mSEL-1L (Suppressor/Enhancer Lin12-like) Protein Levels Influence Murine Neural Stem Cell Self-renewal and Lineage Commitment*

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Murine SEL-1L (SEL-1L) is a key component of the endoplasmic reticulum-associated degradation pathway. It is essential during development as revealed by the multi-organ dysfunction and in utero lethality occurring in homozygous mSEL-1L-deficient mice. Here we show that mSEL-1L is highly expressed in pluripotent embryonic stem cells and multipotent neural stem cells (NSCs) but silenced in all mature neural derivatives (i.e. astrocytes, oligodendrocytes, and neurons) by mmu-miR-183. NSCs derived from homozygous mSEL-1L-deficient embryos (mSEL-1L+/− NSCs) fail to proliferate in vitro, show a drastic reduction of the Notch effector HES-5, and reveal a significant down-modulation of the early neural progenitor markers PAX-6 and OLIG-2, when compared with the wild type (mSEL-1L+/+) NSCs counterpart. Furthermore, these cells are almost completely deprived of the neural marker Nestin, display a significant decrease of SOX-2 expression, and rapidly undergo premature astrocytic commitment and apoptosis. The data suggest severe self-renewal defects occurring in these cells probably mediated by misregulation of the Notch signaling. The results reported here denote mSEL-1L as a primitive marker with a possible involvement in the regulation of neural progenitor stemness maintenance and lineage determination.

Human SEL-1L is an endoplasmic reticulum (ER)3 resident protein (1–3) intensively expressed in neuroepithelial and pancreatic structures (4). Mouse SEL-1L (mSEL-1L) was reported to be abundantly represented throughout the neural tube and dorsal root ganglia with higher abundance in the floor plate starting at embryonic day 10.5 (5). Recently, it has been shown that mSEL-1L gene trap mice were embryonic lethal and displayed severe self-renewal defects occurring in these cells probably due to a systemic endoplasmic reticulum stress, which activated a defective unfolded protein response and impaired protein degradation (7).

SEL1L, in association with HRD1 (E3 ligase), is a key component of the endoplasmic reticulum-associated degradation pathway, acting as a “gate keeper” in the control of newly synthesized soluble and membrane-bound proteins (1, 2). ER quality control mechanisms (8–10) and proteasome degradation (11) are known to regulate proteins implicated in different cellular processes. Several reports have highlighted the role of ubiquitin ligases and proteasome in neural stem cell self-renewal, survival, and commitment (9, 10). The ubiquitin-proteasome-mediated degradation controls the availability of NSC fundamental proteins, such as Nestin (12), REST (repressor element 1-silencing transcription factor) (13–16), N-Myc (17), and components of the Notch/Delta/Numb pathway (18–20). Actually, one of the most important goals in stem cell biology is to unravel the mechanisms that control stem cell self-renewal by modulating molecules with key roles in stemness or cell fate choice (21). The proteasomal degradation pathway is a very complicated regulatory network that integrates cell intrinsic and cell extrinsic signals and partially controls the balance between self-renewal and differentiation. In particular, epigenetic modifiers, no-coding RNA regulators (microRNA: miR-9, let-7b, miR-137, and miR-124) (22), and specific signaling pathways (Notch, FGF, Wnt, Hedgehog, and β-catenin) (23) are emerging as a coordinated machinery that manages NSC fate by activating/repressing specific key regulators. In this study, we explored the possible involvement of mSEL-1L in this network. We found that mSEL-1L protein is selectively expressed in pluripotent embryonic stem cells and in multipotent NSCs, acting as a primitive marker of stemness. We also show that mSEL-1L protein expression is finely controlled by fine microRNA regulation both in vivo during mouse embryonic development and in vitro during NSC differentiation. However, its complete depletion heavily affects self-renewal capacity, by negatively regulating those important genes known to maintain the neural progenitor state. The results here presented reaffirm the previously published concept that mSEL-1L is a “multifaced” protein (4), affecting different biochemical pathways.

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1 The abbreviations used are: ER, endoplasmic reticulum; NSC, neural stem cell; miR, microRNA; qRT-PCR, quantitative real time RT-PCR; NEP, neural progenitor; NS, neural stem; ES, embryonic stem; GFAP, glial fibrillary acidic protein; CNPase, 2’,3’-cyclic nucleotide 3’-phosphodiesterase.

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**EXPERIMENTAL PROCEDURES**

**Cell Lines, Culture Conditions, and Nucleofection—**Murine embryonic stem ES46C cells were cultured on 0.1% gelatin (Sigma) coated in Glasgow minimum essential medium (Sigma) supplemented with 10% FBS, 2 mM l-glutamine, 1 mM sodium pyruvate (Invitrogen), nonessential amino acids (Invitrogen), 100 μM mercaptoethanol, 1000 units/ml leukemia inhibitory factor (Millipore, Billerica, MA). The medium was changed every 2 days. To derive neural precursors, ES46C cells were plated at a density of 6.5 × 10^5 cells/ml and cultured for 7 days in N2/B27 medium consisting of DMEM/F-12 (Invitrogen) and neurobasal medium (Invitrogen) (1:1) supplemented with 1% B27 (Invitrogen), 0.5% N2 (Invitrogen), 50 μM β-mercaptoethanol, 1 mM l-glutamine. The cells were replated on uncoated plastic in a mix of DMEM/F-12 (Invitrogen) and neurobasal medium (Invitrogen) (1:3) supplemented with 1% B27 (Invitrogen), 0.5% N2 (Invitrogen), 50 μM β-mercaptoethanol, 1 mM l-glutamine, and 20 ng/ml FGF-2 (Peprotech, Rocky Hill, NJ).

Mouse neural stem cells were cultured in the expansion medium Euromed-N (Euroclone, Milan, Italy) supplemented with N2 and 20 ng/ml of both EGF (Peprotech) and FGF-2 as described previously (24). For astrocyte differentiation, the cells were plated in expansion medium for 24 h, after which the medium was supplemented with 5% FBS, 1% N2, and 2% B27 (Invitrogen) and cultured for 7 days. Oligodendroglial differentiation was obtained using the Glaser protocol (25): essentially cells were plated on laminin-coated surfaces in expansion medium for 24 h and replaced with DMEM-F12 supplemented with 1% N2, 10 ng/ml FGF-2, 10 ng/ml PDGF (Sigma), and 10 μM forskolin (Sigma) for 4 days. Further differentiation was induced by withdrawal of growth factors for 4 days in the presence of 30 ng/ml T3 hormone (Sigma) and 200 μM ascorbic acid (Sigma). To differentiate the NS46C in neurons, we used the procedure described by Spiliotopoulos et al. (26). Briefly, the cells were subjected to a predifferentiation phase by plating them in Euromed-N medium supplemented with 1% B27, 0.5% N2, and 10 ng/ml FGF-2. Successively, the cells were cultured in a 1:3 mix of DMEM/F-12 and neurobasal medium medium containing 1% B27, 0.5% N2, gradually reducing amounts of FGF-2 (from 10 to 5 ng/ml), and increasing BDNF (Sigma) concentrations (from 20 ng/ml to 30 ng/ml). Terminal maturation was achieved after 21 days. During differentiation, the medium was partially changed every 2–3 days. mSEL-1L stability was assessed by treating undifferentiated or astrocytes committed NS46C cells with cycloheximide (200 μg/ml) for 4 and 7 h, respectively.

NSCs were transiently nucleofected with 250 pmol of pre-miR-183, pre-miR-negative control, siRNA against the exon 3 of mSEL-1L, and siRNA negative control (Applied BioSystems, Foster City, CA) using Nucleofector® technology (Lonza, Basel, Switzerland) according to the manufacturer’s instructions of the mouse neural stem cells kit (Lonza). After 24 h, the transfection medium was replaced with normal expansion medium, and mmu-miR-183 or specific gene expression was appositely evaluated after 48 h.

**Mouse Experiments and Genotyping—**mSEL-1L gene trap mice, previously described in detail (6, 7), were kindly provided by Dr. Q. Long. Adult mice and embryos were genotyped by PCR analysis of tail genomic DNA using the following PCR primers (supplemental Figure S5A): mSEL-1L 14F, 5’-cactctcttgagttacagaccaaa-3’; mSEL-1L, 15R 5’-ccaggtcatatatctgtaga-3’; βGeo R2, 5’-tgccctccagacaagtaga-3’. Primary NSC cultures were established by plating the telencephalic cortex dissociated cells from mSEL-1L−/−, mSEL-1L+/−, and mSEL-1L++ embryonic day 11.5 embryos onto a 96-well laminin-coated plastic plate containing NSC expansion medium.

**Immunofluorescence Analysis—**The cells were fixed in cold 4% paraformaldehyde for 20 min at 4 °C, washed in PBS, and permeabilized with 0.1% Triton X-100 for 10 min at 4 °C and treated with blocking solution (2% donkey serum, 1.5% BSA, 0.5% fish gelatin) for 45 min. The cells were immunostained overnight at 4 °C in blocking solution with antibodies anti-SOX-2 (1:400; Chemicon, Temecula, CA), mouse anti-Nestin (1:100, clone RAT401; Developmental Studies Hybridoma Bank, Iowa City, IA), rabbit anti-Nestin (1:50; Sigma), anti-GFAP (1:1000; Sigma), mouse anti-bIII-tubulin (1:200; Promega, Milan, Italy), rabbit anti-bIII-tubulin (1:100; Sigma), anti-O4 (1:50; Chemicon), anti-OCT-4 (1:100; Santa Cruz Biotechnology, Santa Cruz, CA), anti-cleaved caspase III (1:500; Cell Signaling, Danvers, MA), anti-SEL1L (5 μg/ml), and anti-CN3Pase (1:200; Sigma) (27) and revealed with appropriate secondary antibodies (Rhodamine-Red anti-mouse IgM and anti-rabbit IgG; Jackson ImmunoResearch, West Grove, PA; Alexa Fluor 488 anti-mouse IgG; Molecular Probes, Invitrogen). The nuclei were counterstained with Hoechst 33258, and the samples were mounted with GelMount aqueous mounting medium (Sigma). The images were acquired using a Leica DMI4000B inverted fluorescence microscope linked to a DFC360FX camera (Leica Microsystems, Vienna, Austria). To evaluate the number of immunopositive cells, a number varying from 100 to 1000 cells/slide, depending from each experiment, were scored in a total of five random fields, and then the standard deviation was calculated.

**Western Blot—**The cells and mouse brain sections were lysed in 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, protease inhibitors (Roche Applied Sciences), 1 mM PMSF, 1 mM EDTA, 1 mM sodium fluoride; the protein concentration was determined by BCA assay (Thermo Fisher Scientific, Rodano, Italy). Total cell lysates were resolved on 9% SDS-polyacrylamide gel, blotted onto PVDF membranes, and hybridized with anti-SEL1L (1.6 μg/ml) (27), anti-Nestin (1:1000; Yale University School of Medicine), anti-β-tubulin (2 μg/ml; Sigma), and anti-β-galactosidase (1:1000; Chemicon) antibodies. Hybridizations were performed in sealed bags with X-blot-100 chamber (ISE, Milan, Italy), and proteins were detected with appropriate HRP-conjugated secondary antibodies (Jackson ImmunoResearch) and the ECL system (Thermo Fisher Scientific). Protein expression values were normalized against β-tubulin levels; densitometric analysis was performed using Vision Works LS software (Euroclone).

**RT-PCR—**Total RNA was purified from cells and mouse brain sections using Tri-Reagent (Applied BioSystems) according to manufacturer’s instructions and retro-transcribed with...
Superscript III (Invitrogen). PCRs were performed for 32 cycles. Primer sequences and product sizes are listed in supplemental Table S1.

Quantitative Real Time PCR Analysis—To analyzed miR-183 expression, 100 ng of total RNA isolated with Tri-Reagent were used as template in a two-step TaqMan real time PCR analysis, performed with a TaqMan microRNA reverse transcription kit (Applied BioSystems). Real Time PCRs were assembled in triplicate accordingly with the TaqMan microRNA specific protocol with 3 µl of RT product as template. All of the samples were normalized to sno-202 expression, and the data were compared with those of undifferentiated NS46C cells.

To evaluate the expression of mSEL-1L, GFAP, SOX-2, βIII-tubulin, HES-5, PAX-6, OLIG-2 genes, quantitative real time RT-PCR (qRT-PCR) was performed in triplicate on cDNA obtained from TRIzol extracted total RNA. The data were normalized to GAPDH expression using ΔΔCt method. The primers are listed in supplemental Table S1.

RESULTS

Analysis of mSEL-1L Expression in Murine ES, Neural Precursors, and Expanded NS Cells—A recent study has shown that mSEL-1L null mice exhibited major central nervous system development impairment (7), which resulted in their embryonic lethality, thus precluding an in depth molecular dissection of the key events at the basis of the phenotype.

To investigate whether mSEL-1L played a role during the early phases of neural induction, we used murine ES cells and assayed for mSEL-1L expression during the neuralization process. This phase generates a highly enriched and well defined population of early Sox-1-positive neural progenitors (NEPs) (28) and late Radial Glia-like NSCs (24, 29).

RT-PCR analysis revealed the presence of the mSEL-1L transcript in the ES, NEP, and NS cell populations together with the established stage-specific markers (supplemental Fig. S1A). Because ES46C is a transgenic cell line in which the reporter gene eGFP is under the control of the SOX-1 promoter (28), we monitored the neuralization efficiency following eGFP expression. As expected, only the NEP population was characterized by the presence of eGFP/Sox-2 positive cells that were completely undetectable in ES and NS cultures (supplemental Fig. S1B).

The ES cell population was nearly homogeneous, as indicated by the specific expression of the appropriate stage markers OCT-4 (in embryonic stem cells) and Nestin (in NEPs and NS cells) (Fig. 1A). mSEL-1L was highly and homogenously expressed in the three culture phases (Fig. 1A), with a typical
cytoplasmic and reticular distribution. Co-expression by double immunofluorescence analysis further confirmed that all of the embryonic stem cells were OCT4-positive, and all NEPs expressing Sox-1 were characterized by the specific presence of mSEL-1L (data not shown). Particularly in the NS46C population, mSEL-1L protein co-localized with Nestin (supplemental Fig. S1C), showing a polarized pattern of expression. It is worth noting that ~2–3% of the cell population displayed mSEL-1L and Nestin co-expression but not the same subcellular localization (supplemental Fig. S1C, arrowhead, upper inset); this population is characterized by large polygon-shaped cells, which are yet to be defined but could be indicative of those cells undergoing a loss of multipotency.

Western blot analysis confirmed the presence of mSEL-1L protein in the three stage-specific cell populations, with comparable expression levels (Fig. 1B). In embryonic stem cells, however, two distinct mSEL-1L forms with molecular masses of 95 and 105 kDa were observed; whereas the 95-kDa isoform is the predominant and canonical ER-resident protein, the second could represent an embryonic specific polyglycosylated form (30) whose potential biological significance remains to be established. Overall, these results indicate that mSEL-1L is highly expressed in both pluripotent and multipotent cell populations and remains unmodified during neural lineage restriction process.

mSEL-1L Protein Is Silenced during NSC Trilineage Differentiation—mSEL-1L expression was assessed during NSC differentiation into neuronal, astroglial, and oligodendroglial lineages. Western blot analysis performed at different maturation stages showed the similarity between mSEL-1L and Nestin protein expression, indicating the necessity of mSEL-1L only in immature NSCs (Fig. 1C).

Immunofluorescence analysis confirmed the biochemistry results, showing that in self-renewal conditions mSEL-1L is co-expressed with Nestin (supplemental Fig. S1C) and Sox-2 (Fig. 1D) but lost in mature astrocytes, oligodendrocytes, and neurons (Fig. 1, C and D, and supplemental Fig. S2). Nonetheless, few scattered cells in the cultures retained mSEL-1L immunoreactivity, possibly representing those few cells that maintain an immature phenotype. Overall, the sharply modulated pattern of mSEL-1L during NSCs maturation indicates that its expression is associated with an immature cell status (pluripotency/multipotency), and its down-regulation is required to allow NSC to differentiate.

mSEL-1L Knock-out Leads to Impaired Self-renewal and a Premature Astrocyte Commitment—Primary neural precursors were dissociated from the embryonic day 11.5 forebrain of mSEL-1L wild type, heterozygous, and deficient embryos and in expansion medium with the purpose of generating NS cell lines. It is noteworthy that homozygous mutant embryos exhibited severe growth retardation and a reduction of the cerebral cortex thickness (Fig. 2C). Readily, typical expandable homogeneous Sox-2- and Nestin-positive and GFAP-, CNPase-, and βIII-tubulin-negative NS cell lines were obtained from mSEL-1L+/− (Fig. 2, A and D) and mSEL-1L+/− (Fig. 2, B and D) embryos. As expected, no expandable NS cell line was generated from mSEL-1L−/+ embryos, because of severe proliferation impairment that ultimately resulted in growth arrest and apoptosis (Fig. 2C). After a few rounds of in vitro divisions, mSEL-1L−/+ primary neural cells became predominantly Nestin negative, showing Sox-2 immunonpositivity only in ~40% of the population, but over 50% of the whole culture was positive for GFAP marker expression (Fig. 2, C and D). In addition, mSEL-1L−/+ NSCs were characterized by a high rate of apoptosis, as demonstrated by the expression of the cleaved form of caspase III in ~50% of the cells (Fig. 2, C and D).

Interestingly, mSEL-1L−/+ cells but even more the mSEL-1L−/− neural progenitors exhibited a drastic down-modulation of HES-5 transcript (Fig. 2E), a well established effector of Notch signaling, known to play a critical role in NSC in the maintenance of self-renewal by repressing neuronal differentiation (31). Particularly, mSEL-1L knock-out NSCs were characterized by low levels of expression of the early neural progenitor markers PAX-6 and OLIG-2 when compared with wild type and heterozygous cell lines (Fig. 2E). These results indicate that NSC self-renewal is severely impaired in the absence of mSEL-1L protein, probably by affecting: (i) the Notch pathway, (ii) proliferation efficiency leading to in vitro cell death, and (iii) by an abrupt astroglial commitment.

mSEL-1L+/− NSCs Exhibit Preferential Astrocyte Differentiation—mSEL-1L+/−.derived NSCs, nucleofected with siRNA directed against exon 3, showed that mSEL-1L down-modulation (~40%) determined an increase of GFAP levels of ~5-fold over the control (Fig. 3A). Similarly, mSEL-1L+/− NSCs, characterized by lower levels of mSEL-1L transcript, exhibited a significant increase in GFAP expression up to 5-fold when compared with the wild type cells (Fig. 3B). The maintenance of only one functional mSEL-1L allele (Fig. 3B) or a slight protein down-regulation (Fig. 3A), although promoting increased GFAP expression, is enough to preserve NS cells from the aberrant phenotype as observed in completely depleted mSEL-1L cells. To investigate whether the differentiation process was affected, wild type and heterozygous mSEL-1L NS cells were exposed to lineage-specific culturing conditions as previously described (24–26). Although neuronal maturation efficiency was grossly unaffected in both wild type and heterozygous NSCs (Fig. 3, C and D), showing ~20% of βIII-tubulin-expressing cells (Fig. 3I), glial differentiation was significantly altered. Although both mSEL-1L+/− and mSEL-1L−/− NS cell populations showed basal expression of GFAP during astrocyte differentiation, only ~10% of the wild type cells were strongly positive (Fig. 3, E and I), whereas 20% of heterozygous cells showed intense immunopositivity for this marker (Fig. 3, F and I). In addition, during oligodendroglial differentiation, ~30% of O4-positive cells were detected in the mSEL-1L+/− cell population (Fig. 3, G and I), whereas only 10% of mature oligodendrocytes were detected in mSEL-1L+/− NSCs (Fig. 3, H and I). These results indicate that in heterozygous and RNA-interfered wild type NSCs, mSEL-1L down-modulation determined a significant increase in GFAP expression and that the trilineage maturation of mSEL-1L−/+ cells promoted astrocytic rather than oligodendroglial commitment, without affecting the neuronal differentiation.

mSEL-1L Protein Levels Inversely Correlate with mmu-miR-183 during Trilineage Maturation—Although mSEL-1L protein is rapidly down-regulated during NS trilineage maturation
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(Fig. 1C), its transcript remained highly expressed in all the terminal differentiated neural populations (Fig. 4A), indicating an uncoupled regulation between mSEL-1L protein and its messenger. The significant progressive depletion of mSEL-1L protein during NSCs trilineage maturation led us to assay its stability in cells undergoing astrocyte differentiation. NS46C cells were submitted to astrocyte commitment, and 2 h later, when mSEL-1L protein was found reduced ~25%, cycloheximide was added for 4 and 7 h to inhibit protein synthesis and allow the evaluation of mSEL-1L degradation rate. Undifferentiated NS46C exhibited a reduction in mSEL-1L protein levels of 30 and 40% after 4 and 7 h of cycloheximide treatment, respectively (supplemental Fig. S3) because of the physiological mSEL-1L turnover. In the same way, committed astrocytes,
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mSEL-1L is a member of the proteasome-ubiquitin system and participates in the degradation of specific substrates by recruiting polypeptides on the ER membrane (34). The Caenorhabditis elegans ortholog of mSEL-1L and its E3 ligase (Hr3) were both initially described as negative regulators of Lin-12/Notch activity (35, 36), probably by controlling the stability or export of Lin-12 (37). During mouse embryogenesis, mSEL-1L is actively involved in pancreatic epithelial cell fate determination (endocrine versus exocrine cell differentiation) through Notch signaling (6).

In this study, we show the primitive expression of mSEL-1L in pluripotent embryonic stem cells and in multipotent neural progenitors. We demonstrate that mSEL-1L is abundantly present in the same cell population that expresses: (i) OCT-4, a primordial marker essential in mouse ontology (38); (ii) cycloheximide-treated or untreated, showed almost the same decrease of the protein levels (supplemental Fig. S3), indicating that mSEL-1L down-modulation in differentiated astrocytic cells is likely not due to an increased protein degradation but rather to the inhibition of de novo synthesis.

We thus explored alternative mechanisms that could explain mSEL-1L protein destabilization without affecting mRNA transcription. MicroRNA activation, together with changes in DNA methylation and histone modifications, provide intrinsic epigenetic mechanisms that regulate post-transcriptionally protein synthesis, with implications in stem cell self-renewal and differentiating decisions (32). Hence, we investigated the possible activation of microRNAs that could negatively regulate mSEL-1L mRNA translation. SEL1L gene is characterized by a long 3′-UTR tail (<5 kb) harboring consensus sites for a number of microRNAs (33). Bioinformatics analysis (MicroCosm Targets, Target Scan) suggested mmu-miR-183 as a possible candidate for mSEL-1L silencing, whose consensus sequence is located 183 nucleotides downstream the stop codon (supplemental Fig. S4A).

Analysis of mmu-miR-183 levels in vitro during NS46C tri-lineage differentiation revealed that endogenous mmu-miR-183 was nearly undetectable in self-renewing NS46C cells but significantly increased in mature astrocytes, oligodendrocytes, and neurons (Fig. 4B). Also in vivo, mmu-miR-183 expression (Fig. 4C) was inversely correlated with mSEL-1L protein levels (Fig. 4D); whereas mmu-miR-183 and mSEL-1L mRNA were found in adult olfactory bulbs, hippocampus, thalamus, and cortex (Fig. 4, C and E), the 95-kDa mSEL-1L protein was completely absent in those regions and was detected only in the undifferentiated stem cell sample (NS46C), in which the microRNA was almost undetectable (Fig. 4, C and D).

Moreover, although mSEL-1L mRNA expression is lower in the mSEL-1L−/− NSC line compared with mSEL-1L+/+ (Fig. 3B), the protein level is comparable between the two genotypes (supplemental Fig. S4B). This is probably due to the very low levels of mmu-miR-183 detected in the heterozygous sample that allows efficient mSEL-1L protein expression despite the lack of one functional allele (Fig. 4F). Altogether, these data link mSEL-1L protein expression, but not its mRNA, to mmu-miR-183 by an inverse correlation.

**mMu-MiR-183 Overexpression Negatively Regulates Pax-6 and Olig-2 Expression and Determines an Increased Astrocyte Commitment**—To evaluate the possible regulatory effects of mmu-miR-183 on mSEL-1L protein, NS46C cells were nucleo-fected with pre-miR-183 and analyzed for protein expression. 48 h after nucleofection, when the mature mmu-miR-183 expression increased over 6 × 10^3-fold above control (supplemental Fig. S5B), indicating the proper microRNA processing, mSEL-1L protein levels decreased more than 3-fold (Fig. 5A). Interestingly, this strong decrease of mSEL-1L protein, uncorrelated with significant alterations of its messenger levels, did not drastically alter βIII-tubulin and SOX-2 expression (Fig. 5B). Differently, HES-5 and more markedly PAX-6 and OLG-2 were down-modulated in response to mmu-miR-183 overexpression, whereas GFAP mRNA was up-regulated up to 2.5-fold over the control (Fig. 5B). We also analyzed the effects of mSEL-1L decrease caused by mmu-miR-183 overexpression by immunofluorescence. The number of cells positive for caspase III cleaved form was up-regulated of 3-fold above the control (Fig. 5, C, top panel, and E), whereas the Nestin- and Sox-2-stained cells did not change in response to mmu-miR-183 nucleofection (Fig. 5, C, middle and bottom panels, respectively, and E). βIII-Tubulin-positive cells (Fig. 5, D, top panel, and E) and especially GFAP-positive cells (Fig. 5, D, middle panel, and E) were more represented in the population in which mSEL-1L protein was down-modulated, whereas cells positive for the oligodendrocyte marker CNPase were not detected (Fig. 5, C, bottom panel, and E). Altogether, these data indicate that mmu-miR-183 is a possible negative regulator of mSEL-1L, and its ectopic expression, by acting on mSEL-1L protein, could be partially responsible of a self-renewal alteration and a premature astrocyte commitment.

**DISCUSSION**

**mSEL-1L deficiency induces NSCs apoptosis, loss of stemness, and astrocyte differentiation.** Embryonic day 11.5 mSEL-1L wild type (mSEL-1L+/+) and heterozygous (mSEL-1L+/-) mutant fetuses exhibited a normal brain morphology, whereas the homozygous mutant embryo (mSEL-1L−/−) showed a reduced brain mass and thinning of the cerebral cortex. A and B, SCs directly derived from the telencephalic cortex of embryonic day 11.5 mSEL-1L−/− (A) and mSEL-1L−/− (B) embryos showed immunopositivity for Nestin but not for the activated form of caspase III, whereas no or very few astrocytic, neuronal, and oligodendroglial differentiated cells were observed. The cell population expressed high levels of Sox-2, C, mSEL-1L−/− NSCs were completely devoid of Nestin, but close to half of the cell population was Sox-2-immunopositive. Cells exhibited premature GFAP expression, no βIII-tubulin and CNPase staining, and a discrete rate of apoptosis. D, histogram represents the number of immunopositive cells scored in a total of five random fields for a total of 800 cells/slide for mSEL-1L+/+ and mSEL-1L−/− NSCs. For the mSEL-1L−/− cell line, the entire population consisted of ~100 cells. Although Nestin and Sox-2 were expressed by the entire mSEL-1L−/− and mSEL-1L+/+ cell population, the mSEL-1L-deficient line exhibited no or very few Nestin-positive cells and only 40% of SOX-2 immunopositivity. GFAP was expressed in 50% of mSEL-1L−/− NSCs, but it was undetectable in both the wild type and heterozygous cell lines. Very few βIII-tubulin- and CNPase-expressing cells were observed in the three cell lines. Unlike wild type and heterozygous cell lines, 50% of mSEL-1L−/− cells displayed the cleaved form of caspase III. Photomicrographs were acquired with a Leica 60× objective. E, HES-5, PAX-6, and OLG-2 expression in mSEL-1L−/−, mSEL-1L−/−, and mSEL-1L−/− NSCs was analyzed by qRT-PCR, using SYBR-green detection. HES-5 levels were drastically affected by mSEL-1L depletion, whereas PAX-6 and OLG-2 expression was only partially down-modulated. The experiments were performed in triplicate, the results were normalized for GAPDH expression using the ΔΔCt method, and the standard deviation was calculated.
SOX-2, fundamental to guarantee pluripotent and self-renewing phenotype (39); and (iii) Nestin, a marker of neural stem/progenitor cells (40) (Fig. 1 and supplemental Fig. S1C). The unusual 105-kDa mSEL-1L protein form, never seen in any cell line previously analyzed, could represent the same polyglycosylated protein (embryonic-stage specific) identified by Alvarez-Manilla et al. (30); its role in embryonic cell lineage remains to be explored, because it does not persist in committed cells (Fig. 1B). One of the most unexpected and meaningful data reported here is that during NSC trilineage maturation, but also in some areas of terminally differentiated mouse brain, down-modulation of mSEL-1L protein occurs (Figs. 1C and 4D). During neurodevelopment, following their regional identity, neural progenitors give rise, in a temporally regulated manner, to a mature
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but limited number of neurons, astrocytes, or oligodendrocytes. This process, typical of immature stem cells, requires a high biosynthetic activity to produce those intrinsic and external factors that control cell fate by modulating the expression of stage-specific genes (41). Those molecules, involved in the dynamic gene expression control are then silenced in committed NSC derivates by several molecular mechanisms, one of which is the ubiquitin proteasome-dependent pathway. Although this pathway has long been known to regulate important cellular processes, including cell cycle (42), only recently researchers have begun to address its role in the context of embryonic stem cells, highlighting the critical role of the E3 ligases, proteasomal degradation, and protein stability in the regulation of neurogenesis (16, 43, 44) and stem cell function (10, 45–47). We show that mSEL-1L is highly expressed in primitive cell lineages, reflecting a possible role in the abatement of factors involved in neurogenesis, perhaps by targeting them to the endoplasmic reticulum-associated degradation pathway and favoring stemness preservation. In vitro data collected in our laboratory (data not shown) exclude, however, the activation of the unfolded protein response gene responses usually sustained in ER overloaded cells. Interestingly, in NS46C, mSEL-1L and Nestin co-express and co-localize in most of the undifferentiated cells (Fig. 1C and supplemental Fig. S1C). However, in a subpopulation (2–3%) of cells characterized by round-shaped, short extensions and large nuclei, mSEL-1L is starting to be turned off. These cells exhibit low levels of Nestin and probably represent a transient multipotency-losing population, in which mSEL-1L does not sublocalize with Nestin. During NSC differentiation, loss of Nestin is an early event mediated by the ubiquitin-proteasome system and by Notch (12), but it still remains to verify whether mSEL-1L in association with the E3 ligase HRD1 could facilitate Nestin degradation.

Phenotype analysis of mSEL-1L deficient NSCs leads to some questions: (i) Is mSEL-1L involved in the maintenance of a cor-

FIGURE 3. mSEL-1L+/− NSCs preferentially differentiate in astrocytes and exhibit impairment of oligodendroglial commitment. A, mSEL-1L+/− NSCs were nucleofected with 250 pmol of siRNA against mSEL-1L and of a negative control. After 48 h, mSEL-1L and GFAP expression was evaluated by qRT-PCR. The experiments were performed in triplicate, the results were normalized for GAPDH expression using the ΔΔCt method, and the standard deviation was calculated. mSEL-1L down-modulation was associated to GFAP overexpression. B, the histogram represents mSEL-1L and GFAP expression in mSEL-1L+/− NSCs, related to the wild type line. The analysis was performed by qRT-PCR using SYBR-green detection method in triplicate, and the data were normalized to GAPDH expression, using the ΔΔCt method. The heterozygous cells obviously exhibited low levels of mSEL-1L and were characterized by an increased expression of GFAP when compared with control cells. C and D, mSEL-1L+/− and mSEL-1L+/− NSCs, when subjected to neuronal differentiation, generated approximately the same number of βIII-tubulin immunopositive cells. E and F, when induced to astrocytic maturation, the heterozygous cells were more GFAP immunoreactive than the wild type differentiated cells. G and H, during oligodendroglial maturation, the wild type population produced more O4-expressing cells with respect to mSEL-1L−/− NSCs. Photomicrographs were acquired with Leica 60 × objective. I, histogram represents the number of immunopositive cells scored in five random fields of ~600 mSEL-1L+/− and mSEL-1L−/− NSCs/slide, and the standard deviation was calculated. GFAP intense immunopositive cells represented ~10 and 20% of the astrocyte differentiated wild type and heterozygous cell lines, respectively. During oligodendroglial maturation mSEL-1L+/− NSCs originated 3-fold more O4-positive cells than those detected in mSEL-1L−/−. The number of βIII-tubulin-positive neurons was approximately the same in mSEL-1L+/− and mSEL-1L−/− cell lines.

FIGURE 4. mSEL-1L protein levels inversely correlate with mmu-miR-183 expression. A, RT-PCR showed that during NS46C trilineage maturation, whereas GFAP and βIII-tubulin mRNAs were up-regulated, mSEL-1L expression did not change during astrocyte (7 days), oligodendrocyte (7 days), and neuron (21 days) maturation. GAPDH was used as loading control. B, TaqMan assay performed on the same differentiated cells described in A showed an increased expression of mmu-miR-183 when compared with undifferentiated cells. The values were normalized relatively to snoRNA202 levels; the data were presented as the averages of three independent experiments. C, dissected tissue fragments collected from different mouse brain areas (olfactory bulbs, hippocampus, thalamus, and cortex) were analyzed for mmu-miR-183 expression by specific TaqMan assay, using snoRNA202 as normalizer; the microRNA was highly expressed in all adult brain sections. The data reported the averages of three independent experiments and are presented as fold increase compared with undifferentiated cells. For all the experiments, the standard deviation was calculated, and the significant differences were indicated with asterisks (p < 0.05, t test). D, mSEL-1L 95-kDa protein expression detected in the same adult brain areas described for C did not reflect the mRNA levels (E) and was expressed only in undifferentiated NS cells. β-Tubulin was used as protein loading control. mSEL-1L transcript was evaluated in the brain total RNA by RT-PCR, using actin as a housekeeping gene. F, the histogram represents the expression levels of mmu-miR-183 in mSEL-1L+/− NSCs compared with the wild type cell line. The same experiment was performed in triplicate by TaqMan assay, using snoRNA202 as normalizer.
rect “stem-program”? (ii) Is mSEL-1L a direct regulator of Notch signaling? This pathway has been described to be essential in cell fate determination, tissue patterning and morphogenesis, cell differentiation, proliferation, and cell death (48). Although a direct link between mSEL-1L and Notch has been demonstrated only in C. elegans (35), our results show a signif-
significant down-modulation of the Notch effector HES-5 in neural progenitors deprived of mSEL-1L, negatively affecting the self-renewal. This is a vital process that allows stem cells to perpetuate through the control of complex signaling pathways (e.g. Wnt, FGF, and Notch), transcriptional regulators (e.g. Bmi1, a senescence repressor and self-renewal-promoting protein), or repressors (e.g. HES-1, HES-5) and cell-matrix interactions (e.g. integrins) (49). Ongoing studies in our lab are trying to unravel the specific mechanism by which mSEL-1L loss influences Notch pathway and NSC self-renewal/differentiation, how the self-renewal defects could alter NSC differentiation and the ability to properly differentiate, and whether the exhaustion of the progenitor pool could affect mSEL-1L-deficient mice development.

Contrary to the homozygous mutant mSEL-1L NSCs, heterozygous cells survive and maintain an apparently “normal” self-renewing phenotype but are characterized by basal higher levels of GFAP. The same increase in the astrocytic marker is also shown when wild type cells were subjected to mSEL-1L siRNA-mediated interference. The astrocyte differentiation process is under the control of complicated and poorly understood molecular mechanisms, among which is the TGF-β/SMAD pathway (50, 51). Interestingly, because overexpression of human SEL1L determines a strong down-modulation of the four SMAD signal transducers (55), it seems reasonable to assume that lower levels or absence of mSEL-1L may lead to alterations of the TGF-β/SMAD pathway.

The results reported here support the hypothesis that mSEL-1L, being part of the endoplasmic reticulum-associated degradation physiological processes, may be implicated in neural stem cell fate decisions, probably by controlling the turnover of stage-specific factors. The C. elegans ortholog sel-1 is involved in the cell nonautonomous decision-making between two gonad cells; the choice between anchor cell and ventral uterine cell fates is stochastically controlled by the activity of Lin-12/Notch pathway (35). Variations in gene expression in a precursor cell must be under strict control, because cell fate decision depends on stochastic levels between different proteins. Immediate and fine tuned expression of proteins has been associated with activation/silencing of specific microRNAs able to inhibit the translation of a series of mRNAs for transcription factors (53). Following this concept, mSEL-1L protein, being prematurely expressed in ES cells, must be accurately controlled, and here we show that mmu-miR-183 is at least partially responsible for its post-transcriptional regulation (Fig. 4). It is a well-established concept that stem cell molecular programming and reprogramming are under the control of an orchestrated action of extrinsic signals and intrinsic factors (54), and microRNAs are emerging as important post-transcriptional factors that regulate the dual capacities of stem cells (55). The sophisticated action operated by mmu-miR-183 on mSEL-1L protein guarantees its suppression, allowing the cellular system to proceed to the differentiation pathway. In NSCs generated from the mSEL-1L heterozygous mouse brain, to guarantee stochastic levels of mSEL-1L protein in the absence of one functioning allele, the cellular system stimulates a drastic reduction of mmu-miR-183 expression, preserving mSEL-1L mRNA translation (Fig. 4F). Moreover, when in self-renewing condition mSEL-1L protein is forcedly down-modulated by mmu-miR-183 overexpression, a premature increased GFAP expression, a discrete enhanced cell death, and a strong down-modulation of neural progenitor markers PAX-6 and OLIG-2 were observed (Fig. 5). This might be due to inhibition of the Notch pathway as revealed by the partial down-modulation of HES-5. A more profound effect is observed in neural progenitors completely depleted of mSEL-1L, where HES-5 level are almost undetectable, suggesting a direct correlation between inhibition of Notch pathway and mSEL-1L protein silencing. Interestingly, mmu-miR-183 ectopic expression induces a even more intense reduction of PAX-6 and OLIG-2 expression than mSEL-1L complete depletion. mmu-miR-183 pleiotropic effects on self-renewal can explain the strong impact on these genes that are crucial in neural/stem/progenitor maintenance. In fact, besides mSEL-1L, miR-183 targets other proteins fundamental in the preservation of stemness such as β1-integrin (56) and Bmi1 (57). Because of their ability to simultaneously control several target genes, microRNAs are probably the best candidates to regulate such complex processes as self-renewal and cell fate decision. β1-Integrin, in vivo, confers to NSCs the ability to receive and elaborate messages from the “stem cell niche” and directs neural progenitors to undertake the correct fate, preserving their stemness or promoting the differentiation and migration in the developing cortex. As demonstrated for mSEL-1L, β1-integrin absence in neural progenitors can compromise stemness maintenance, determining differentiation or cellular death (58). Bmi1 is a transcriptional repressor belonging to the polycomb family, which preserves self-renewal by repressing the cyclin-dependent kinase inhibitor p16Ink4a and p19Arf (49). Bmi1 and Ink4a/Arf deficiency in NSCs determines alterations in collagen-related gene expression, increasing secretion of extracellular matrix, and enhancing β1-mediated binding to cellular matrix (59). Interestingly, human SEL1L was
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proposed to be an important element in extracellular matrix interactions (60), likely because of its capacity to alter the expression of molecules involved in extracellular matrix and cytoskeletal remodeling (52, 61). It is noteworthy that mSEL-1L (regulating Notch signaling), β1-integrin (mediating specific cell-matrix interactions), and Bmi1 (controlling stem cell fate) might belong to the same biochemical pathway regulated by the refined action of miR-183; perhaps together they synergically participate in self-renewal maintenance by creating a specific "niche" environment able to control NSC proliferation, adhesion, and migration.

Very recently it was reported that in a completely differentiated neural system such as in adult brain, mSEL-1L expression is confined to the dentate gyrus of the hippocampus (62), a specific microenvironment rich in stem cells (63), in which mSEL-1L could preserve progenitor cell renewal. The mSEL-1L role is not unique to NSCs, but it is also important in other immature cell types such as mesenchymal (64), splenic (65), murine embryonic (30), and endoderm-derived parietal epithelial stem cells (6). To our knowledge, this is the first report dealing with mSEL-1L in NSC and paves the way to further studies to identify ulterior proteins acting in synergism with mSEL-1L to preserve stemness during development.

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Additional text included in the image: "mSEL-1L Expression in Neural Stem Cells proposed to be an important element in extracellular matrix interactions (60), likely because of its capacity to alter the expression of molecules involved in extracellular matrix and cytoskeletal remodeling (52, 61). It is noteworthy that mSEL-1L (regulating Notch signaling), β1-integrin (mediating specific cell-matrix interactions), and Bmi1 (controlling stem cell fate) might belong to the same biochemical pathway regulated by the refined action of miR-183; perhaps together they synergically participate in self-renewal maintenance by creating a specific “niche” environment able to control NSC proliferation, adhesion, and migration. Very recently it was reported that in a completely differentiated neural system such as in adult brain, mSEL-1L expression is confined to the dentate gyrus of the hippocampus (62), a specific microenvironment rich in stem cells (63), in which mSEL-1L could preserve progenitor cell renewal. The mSEL-1L role is not unique to NSCs, but it is also important in other immature cell types such as mesenchymal (64), splenic (65), murine embryonic (30), and endoderm-derived parietal epithelial stem cells (6). To our knowledge, this is the first report dealing with mSEL-1L in NSC and paves the way to further studies to identify ulterior proteins acting in synergism with mSEL-1L to preserve stemness during development."