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miRNAs Stem Cell Reprogramming for Neuronal Induction and Differentiation

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Abstract Mimicking the natural brain environment during neurogenesis represents the main challenge for efficient in vitro neuronal differentiation of stem cells. The discovery of miRNAs opens new possibilities in terms of modulation of stem cells lineage commitment and differentiation. Many studies demonstrated that in vitro transient overexpression or inhibition of brain-specific miRNAs in stem cells significantly directed differentiation along neuronal cell lineages. Modulating miRNA expression offers new pathways for post-transcriptional gene regulation and stem cell commitment. Neurotrophins and neuropoietins signaling pathways are the main field of investigation for neuronal commitment, differentiation, and maturation. This review will highlight examples of crosstalk between stem-cell-specific and brain-specific signaling pathways and key miRNA candidates for neuronal commitment. Recent progress on understanding miRNAs genetic networks offers promising prospects for their increasing application in the development of new cellular therapies in humans.

Keywords Stem cell · miRNA · Neuronal differentiation · Neurogenesis · Cytokines · Neurotrophins · Neuropoietin · Cell reprogramming · Cell therapy

Introduction

Stem cell research may change the clinical approach for the treatment of central nervous system diseases and injuries [1, 2]. Safe and efficient in vitro and in vivo technologies are necessary for stem cell induction into functional neurons. The main obstacle in neuronal reprogramming of adult stem cells is low yield of generated neuroblasts. Efficient generation of neural stem cells in vitro and their differentiation into functional neurons require detailed knowledge about the molecular and cellular factors activating the key signaling pathways responsible for cell survival and neurogenesis such as Akt/PI3K [3], mitogen-activated protein kinase (MAPK) [4], BMP [5], Jak/STAT [5], Wnt [6], and Notch [7] as well as their target genes regulating cell survival and neurogenesis process (Fig. 1a, b).

Several methods have been used for neuronal induction and differentiation of pluripotent and multipotent stem cells. Vierbuchen and colleagues described a very attractive method for direct neuronal reprogramming of adult rodent fibroblasts by tissue-specific transcription factors [8]. In this study, three transcription factors, Mash1, Brn2, and Myt1l, were introduced into rodent fibroblasts with lentiviral vectors, with subsequent differentiation into functional neurons. Stem cells can be also induced into neuronal phenotypes by defined cytokines and morphogenes, for instance: retinoic acid (RA) [9, 10], basic fibroblast growth factor and forskolin [11], brain-derived neurotrophic factor (BDNF) [10], butylated hydroxyanisole [12], glial-derived neurotrophic factor, sonic hedgehog (SHH) [13, 14], or cyclic adenosine monophosphate (cAMP) [15]. Cytokines are divided into seven major families of interleukins, chemokines, tumor necrosis factors, interferons, growth factors, neurotrophins and neuropoietins. In this review, we focus on the crosstalk between the signaling networks activated by two last families involved in the in vitro neuronal differentiation of stem cells and in vivo neurogenesis process (Fig. 1a, b) and strategies for microRNA (miRNA) reprogramming of stem cells.

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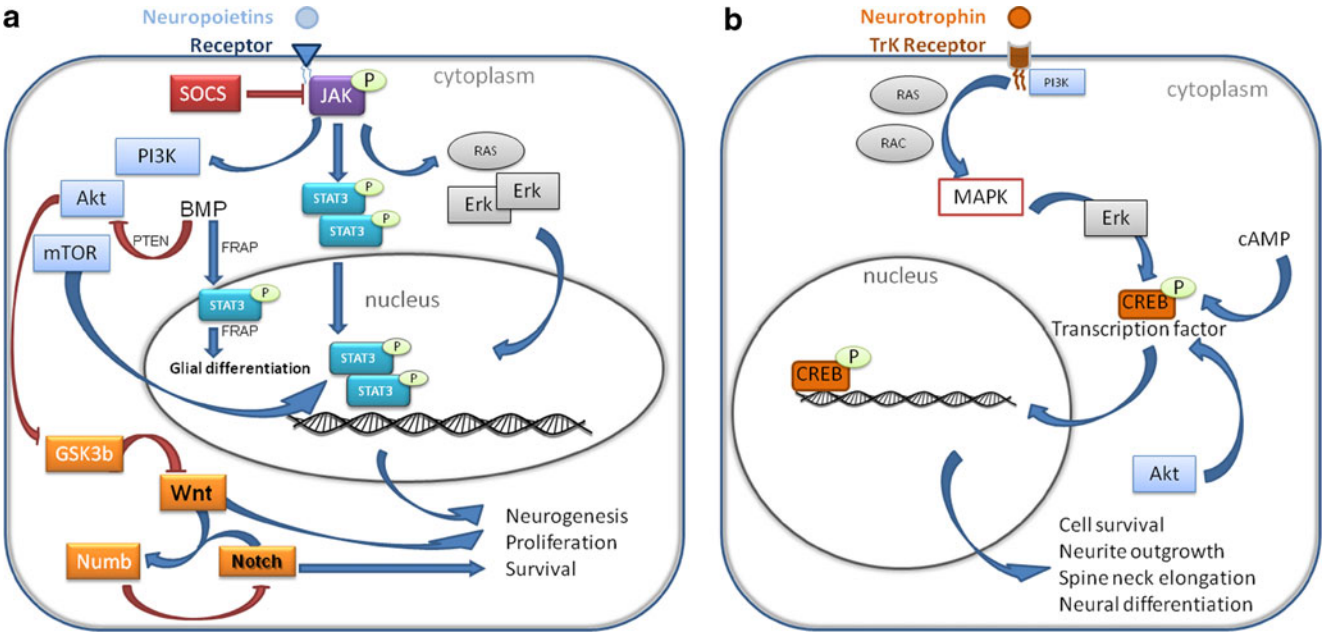

Fig. 1 **a** Schema of the neuropoietin cytokines signaling through the JAK/STAT and Wnt/Notch pathways. **b** Schema of the neurotrophins signaling through the MAPK and CREB pathway

Cytokines such as IL-6, leukemia inhibitory factor (LIF), CNTF, and oncostatin M (OSM) of the neuropoietins family, following their binding to the homo- or hetero-dimer with gp130 receptor, transduce the signal via the Janus kinase (JAK)/STAT, extracellular signal-regulated kinase (ERK), and Akt/PI3K pathways and cAMP-binding transcription co-factor (CREB) to promote cell growth and survival (Fig. 1a). The neurotrophins represent a group of factors involved in cell survival and differentiation and thus mediates important effects in the central and peripheral nervous system [16]. In vitro studies show that BDNF and nerve growth factor (NGF) act on neural stem cell through intracellular cascades including the MAPK, ERK, Akt/PI3K, and PLCγ pathways (Fig. 1b). The Akt/PI3K signaling is very important in transduction of survival and differentiation signaling. This pathway can be negatively regulated by BMP signaling via PTEN protein [3]. BMP signaling is a well-known inhibitor of neuronal differentiation and can interact with Jak/STAT signaling via FRAP (FKBP12/rapamycin-associated protein) kinase [5]. The Akt/PI3K signaling may also activate Wnt canonical pathway by inhibitory phosphorylation of GSK3β kinase [6]. Wnt pathway can cross-talk with Notch signaling (the two most important pathways in neurogenesis) via Numb protein, whose expression is regulated by Wnt-dependent TCF/LEF transcription co-factors [17]. The Numb protein plays a very important role in neural stem cells regulation via control of asymmetric cell division. It can be accumulated in the apical or basal region of neural stem cells and following cell division remain only in one of the daughter cells. The stem cell containing Numb protein remains undifferentiated since Numb negatively regulates Notch signaling pathway responsible for neuronal fates induction.

miRNAs [18, 19] have been reported as having a significant role in epigenetic regulation of brain development [20], neurogenesis [21], and neuronal fates specification [22-24]. miRNAs are key factors for regulation of gene expression at the mRNA level (Fig. 2). We will describe

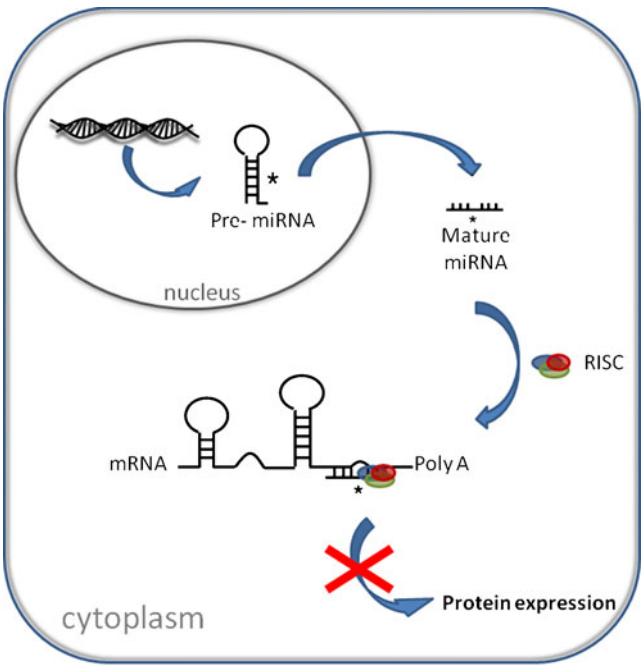

Fig. 2 Schema of the general miRNA pathway in cells leading to the translation inhibition of the miRNA mRNA target

herein the role of small RNA molecules during neurogenesis as well as the role of miRNA molecules in stem cells differentiation into neuronal cells in vitro. miRNAs are multi-target epigenetic tools capable of turning the molecular machinery of signaling pathways towards neuroepithelial developmental lineages for neuronal reprogramming and differentiation by extrinsic cytokines, morphogenes, or genes. This review paper will provide a comprehensive overview of recent findings on miRNA regulation of neuronal differentiation and neurogenesis, cross-talks between these pathways, and significant regulatory role of miRNA molecules.

Mechanisms of miRNAs Activity

In 1998, the American scientists Andrew Fire, Craig Mello, and colleagues, discovered that small double-stranded RNA can mediate efficient gene silencing [25]. This research on RNA interference has since been awarded with the 2006 Nobel Prize in Physiology or Medicine. To the date, the number of identified mature miRNAs increased to over 1,200 in the human genome database [26].

miRNAs are small endogenous non-coding RNAs of 21 to 24 nucleotides long, expressed by many eukaryotic organisms. In the genome, microRNAs can be located in either introns, exons, or in intragenic regions [27]. miRNAs regulate gene expression post-transcriptionally through the degradation and/or interference with their mRNA targets (see Fig. 2; [28, 29]). In the nucleus, those small RNAs are obtained from the genome through an initial transcription that gives birth to long primary transcripts known as pri-miRNAs. The miRNA process continues with the Drosha nuclear RNase enzyme complex, which cleaves the pri-miRNA into a hairpin-loop pre-miRNA sequence [30-32]. After exportation into the cytoplasm by exportin 5, the majority of the pre-miRNAs are further processed by an RNase-III-type enzyme Dicer into a small double stranded intermediate [32-34]. One strand of this microRNA is loaded onto RNA-induced silencing complex (RISC) [35-38] to direct post-transcriptional repression, when the other strand is degraded [18]. However, recent studies in mice ES cells showed that some miRNAs, such as miR-451 might enter the RISC through an alternative biogenesis pathway independent from Dicer [39]. Occasionally, miRNAs promote the association of proteins to specific AU-rich elements sequences and thus enhance mRNA translation [40].

An important property of miRNAs consists in its ability to target different genes, because of the short 7–8-base pair sequence complementarity required between the miRNAs and the 3′ untranslated region (UTR) mRNA targets. Further to this, only partial complementarity is sufficient for miRNA-induced silencing. Single miRNAs are thus predicted to potentially regulate hundreds of different targeted genes simultaneously and thus may share the regulation of common cellular processes [41, 42].

In addition to their role in transcriptional regulation, post-transcriptional control by small non-coding RNAs is central for many biological and disease processes in vivo and in vitro. miRNAs were shown to modulate the metabolism of both animals and plants [43-45]. miRNAs are further involved in several biological processes such as development, cell proliferation, differentiation, apoptosis, and immunity, reviewed in [18, 46].

Among other roles, miRNAs molecules were found to control and regulate eye and neural crest development [47]. Many studies revealed miRNAs involvement during neural development [48-51]. Many papers exposed the importance of epigenetic regulation in brain development and neurological disorders [52, 53] and revealed that deregulation of microRNAs expression can mediate the pathogenesis of various diseases, including cancer and tumorigenesis [54].

miRNAs Expression During Neurogenesis and Neuronal Differentiation

ESC-specific microRNAs were reported both in human and mouse ESCs lines [55, 56]. Both species share a common miRNA cluster of pluripotency constituted by miR-302b, miR-302c and miR-367 [57]. Human ESC could be characterized by a miRNA expression signature composed of 31 miRNAs [58].

Specific miRNAs are known to play important roles in modulating the proliferation and differentiation of many types of stem cells in many tissues and species, for instance zebrafish, *Drosophila*, mouse, and human embryonic stem cells or adult neural stem cells [59-62]. Among others, microRNAs which target Nanog, Oct4, and Sox2 coding regions modulate embryonic stem cell differentiation [63]. Recently, several studies reported the key role of miRNAs during neurogenesis and neuronal differentiation [64-67].

While some miRNAs like miR-16 are ubiquitously expressed in human tissues, the expression of other miRNAs is tissue specific and is frequently restricted to a specific developmental stage [68]. During brain development, expression pattern of many classes of miRNAs were observed to follow temporal waves [69, 70], suggesting their role in regulating developmental processes.

Mir-9, -124, -128, -132, -134, and-138 were reported as brain-specific miRNAs [71]. MiR-9 and miR-124 are the highly brain-enriched miRNAs induced upon ES cells neuronal differentiation [72].

Sempere and colleagues compared the miRNA profile between embryonal carcinoma cells mouse P19 and human

NTera-2 clone D1 after differentiation into neurons upon RA treatment [73]. This study further demonstrated that some miRNAs were expressed in the brain while others were induced by RA differentiation, including miR-9, miR-124, miR-125, miR-128, and miR-135. Taken together, these studies confirm the importance of miRNA as key regulators of neuronal gene expression during nervous system development and neuronal differentiation.

MiR-9 is a brain-specific miRNA whose nucleotide sequence is conserved across the species even if its functions differ from one species to another. MiR-9 controls the balance between the proliferation and differentiation of neural stem cells. Zhao with colleagues showed that miR-9 and the TLX nuclear receptor. TLX maintains the undifferentiated state and self-renewal properties of neural stem cells mainly through the canonical pathway of Wnt and its downstream effector β-catenin acting on cell cycle proteins expression such as cyclin D1 [74], forming a feedback regulatory loop in mouse [23]. Promoting cell self-renewal with the increase of TLX expression level, this feedback loop lead to neural cell differentiation when a higher miR-9 expression ensure cell fate transition [75]. Interestingly, the opposite effect was observed in human embryonic stem cells-derived neural progenitor cells (NPCs) [76]. In those human cells, miR-9 main target is a microtubule-associated protein named stathmin that plays a role in cellular proliferation and migration orientation. MiR-9 and stathmin's expression are inversely correlated. When miR-9 promotes NPCs proliferation, this in parallel limits cell migration to injury sites by direct modulation of stathmin at the post-transcriptional level [77]. By targeting the TLX receptor or stathmin, miR-9 can therefore play opposite functions and direct cell fate in a temporal way.

Starting from a quite undetectable expression level during neurogenesis, miR-124 expression progressively increases during neuronal progenitors differentiation and finally becomes the most abundant miRNA in the adult brain [71]. This expression profile suggests a regulatory implication of miR-124 in the transition progenitor to neuronal genes and therefore in the switch between non-neuronal to neuronal gene expression [78].

Li-Chun Cheng, Erika Pastrana and colleagues [64] showed in mouse adult brain that miR-124 is expressed at low level in the subventricular zone. Mostly expressed in mature neurons throughout the brain and in the sensory neurons of the olfactory bulb, miR-124 is absent from glial cells (oligodendrocytes or astrocytes). In contrast, miR-124 in Sprague-Dawley rat brain is one of the most abundant miRNA oligodendrocyte progenitor cells; but its expression level decreases during cell maturation, which correlates with its role in neuronal cell specialization [79].

Interestingly, microglial cells also express miR-124 in the CNS. This unique hematopoietic cell lineage remains in a quiescent state, watching the CNS microenvironment, unless encountering neuromodulator molecules and receiving signal from surrounding cells such as neurons or astrocytes [80]. In consequence, microglia gets activated and undergoes morphological and phenotypical changes to acquire "brain macrophages" characteristics and ensure the maintenance and restoration of the CNS [81]. Microglia has been identified as a key player in inflammatory response, autoimmune and neurodegenerative diseases such as multiple sclerosis and Alzheimer's disease [82, 83].

Using the experimental autoimmune encephalomyelitis mouse model, Ponomarev and colleagues demonstrated that miR-124, highly expressed in the CNS microglia, is able to maintain its quiescent status and contain macrophage activation, as well as to protect from diseases development by regulating the expression of many genes [84, 85]. Those findings suggest that miR-124 could be involved in many other inflammatory diseases requiring macrophage activation.

Both miR-9 and miR-124 target members of the anti-neural transcriptional repressor REST complex and thus allow the expression of neuronal genes. Another direct target of miR-124 is the PTBP1 mRNA. During neuronal differentiation, the miR-124 binding decreases PTBP1 expression level and triggered brain-specific alternative pre-mRNA splicing. This leads to the transition from non-neuronal to neuronal specific profile [86].

Both miR-124 and miR-132 exert their growth-promoting effect by regulating the activity of small Rho GTPases, which are crucial regulators of the dendritic actin cytoskeleton.

MiR-132 is a brain-enriched miRNA target of the cAMP-response element binding protein (CREB) transcription factor. CREB, by inducing immediate early genes, is involved in the regulation of axonal and dendritic development and particularly in peripheral neurons where it is a candidate in NGF-induced axonal outgrowth regulation. Is has been shown that miR132 plays a role in early neuronal morphogenesis by downregulating the expression of p250GAP, a NMDA receptor-associated RhoGAP protein that regulates neurite outgrowth via Rac/cdc42 signaling. P250GAP also regulates neuronal differentiation by interacting with a glutamate receptor subunit, the neuronal PDZ protein PSD-95 (postsynaptic density 95), and β-catenin involved in the function of miR-9 [87]. Thus, neurotrophins like BDNF and NGF trigger CREB-induction of miR-132 expression that in turn downregulate p250GAP inducing neuronal survival, dendritic and neurite outgrowth and development [88, 89].

Neuronal activity induces the activation of the transcription factor MeF2 that in turn increases the transcription of a miRNA cluster containing miR-134 [90]. In distinct compartments of dendritic cells, this miRNA can target and inhibit different mRNAs.

In the dendritic RNA granules, miR-134 negatively controls dendrite growth and activity-dependent remodeling by specifically targeting the RNA-binding protein Pumilio (also known as PuM2).

At the dendritic spines of mature hippocampal neurons, miR-134 inhibits the translation of an mRNA encoding a protein kinase, Limk1, involved in actin filament polymerization. Therefore, miR-134 negatively regulates dendritic spine growth and the size of postsynaptic sites [20, 43, 91].

MiR-135 was identified as a cortex-specific miRNA in mouse brain [71]. A study on classic Hodgkin lymphoma (cHL) patients revealed that miR-135a affects apoptosis and proliferation. In cHL, L-1236, and HDMYZ cell lines, miR-135a directly targets JAK2, a member of the JAK/STAT signaling pathway. Through the decrease in JAK2 protein levels, miR-135 is involved in the control of this signaling pathway and promotes apoptosis and lower proliferation levels through the regulation of the Bcl-xL antiapoptotic gene expression [92]

A Cross-Talk Between Neurotrophins and miRNAs

During brain development, many signaling pathways cross-talk to generate mature and functional neuronal tissues. Among other growth factors, neurotrophins play important roles in neural stem cells, during neuronal differentiation and development, and in mature neurons. BDNF, NGF, neurotrophin 3 (NT-3) and neurotrophin 4 (NT-4) are important members of the neurotrophin family [93].

The presence of those neurotrophins in the cellular environment results in the activation of members of the tropomtosin-related kinase (Trk) receptor family, respectively TrkB, TrkA, and TrkC, followed by several intracellular cascades including the MAPK/ERK1/2, PI3K (phosphatidylinositol 3-kinase), PLCγ (phospholipase C-gamma) pathways and their downstream effectors [94]. In NSC, all BDNF, NGF, NT-3, and NT-4 were shown to activate the PI3K/Akt pathway and the MAPK pathway [95-97].

Neurotrophins Signaling Pathways

The neurotrophin factor BDNF is essential for many neuronal aspects, including proliferation, differentiation and survival of neuronal stem cells in the CNS. Following its binding to the TrkB receptor, several canonical pathways such as the Erk/MAP kinase AKT, CREB and JAK/STAT3 are activated [98]. In addition, BDNF promotes synaptic maturation and modulates synaptic plasticity, including long-term potentiation (LTP) [99]. In cultured rat hippocampal neurons, it was demonstrated that depending on the delivery of BDNF, distinct molecular pathways were induced and influenced cell survival, morphology, and functions. Thus, acute application of BDNF triggered a transient increase in TrkB resulting in a transient Ras-MAPK signaling and CREB phosphorylation, promoting neurite elongation. Conversely, the gradual increase of BDNF elicits sustained activation of TrkB receptor that results in a sustained Rap1-MAPK signaling associated to a long-lasting CREB activation. This pathway facilitates neurite branching and spine–neck elongation in early development [100].

NGF acts by activating the transmembrane tyrosine kinase A (TrkA) homodimer receptor or the heterodimer made up of TrkA and the p75 neurotrophin receptor (p75NTR), thereby inducing downstream signaling leading to increased survival [101]. Contrary to the TrkB receptor that is broadly expressed in the brain, TrkA expression is restricted to neuronal-specific population such as cholinergic cells in the basal forebrain. Those cells express TrkA in abundance and exogenous application of NGF leads to increased cholinergic input to the hippocampus. Frielingsdorf and colleagues have shown that NGF increased hippocampal cholinergic activity and thereby promoted the growth, differentiation, and survival of cholinergic neurons in the basal forebrain [102]. NGF was found not to affect proliferation of progenitor cells in the dentate gyrus granule cell layer. NGF is known to promote survival of newly born neurons and more generally neurogenesis in adult rat hippocampus [102].

The neurotrophins' downstream PI3K-Akt signaling pathway is essential for neuronal survival as well as for the development of dendrites. Kumar and colleagues demonstrated that Ras signals via both PI3K-Akt and MAPK pathways that cooperate to promote elongation and dendritic filopodia-like protrusion growth in dissociated postnatal hippocampal CA1/CA3 neuronal cultures [103]. Those two pathways then converge to the mammalian target of rapamycin, mTOR positively regulated by insulin/IGF-1 signaling and known to control many cellular processes such as neuronal proliferation, cell size, and synaptic plasticity [104, 105]. In embryonic NSCs, mTOR activation is improved by the Notch pathway [106] and by the pro-apoptosis factor CD95 though the PI3K/Akt pathway in adult NSCs. Activated mTOR complex then promotes global mRNA translation, increased survival, and enhanced neuronal differentiation of NSCs [104, 107]. In a more recent in vitro study of primary cultures of mouse neural stem/progenitor cells, EGF and FGF2 activation of PI3K and mTOR were shown to contribute independently but concomitantly to the maintenance of the stem cell state of neural stem/progenitor cells [108].

Ras-dependent activation of PI3K-Akt depends on mTOR function and is the major pathway by which neurotrophins convey cell survival-promoting signals.

miRNAs Role in Neurotrophins Pathway Signaling Regulation

In addition to regulating gene expression and neuronal differentiation via the activation of transcription factors, BDNF was also found to affect miRNA expression levels. Interestingly BDNF has a significant positive impact on the brain-specific miRNA miR-132 whereas miR-9, miR-124, miR-128a, miR-128b, miR-134, miR-138, and miR-16 were not affected by the presence of this neurotrophin in cultured cortical neurons [88].

Thus, among other targets, BDNF triggers the rapid induction and persistent expression of mature miR-132 that was shown to positively regulate neuronal morphogenesis and promote neuronal outgrowth [22, 89, 109]. A study in PC12 cell line and neonatal rat cortical neurons reveals that miR-132 is enriched in neurons. Moreover, miR-132 is a member of the intronic miR-212/miR-132 cluster of a non-coding immediate early gene. Those are the most responsive microRNAs to BDNF treatment. It was demonstrated in cultured primary cortical mouse neurons and primary murine fibroblast cultures, that miR-132 and 212 were regulated by the ERK1/2 pathway with or without involvement of the nuclear mitogen and stress-activated serine/threonine protein kinase MSK [110, 111]. Further to this, BDNF in neurons was shown to induce MSK activation through ERK1/2. MSK, by successive phosphorylation, activates the CREB downstream transcription factor (Fig. 3). This cascade of activation through ERK1/2 and CREB cooperation thus enhance the transcription of pri-miR-212/132 [112, 113]. miR-132 acts via the Rac1/cdc42 signaling by decreasing the level of p250GAP, a member of the Rac/Rho family of GTPase-activating protein that also interacts with the glutamate receptor NR2A/B subunits, PSD-95, and β-catenin. This miR132/p250GAP pathway also promotes spine formation by modulating the Kalirin7-Rac1 signaling [22].

In cultured hippocampal neurons, contrary to cultured cortical neurons, another miRNA activity is influenced by synaptic BDNF release. MiR-134, localized in the synapses of the dendrites, negatively regulates dendritic spine growth and the size of postsynaptic sites. When BDNF is released upon synaptic stimulation, it binds to the TrkB receptor whose dimerization activates downstream cascades such as the Ras-PI3K-Akt-mTOR signaling pathway. The latter plays a role in dendrite formation and regulation of spine growth in cultured hippocampal neurons [103]. miR-134 function is thus blocked and Limk1 inhibition is released, promoting spine growth and development [20].

NGF protects neurons from the pro-apoptotic effects of BIM, a member of the BH3-only proapoptotic subfamily of the Bcl-2 family of proteins. Via the MEK/MAPK pathway, NGF acts on BIM by concomitant acute phosphorylation inhibition and by its longer-term repression of expression in neuronal PC12 cells [114].

NGF-deprived neonatal sympathetic neurons are a common model for neuronal programmed cell death (PCD) studies. Neuronal PCD requires de novo protein synthesis, mitochondrial cytochrome *c* release and caspase activation and can be stopped by neuroprotective agents, such as KCl and cAMP. A general characteristic of this death is the upregulation of BIM that, in its active form, is an integral membrane protein of the mitochondria that, in parallel with the c-Jun N-terminal kinase (JNK) pathway,

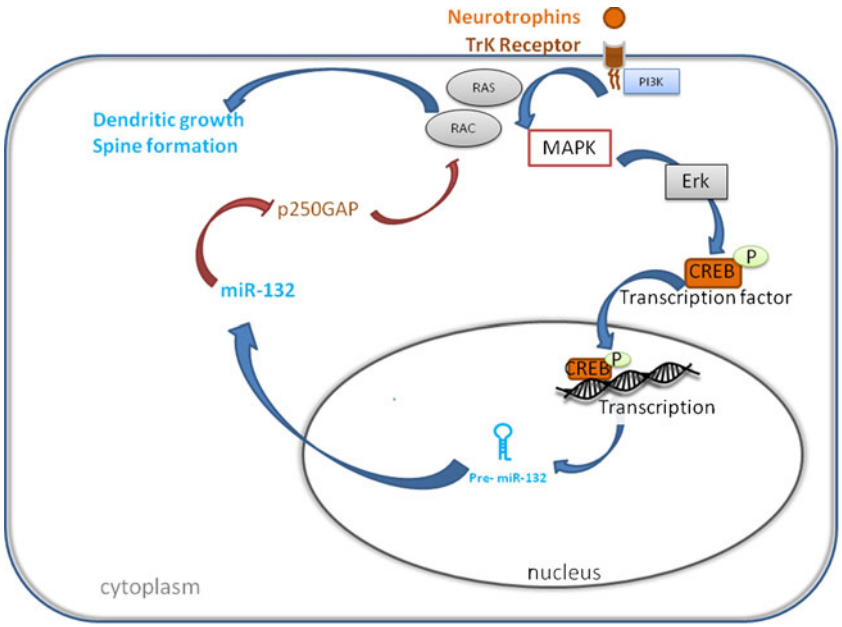

Fig. 3 Schema of the neurotrophin and miRNA pathways crosstalk. BDNF, through sustained activation of the MAPK/ERK1/2 pathway, induces CREB-dependent miR-132 transcription. RISC-miR-132 fixes its target mRNA p250GAP, inhibiting p250GAP protein expression, and consequently promoting spine development

regulates cytochrome *c* release and therefore sympathetic neuronal apoptosis under physiological conditions [115, 116]. When NGF stimulation occurs, activation of ERK1/2 causes phosphorylation of BIM proteins and inhibits their function, resulting in cell survival. Terasawa and colleagues showed that NGF, through sustained activation of the ERK1/2 pathway and de novo protein synthesis, induced the expression of miR-221 and 222 [117]. Furthermore, BIM is a plausible target of those two miRNA derived from the same pri-miRNA and encoded in tandem on the X chromosome (Fig. 3).

miRNAs Regulation of Neuropoietins Cytokine Family and Associated Signaling Pathways in Neurogenesis and Neuronal Differentiation

In response to cytokines such as interleukin-6 (IL-6), LIF or OSM, and growth factors, a transcription activator family is regulated, named the signal transducer and activator of transcription (STAT) family. Active STATs form homo- or heterodimers that translocate to the cell nucleus and thus play a central role in neuropoietin-type cytokine signaling.

1. Neuropoietins cytokine signaling via JAK/STAT pathways and SOCS/PIAS inhibitors

Gp130/OSMRβ receptor complex is dedicated to OSM while the gp130/LIFR complex receptor is shared by LIF and OSM [118]. Cytokines signal transit via the two main signaling pathways JAK/STAT and MAPK. Fixation of the cytokines to the corresponding receptor activates multiple members of the JAK family such as JAK1, JAK2, and Tyk2 in the case of OSM. Then, the signals convey to the STATs family members such as STAT1, STAT3, and STAT5 in many types of cells. The MAPK second signaling pass through the activation of the guanine nucleotide-binding protein Ras and the serine/threonin kinase Raf to promote the three following MAPK cascades: ERK, JNK, and p38 protein network. [119].

The JAK/STAT pathway notably involves the STAT3 protein. STAT3 mediates expression of growth-promoting gene products and plays a role in neuronal development and survival. Activation of STAT3 pathway has been shown to inhibit neuronal terminal differentiation and favors differentiation of neural precursors along a glial lineage whereas suppression of Stat3 directly induces neurogenesis and inhibits astrogliogenesis in neural stem cells [120].

The STAT3 transcription factor is assisted in its role by the anti-apoptotic pro-oncogene Bcl-2 [121]. In parallel, Bcl-2 activates the cAMP-response element binding protein CREB, and modulates calcium signaling. The JAK/STAT pathway can be regulated by several negative-feed-back mechanisms including the suppressors of cytokine signaling (SOCS) and protein inhibitor of activated STAT (PIAS) proteins [118, 122]. In many cell types, the expression of the endogenous SOCS proteins is itself induced by IL-6 cytokines family members such as OSM. For instance SOCS1 specifically inhibits the activity of JAK2, SOCS3 binds to gp130 and both plus SOCS5 are capable of inhibiting IL-6, IL-11, and LIF and the biological effect of OSM [118]. Members of the SOCS family are important modulators of the immune and inflammatory response [123, 124], cell survival and differentiation [125], and acts as feedback inhibitor of the JAK/STAT3 pathway. Concerning the PIAS family, PIAS1 in known to inhibit IFN-γ-induced STAT1 signaling while the PIAS3 inhibits STAT3-mediated gene expression after neuropoietins stimulation [118].

2. Neuropoietins cytokine signaling via the Notch pathway and Hes/Hey inhibitors

In parallel with the JAK/STAT pathways, the Notch pathway signaling is crucial for the nervous system development. Depending on the cellular context, Notch receptors and ligands signaling influences cell fate decisions in a positive or negative manner. Notch acts on cell self-renewal and differentiation as well as on cell proliferation and apoptosis [126]. In order to determine mammalian stem cells lineage decision, Notch activates the expression of key lineage specific transcription factors such as Hes5, Pax6, Sox9, and Id proteins in mouse ES [127].

Conversely and at the same time, Notch can restrict neural differentiation by targeting the genes of the helix–loop–helix repressors called hairy/enhancer of split (Hes)/Hey family which repress the expression of pro-neural genes and thus blocks stem cell differentiation [127]. Schwanbeck and colleagues proposed a model for the context-dependent function of Notch signaling in stem cell differentiation [126]. Notch would activate specific transcription factors to assure lineage determining when in parallel a feedback loop involving Hes/Hey transcriptional repressors would favor lineage specific stem and progenitor cells expansion [126]. In primary cultures of rat cortical neurons, inhibition of the Notch response using DAPT, a γ-secretase inhibitor, as well as the activation of the SHH response results in enhanced neuronal differentiation [128, 129].

In the murine telencephalon, astrocytic differentiation of neural progenitors requires Notch and an active JAK/STAT pathway. Notch favors the activation of the JAK/STAT pathway through the demethylation of astrocyte-specific promoters. This is an essential step for the induction of the astrocyte fate by the JAK/STAT pathway triggered by the nuclear factor I A [130].

During development, the Notch pathway is instrumental for cell binary fate decision [130], opposing neural to epidermal lineages during early fate decisions. However,

during late fate decisions, Notch can influence the balance to promote specialization into the different subtypes of neural cells.

More than influencing this binary fate decision in the developing nervous system, the active form of Notch can protect against apoptosis by several mechanisms including activation of PI3K-PKB/Akt signaling [131-133]. In addition, Notch plays a role in brain tumor biology as Notch receptors were found to be overexpressed in many different brain tumor types [134].

3. miRNAs in the neuropoietins pathway

Cytokines binding to their specific receptors (for instance OSM to the Gp130/OSMRβ receptor complex) leads to the activation of several downstream proteins by a cascade of phosphorylation [118-135]. Among others proteins, conformational changes of the receptor allows the phosphorylation of JAK2 proteins that afterwards set off STATs phosphorylation and dimerization [136]. Among the members of the STAT family, STAT3 and STAT5 are the preferred downstream targets of phosphorylated JAK2 [137]. However, a miRNA has been identified in cHL cell lines to control the cascade of protein activation through the JAK/STAT pathway. MiR-135a was shown to target the mRNA encoding JAK2 and thereby to regulate the downstream protein network including STAT5 and the pro-survival protein Bcl-xL that plays an important role in tumor pathogenesis [92].

Brain-specific miR-124a and miR-9 were shown to affect STAT3 phosphorylation levels influencing neural lineage differentiation in the ES cell-derived cultures. During neuronal development, STAT3 plays an essential role by promoting neural precursors along a glial lineage in spite of neuronal terminal differentiation. In ES-derived neural precursor cells, miR-9 inhibition leads to an increase in STAT3 active form expression level while miR-124a and miR-9 overexpression reduced the level of phosphorylated STAT3. Using anti-sense miRNAs, it was shown that early overexpression of miR-124a in neural progenitors prevented gliogenesis, whereas miR-9 expression contributed to neurogenesis [72].

Many studies on *Drosophila*, zebrafish, and *C. elegans* demonstrated that miRNAs directly regulated Notch target genes. First identified in *Drosophila* in the 3′ UTRs of Notch pathway target genes encoding basic helix–loop–helix (bHLH) repressors and Bearded family proteins are three 3′-UTR sequence motifs, the K box (cUGUGAUa), the Brd box (AGCUUUA), and the GY box (uGUCUUCC) which mediate negative post-transcriptional regulation. Those boxes are complementary to the 5′-ends of various *Drosophila* miRNAs. The GY box has the particularity to mediate miRNA fixation through the proneural box (AUGGAAGACAAU), a motif located in the 3′-UTRs of transcripts encoding proneural bHLH factors that act in the Notch pathway [138]. miRNA targeting specifically those three boxes have been identified: GY-box-containing 3′-UTRs are inhibited by miR-7, those with Brd boxes by miR-4 and miR-79 that are both expressed at high levels during embryonic development, and those with K boxes by miR-2 and miR-11. Two enhancer of split-Complex (E(spl)-C) genes with GYboxes have since been validated as miR-7 targets [139].

Members of a specific subset of E(spl)-related repressors, named the Hey genes, contain a preponderance of Brd-,

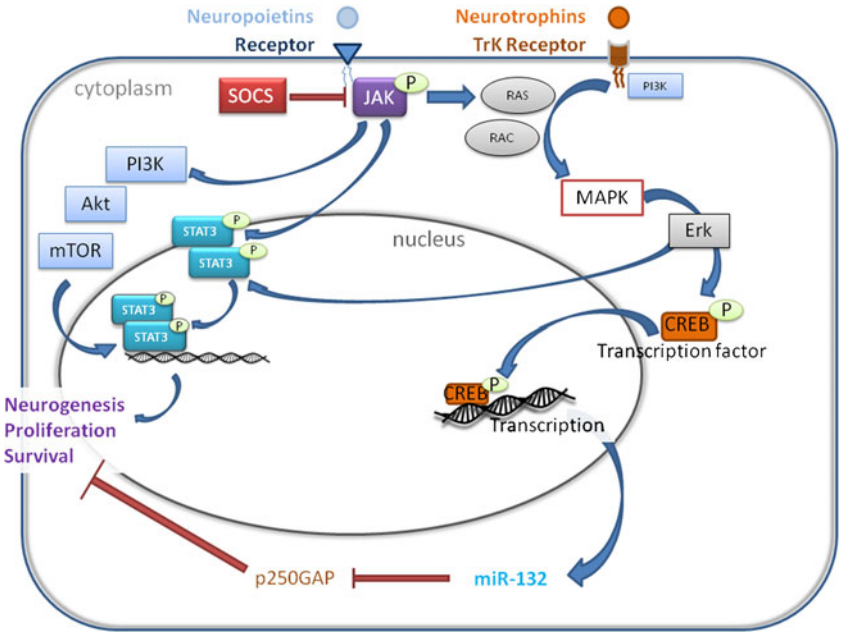

Fig. 4 Schema of the neurotrophin, neuropoietins, and miRNA pathways crosstalk. Schema of the neurotrophin and miRNA pathways crosstalk. BDNF, through sustained activation of the MAPK/ERK1/2 pathway, induces CREB-dependent miR-132 transcription. RISC-miR-132 fixes its target mRNA p250GAP, inhibiting p250GAP protein expression, and consequently promoting spine development. Concomitantly the neurotrophin pathway, via the JAK/STAT signaling pathway enhances gene translation in favor of neurogenesis, proliferation and cell survival

GY-, and K-boxes in their 3′-UTRs. This appears to be the case in a variety of mammals including humans, suggesting that miRNA-mediated regulation of Notch target genes may be a conserved feature [126, 140]

Upon treatment with either DAPT or cyclopamine [141], identified miRNAs whose expression changed upon inhibition of the Notch and Hedgehog pathways. Therefore, they identified a series of negatively or positively modulated microRNAs from which some are involved in the Notch pathway and key neuronal processes. This is the case of miR-204, miR-29b, miR-29a, miR-296, miR-155, and miR-130b.

Figure 4 illustrates the crosstalk between the three pathways presented in this review with regard to the both cytokine signaling pathways (i.e., neurotrophin and neuropoietin), with the miRNA network regulatory system. Combining many intra- and extracellular factors, cell fate depends on the balance between proliferation, survival or apoptosis, and at times cell differentiation.

Conclusions and Perspectives

The discovery of miRNAs gives rise to new possibilities in terms of epigenetic modulation of stem cell lineage differentiation and specification for future cell therapy. Currently, the main challenge is identification of specific targets of miRNA molecules and the downstream mechanism of its activity. This knowledge will allow for more precise regulation of stem cell differentiation fates and better diagnostics of the many disorders of the central nervous system.

Many studies demonstrated that in vitro transient overexpression or inhibition of brain-specific miRNAs in stem cells significantly directed differentiation along neuronal cell lineages conversely with the glial lineage and thus affect the balance between neurogenesis and gliogenesis. Therefore, modulating miRNA expression offers new pathways of post-transcriptional gene regulation and can guide stem cell commitment. Identification of brain-specific miRNAs responsible for neurogenesis offers good clues for the selection of miRNA candidates to get stem-cell-derived neurons applicable in personalized regenerative medicine, or, to reprogram endogenous neuroblast differentiation fates in vivo.

From a clinical perspective, miRNA offers a promising tool for in situ silencing of aberrant gene expression as an alternative for the siRNA technique. miRNA modulation also offers a promising tool in stem cell reprogramming for regeneration of injured CNS. Regulation of cell fates by epigenetic tools will be safer in comparison to direct reprogramming by overexpression of transcription factors. Cell reprogramming into neuronal fates could be especially effective with easily accessible adult mesenchymal stem cells [142-144] expressing already a number of neuro-epithelial genes (neurotrophin receptors TrkA and B and their neurotrophin ligands, the neuronal transcription factors of bHLH family, STAT3 and CREB among others) ([145]; Jurga et al., unpublished data). Moreover, MSC are already in clinical use to treat a number of neural disorders, e.g., ischemic stroke (NCT00875654, NCT01091701), spinal cord injury (NCT01162915, NCT00816803), and Parkinson's disease (NCT00976430; all these trials' details available on www.clinicaltrials.gov).

Increasing our fundamental knowledge of the miRNA genetic network offers good perspectives where miRNAs may be exploited to develop new, safer cellular therapies, and diagnostic tools in neuroscience.

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