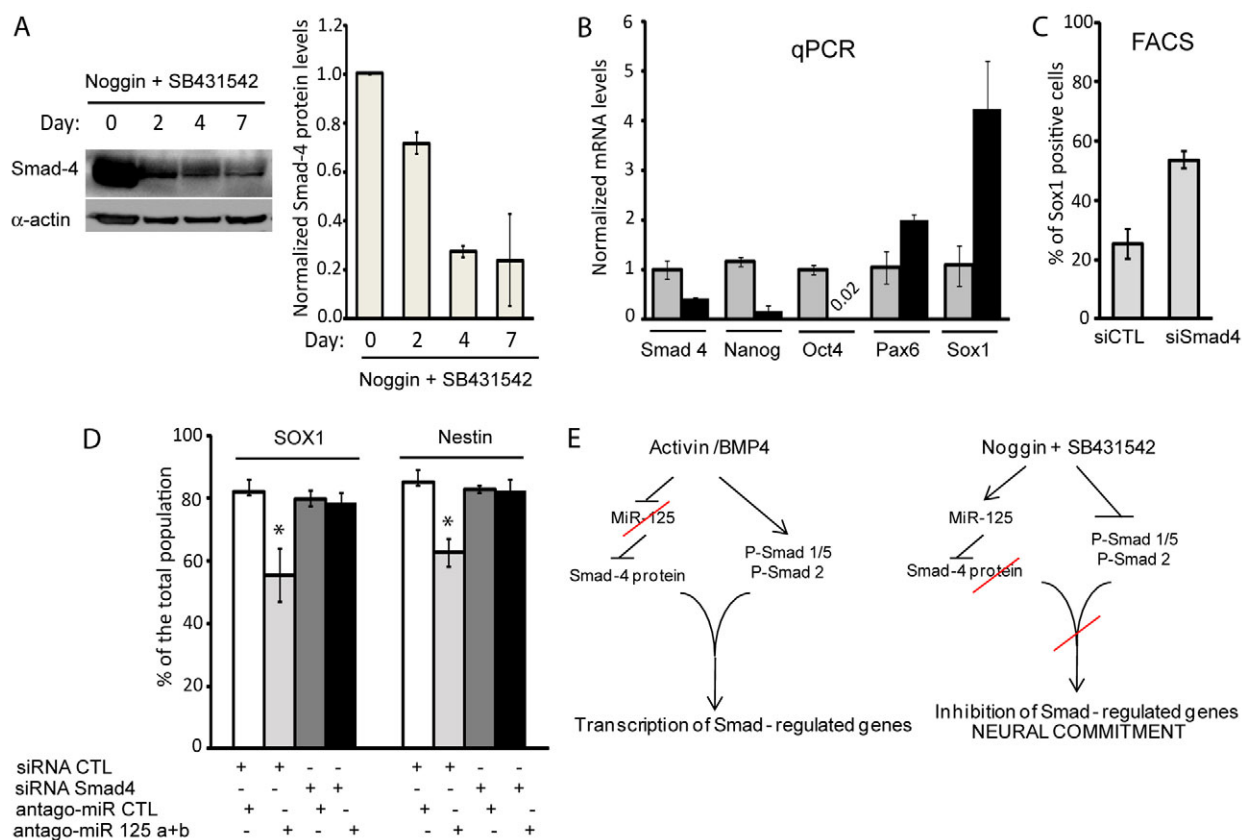


Fig. 7. Targeting of SMAD4 is instrumental in miR-125 function in promoting neural differentiation. (A) SMAD4 protein levels decrease as neural differentiation proceeds. Neural differentiation of hESCs was induced with noggin and SB431542 and the levels of SMAD4 protein were quantified over time by western blot analysis. Alpha-actin was used to ensure equal loading. The quantification of three independent experiments was carried out using ImageJ software. (B) SMAD4 knockdown cells showed a higher propensity to enter the neural lineage. siRNAs were transfected into hESCs in order to knock down SMAD4. SMAD4 knockdown hESCs were then placed in a culture medium that includes self-renewal-supporting factors (N2B27 medium without feeder cells). Expression of pluripotency genes or of key neural lineage markers was quantified 7 days after transfection by qPCR. (C) The percentage of cells expressing the neural marker SOX1 was evaluated by FACS in parallel. Cells transfected with a non-targeting control siRNA (siCTL) were used as a reference. (D) miR-125 activation is no longer necessary to control neural induction in SMAD4 knockdown cells. Neural differentiation was induced with noggin and SB431542 in hESCs co-transfected with different combinations of siRNA and antago-miR. The resulting phenotype of the cells was quantified 7 days after transfection by FACS. * $P < 0.01$, one-tailed Student's *t*-test compared with siRNA-CTL and antago-miR CTL-transfected hESCs. Error bars indicate s.d. (E) Model of the mechanisms by which miR-125 secures the neural lineage commitment decision by modulating the level of the central regulator SMAD4 in response to variation in levels of extracellular activin and BMP.

wild-type cells. This clearly placed SMAD4 as a central regulator of cell fate decisions and, consequently, pointed to miR-125 isoforms as major actors in those processes as they repress SMAD4 expression. The demonstration that miR-125 directly binds and represses SMAD4 points to a cell-autonomous mechanism of action. However, our study does not exclude the possibility of an additional contribution of a non-cell-autonomous effect of miR-125 on neural induction. Bystander effects of miRNAs have recently been reported, mediated either by direct transfer between neighboring cells through gap junctions (Kizana et al., 2009) or, more indirectly, by secretion of miRNAs in exosomes (Valadi et al., 2007). If this phenomenon operates in our experimental set-up, its extent and nature remain to be determined.

The activation of miR-125 isoforms in hESCs was shown in this study to be directly repressed by activin and BMPs. The mechanism at the base of miR-125 repression by activin and BMP remains to be elucidated. The fact that optimal repression was obtained when hESCs were exposed to both growth factors raises two possibilities: the stimulation of both TGF β -type

receptors might activate two different signaling pathways that act complementarily; alternatively, the same signaling pathway might be activated but optimal strength to repress miR-125 is only achieved when both types of receptor are stimulated. Activin has been identified as a key cytokine in maintaining pluripotency by controlling core pluripotency genes, including *NANOG*, *SOX2* and *OCT4* (Vallier et al., 2009). This, in turn, contributes to the high level of expression of the RNA-binding protein LIN28, a known repressor of pro-differentiation miRNAs including let-7 and miR-125 (Rybak et al., 2008). In parallel, BMPs have been shown to repress particular sets of miRNAs through activation of the JAK-STAT pathway (Sahni et al., 2010). These two complementary pathways might act together to fully repress a selected miRNA set.

Stimulation of activin or BMP receptors leads to the activation of R-Smad proteins. In addition to their role as transcription factors, R-Smads were recently highlighted as RNA-binding proteins capable of directly regulating the processing and maturation of pri-miRs within the DROSHA complex (Davis et al.,

2008). To date, only the upregulation by Smad proteins of a discrete set of 20 miRNAs, including miR-21, has been reported. Smad binding to a consensus CAGAC motif located 10 bp from the terminal loop is necessary and sufficient to promote pri-miR processing by the DROSHA complex (Davis et al., 2010). It is still not known whether R-Smad can, in parallel, repress other miRNA processing. This duality of miRNA regulation has been reported for another signaling pathway common to both activin and BMP receptors: the MAPK phosphorylation cascade. Upon activation, the MAPK pathway ultimately leads to the phosphorylation of one of the components of the miRNA-generating machinery: the HIV TAR RNA-binding protein TRBP (TARBP2). Phospho-TRBP mediates enhanced biogenesis of selected miRNAs, including many growth-promoting miRNAs, although its activation is also associated with repression of the tumor suppressive miRNA let-7 (Paroo et al., 2009). Interestingly, let-7 and miR-125 are regulated within the same feedback loop in embryonic carcinoma cells, another type of pluripotent cell (Rybak et al., 2008). One may hypothesize that the mechanism of MAPK-mediated repression highlighted for let-7 might be shared by miR-125. In addition, the MAPK signaling pathway has also been reported to directly repress miRNA transcription through activation of the transcriptional repressor RREB1 (Kent et al., 2010). It is likely that other cellular systems similarly target the miRNA pathway in order to achieve biological responses.

Molecular mechanisms suggested on the basis of our results are summarized in Fig. 7E. According to this scheme, the presence of activin and BMP4 in the culture medium totally blocks the activity of the miR-125 isoforms and promotes significant levels of SMAD4 expression. This, in addition to the phosphorylation of SMAD2/3 and SMAD1/5, opens up the fate choice into one of either self-renewal or differentiation into BMP-induced lineages such as neural crest and epidermis. By contrast, when BMP and activin receptors are concomitantly antagonized, miR-125 isoforms are strongly activated. The consequent downregulation of SMAD4, together with the blockage of Smad phosphorylation, secures irreversible commitment toward the neural lineage. According to such molecular mechanisms, variations in the expression of miR-125 isoforms might serve as a sensor for TGF β -like molecules in the hESC environment, with a consequential role in cell fate commitment.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material available online at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.073627/-DC1>

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