Molecules and Cells

Minireview

Neurodevelopmental Aspects of RASopathies

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***RAS* gene mutations are frequently found in one third of human cancers. Affecting approximately 1 in 1,000 newborns, germline and somatic gain-of-function mutations in the components of RAS/mitogen-activated protein kinase (RAS/MAPK) pathway has been shown to cause developmental disorders, known as RASopathies. Since RAS-MAPK pathway plays essential roles in proliferation, differentiation and migration involving developmental processes, individuals with RASopathies show abnormalities in various organ systems including central nervous system. The frequently seen neurological defects are developmental delay, macrocephaly, seizures, neurocognitive deficits, and structural malformations. Some of the defects stemmed from dysregulation of molecular and cellular processes affecting early neurodevelopmental processes. In this review, we will discuss the implications of RAS-MAPK pathway components in neurodevelopmental processes and pathogenesis of RASopathies.**

Keywords: neurodevelopment, RAS, RASopathy

INTRODUCTION

RAS proteins function as a signal relay molecules that can transmit receptor activation by external stimuli such as growth hormones or environmental stress to downstream effectors leading to major cellular responses such as proliferation, survival and differentiation (Bourne et al., 1990). Somatic gain-of-function mutations in *RAS* genes are found in one third of human cancers (Li et al., 2018) and thus RAS pathway has been extensively studied in the context of oncogenesis. Since the discovery of *NF1* mutations in neurofibromatosis 1, germline mutations in the components of RAS signaling pathway also have been found in some congenital disorders such as Noonan, LEOPARD (Lentigines, Electrocardiographic conduction abnormalities, Ocular hypertelorism, Pulmonic stenosis, Abnormal genitalia, Retardation of growth, Deafness), Cardio-facio-cutaneous and Costello syndromes suggesting that aberrant RAS signaling may contribute to the pathogenesis of developmental disorders as well. Genes mutated in these diseases include *HRAS*, *KRAS*, *BRAF*, *NF1*, *SOS1*, *PTPN11* (which encodes SHP2), and *MEK* (Fig. 1C). The developmental disorders associated with RAS pathway mutations, collectively known as RASopathies, share clinical features such as craniofacial, cardiac, cutaneous, musculoskeletal and ocular abnormalities. Neurological abnormalities including neurocognitive impairment, hypotonia, macrocephaly, and seizure are also present to varying degrees (Rauen, 2013).

RAS proteins, *KRAS*, *HRAS,* and *NRAS*, are small GTPases cycling between an active guanosine triphosphate (GTP)-bound and inactive guanosine diphosphate (GDP)-bound conformations. RAS proteins are tightly regulated by GTPase activating proteins (GAPs) and guanine nucleotide exchange factors (GEFs). When extracellular stimuli activates receptor tyrosine kinases (RTKs), docking sites for adaptor molecules and signal-relay proteins, such as *GRB2* and *SHP2*, are created. GEFs (e.g., SOS1) are then recruited and displace GDP from RAS allowing RAS to bind to GTP, which is abundant in the cytosol (Fig. 1C). GTP-bound RAS can activate a large

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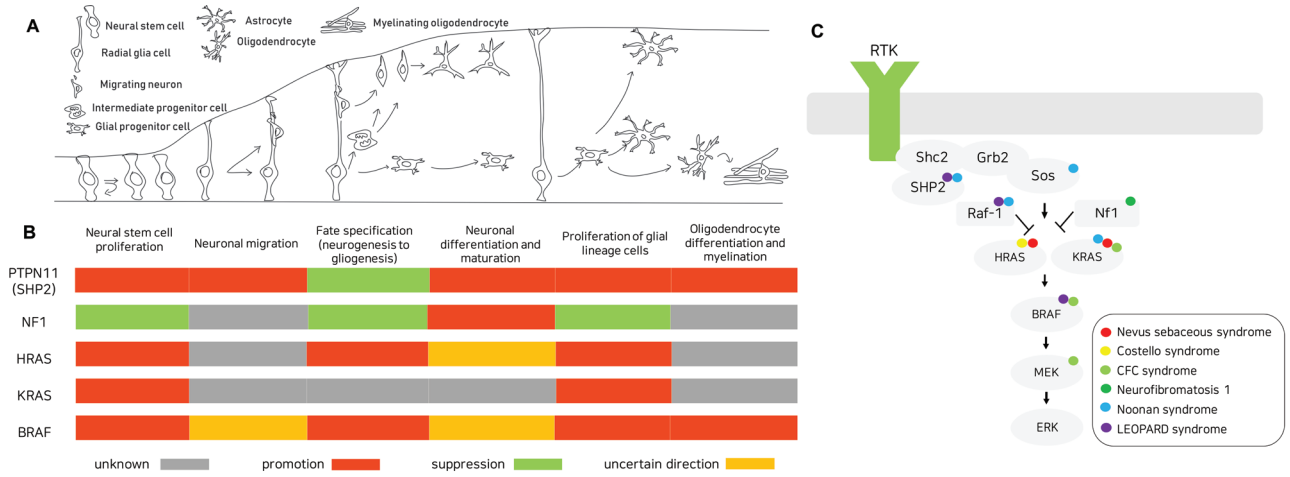

Fig. 1. The role of RAS pathway in neurodevelopmental process. (A) The simplified view of the corticogenesis. For more detail information, refer to Hanashima and Toma (2015); Paridaen and Huttner (2014). The neurodevelopmental process progresses from left to right. Neuronal differentiation, proliferation of glial lineage cells, and oligodendrocyte differentiation and myelination occur at the same time in different cell types. (B) The role of components in the RAS/MAPK pathway in neurodevelopmental process. Each column is listed in the chronological order roughly, matching with Figure 1A. Red represents that the corresponding proteins promotes the step of column, in contrast, green means that the corresponding proteins suppresses the step of column. Gray indicates that the relationship of gene and each step is unknown. Yellow indicates that the genes are involved in each step, but it is uncertain that those genes promote or suppress the process. Of note, NF1 is a negative regulator of RAS/MAPK pathway, unlike the other genes. (C) The RAS/MAPK pathway and the disorders involving somatic/germline mutations of related genes. RTK, receptor tyrosine kinase; Nf1, neurofibromatosis type 1.

number of effector pathways including RAF family of proteins. The mitogen-activated protein kinase (MAPK) pathway is best-characterized RAS effector pathway.

Previous studies have shown that majority of mutations found in RASopathies are not as robustly activating as those associated with human cancers which may explained by embryonic lethality resulting from the germline mutations. More recently, however, the same oncogenic somatic mutations in *HRAS* and *KRAS* are found in nevus sebaceous syndrome (NSS) suggesting that the broader involvement of RAS signaling pathway in the pathogenesis of developmental disorders (Groesser et al., 2012). These findings support the general perspective that the degree and duration of enhanced and/or dysregulated RAS signaling as well as cell types should be considered for its role in oncogenesis and developmental disorders. In this review, we will focus on the developmental functions of several genes associated with RASopathies including *NF1*, *PTPN11*, *SOS1*, *HRAS, KRAS*, *RAF1*, and *BRAF*, and their roles in the pathogenesis of RASopathies. For simplicity, we used gene sets as human gene symbols.

NF1 AND NEUROFIBROMATOSIS TYPE I

Neurofibromatosis type I is autosomal dominant disorder caused by loss-of-function-mutations in *NF1* (Castle et al., 2003). *NF1*, functions as a GAP, negatively regulates RAS pathway. Individuals with neurofibromatosis type 1 are predisposed to specific cancers, and showed neurological deficits including cognitive disability, cerebrovascular defects, malformations (Williams et al., 2009). Some individuals with neurofibromatosis type 1 show brain structural abnormalities such as macrocephaly, increased white matter size, and polymicrogyria (Cutting et al., 2002; Karlsgodt et al., 2012; Ruggieri et al., 2011). Since *NF1* is frequently associated with human cancers, initial studies have focused on its roles in cell proliferation. In 1999, Gutmann and his colleagues reported that *NF1* haploinsufficiency promotes astrocyte proliferation (Gutmann et al., 1999). Later studies have identified the cell type- and brain region-specific function of *NF1* loss. Studies with *NF1* knockout and conditional knockout mice have been shown that depletion of *NF1* results in increased proliferation of neuroglial progenitor cells, noticeably in astrocyte and oligodendrocyte (Bajenaru et al., 2001; 2002; Dasgupta et al., 2003). In *NF1*-deficient brains, the number of proliferative cells in the rostral migratory system, corpus callosum, cortex, striatum, and subventricular zone is higher than control at the early stage, but most of them differentiate to glial linage (Wang et al., 2012). As a result, neurofibromatosis type 1 animal models showed increased gliogenesis at the expense of neurogenesis. NF1 functions through selective use of downstream RAS effectors. While MEK signaling is involved in the neuroglial progenitor proliferation, PI3K/AKT pathway is involved in neural stem cell proliferation (Chen et al., 2015).

NF1 plays a vital role in neuronal differentiation and morphology (Hegedus et al., 2007; Lee et al., 2010; Zhu et al., 2001). It has been reported that activation of the RAS pathway promotes neurite outgrowth (Arendt et al., 2004; Gärtner et al., 2004a); however, studies have shown that *NF1*-deficient neurons have shorter neurites (Dasgupta and Gutmann, 2005; Hegedus et al., 2007). NF1 regulation of neurite outgrowth seem independent to RAS-MAPK pathway but dependent to PKA-RhoA-ROCK pathway (Brown et al.,

2012). The increase of gliogenesis and immature astrocyte shown in *NF1*-deficient models can explain why individuals with neurofibromatosis type 1 are susceptible to cancers in central nervous system, especially gliomas. Disrupted balance between neurogenesis and gliogenesis may also associated with other clinical manifestation related to central nervous systems.

PTPN11 (SHP2), SOS1, RAF1 AND NOONAN SYNDROME

Noonan syndrome is autosomal dominant disorders affecting one in 1,000 to 2,500 and characterized by distinctive facial features, short stature, chest deformity, congenital heart disease and, in some instances, neurological manifestations (Duenas et al., 1973; Romano et al., 2010). *PTPN11* (which encodes SHP2) mutations explain nearly half of the cases, besides mutations in *SOS1*, *RAF1*, and *KRAS* (Schubbert et al., 2006). The degree of cognitive impairment varies from person to person, but individuals with a mutation in relative upstream of RAS pathway such as *SOS1* or *PTPN11* show mild or no cognitive impairment (Cesarini et al., 2009; Rauen, 2013). *PTPN11* mutations associated with Noonan syndrome are frequently found in the residues that are crucial for auto-inhibited closed conformation resulting elevated phosphatase activity, thus increased RAS signaling, while LEOPARD syndrome (Noonan syndrome with lentigines)-associated *PTPN11* mutants exhibit reduced catalytic activity (Keilhack et al., 2005; Tartaglia et al., 2006). Expression of *PTPN11* mutations found in Noonan syndrome promotes neurogenesis and suppress astrogliogenesis (Gauthier et al., 2007). Conversely, loss of *PTPN11* results in increase gliogenesis but reduce neurogenesis (Ke et al., 2007; Zhu et al., 2018). It has been shown that *PTPN11* functions through multiple downstream signaling to control gliogenesis and neurogenesis. After rescue experiment using pSTAT3 inhibitor and MEK inhibitor, Gauthier et al. (2007) and Ke et al. (2007) showed that *PTPN11* inhibits astrogliogenesis through GP130-JAK-STAT3 pathway and stimulate neurogenesis through MEK-ERK pathway, and these pathways are reciprocal.

Differential regulation through selective use of downstream signaling also has been shown in oligodendrocyte development. When *PTPN11* was deleted in oligodendrocytes in ventral telencephalic region and spinal cord, the proliferation of oligodendrocyte decreased and precocious maturation observed (Ehrman et al., 2014; Zhu et al., 2010). The expression of gain-of-function mutant induced to oligodendrocyte progenitor cell (OPC) proliferation with abnormal myelination (Ehrman et al., 2014). These results suggest that *PTPN11* regulates the oligodendrocyte proliferation and maturation. Noticeably, *PTPN11* function through the MAPK-ERK pathway in OPC maturation but not important for survival and proliferation of OPC. The proliferation of OPC by *PTPN11* seems to be controlled through a different pathway such as AKT signaling (Fyffe-Maricich et al., 2011; Ishii et al., 2012; Liu et al., 2011). *PTPN11* not only involves in neurogenesis but also promotes neuronal differentiation, neurite outgrowth and migration (Gauthier et al., 2007; Huang et al., 2012). The increased neurogenesis, abnormal myelination by gain-of-function mutation of *PTPN11* may explain why some individuals with Noonan syndrome showed neurological manifestations such as motor dysfunction and epilepsy. However, correlation between variability in cognitive dysfunction and mutated genes is not well understood.

SOS1, another causative gene of Noonan syndrome, is GEF of RAS (Chardin et al., 1993). Gain-of-function mutation of *SOS1* is usually found in patients with Noonan syndrome, and most of them are located in PH domain, which can inhibit the formation of auto-inhibitory conformation and results in the activation of RAS/MAPK pathway (Sondermann et al., 2004; Tartaglia et al., 2007). *SOS1* mutation-positive patients with Noonan syndrome have been reported to show neurological abnormalities such as mild cognitive impairment and spinal nerve enlargement (Perrino et al., 2018; Santoro et al., 2018). However, the role of *SOS1* in the neurodevelopment process has been poorly understood. *SOS1* has been reported to be involved in neurite outgrowth by nerve growth factor (NGF) stimulation, forming RAC1/Cdc42 complex in PC12 cells (Aoki, 2005). Tian et al. (2004) showed that *SOS1* is highly expressed in the neonatal cortical tissue and they can activate RAS/ERK/CREB signaling by N-methyl-D-aspartate (NMDA) glutamate receptor through Shc-Grb2 interaction in neonatal cortex. Since NMDA receptor signaling is important for synaptic plasticity, the alteration of this signaling pathway can be related to cognitive delay in Noonan syndrome (Hunt and Castillo, 2012). However, how Noonan syndrome-specific *SOS1* mutations affect on the neurodevelopment has not been actively studied. Considering that *SOS1* positive patients show relative better language ability and adaptive behavior in the patients with other mutations, dysregulation of *SOS1* may show relatively mild neurodevelopmental defects (Pierpont et al., 2009; 2010).

Gain-of-function mutation of *RAF1* is also shown in the Noonan syndrome. Most of these mutations inhibit S621 and S259 phosphorylation, which is important for closed conformation and can act as a binding site of 14-3-3 to maintain inactive state (Kobayashi et al., 2010). About 55% of *RAF1*-positive patients with Noonan syndrome showed intellectual disability, and 95% of them showed relative macrocephaly (Kobayashi et al., 2010). This indicates that *RAF1* is crucial for normal brain development; however, the function of *RAF1* in the neurodevelopment has not been actively studied. *RAF1*-deficient mice show an increase of cell proliferation and apoptotic death, and abnormal differentiation in the hippocampus (Pfeiffer et al., 2018). This may be related to cognitive impairment in Noonan syndrome.

HRAS AND COSTELLO AND NEVUS SEBACEOUS SYNDROMES

Costello syndrome is an autosomal dominant disorder caused by germline mutation of *HRAS* (Aoki et al., 2005). The typical symptoms are severe failure to thrive, cardiac abnormalities, papilloma, and malignant tumors, short stature, hyperkeratinosis and neurological abnormalities including hypotonia, macrocephaly, developmental delay, and intellectual disability (Aoki et al., 2005; Rauen, 2013; Sol-Church et al., 2009). Some individuals with Costello syndrome show brain struc-

tural abnormalities such as poor grey-white matter differentiation, small corpus callosum and small brain stem (Delrue et al., 2003). The *HRAS* mutations occurred in Costello syndrome located in glycine 12 and glycine 13, the frequent oncogenic gain-of-function mutations activating RAS-MAPK pathway (Aoki et al., 2005).

HRAS plays crucial functions during brain development. *HRAS* promotes proliferation of neural stem cells and involved in neuronal morphological development. Induced pluripotent stem cells (iPSCs) derived from Costello syndrome showed increased production of cortical neurons associated with extended progenitor phase (Rooney et al., 2016). Transgenic mice with *HRAS* gain-of-function mutation show aberrant cortical lamination and abnormal neuronal morphology such as cytomegaly and short neurite length (Rooney et al., 2016). *HRAS* activation after postnatal stage also leads to neuronal hypertrophy and more complex dendritic structure and enlarged axons (Gärtner et al., 2004b; Seeger et al., 2003). Another study showed that *HRAS* localize to axon growth cone with PI3K during the formation of axon (Fivaz et al., 2008). *HRAS* also regulates the astrogliogenesis. During transition from neurogenesis to gliogenesis, Paquin et al. (2009) showed that variants found in Costello syndrome suppress neurogenesis but promote astrogenesis. Increased astrogenesis is further maintained at the postnatal stage. Noticeably, astrocyte-specific expression of *HRAS* gain-of-function mutation could influence neuronal morphogenesis by extracellular component (Krencik et al., 2015). These studies suggest non-cell autonomous effects caused by *HRAS* somatic gain-of-function mutation during the brain development.

Somatic mutations of *HRAS* and *KRAS* in the same loci are also found in a group of neurocutaneous disease called NSS which is characterized by sebaceous nevus associated with other abnormalities in brain, eyes and bones (Groesser et al., 2012). Interestingly, the mutations were predominantly found in lesions and associated secondary tumors but not in nonlesional tissues. Studying the function of the oncogenic *HRAS* and *KRAS* mutations in developing brain may provide patho-developmental mechanisms of NSS.

KRAS, BRAF, MEK1, MEK2 AND CARDIO-FACIO-CUTANEOUS SYNDROME

Cardio-facio-cutaneous syndrome is dominant congenital disorder typically characterized by distinctive facial appearance, heart defects and intellectual disability, short stature and skin abnormalities (Niihori et al., 2006). *KRAS* mutations are found in Cardio-facio-cutaneous syndrome and Noonan syndrome. Somatic mutations of *KRAS* also found in NSS (Groesser et al., 2012). However, the location of germline and somatic mutations are distinct in pattern. Oncogenic gain-of-function mutations are frequently found in NSS while more functionally mild mutations are identified in Noonan and cardio-facio-cutaneous syndromes (Groesser et al., 2012; Schubbert et al., 2006; 2007). This may explained by embryonic lethality due to strong germline gain-of-function mutations (Tuveson et al., 2004). Unlike to *NF1*, *PTPN11*, and *HRAS*, function of *KRAS* have not been well studied in the neurodevelopment. Kubara et al. (2018) has been shown that activation of *KRAS* is required for self-renewal of iPSC. In their study, *KRAS* activation by p.G13C heterozygote mutation suppresses neuronal differentiation suggesting its role during the neurodevelopment. *KRAS* activation but not *HRAS* and *NRAS* increases neural stem cell proliferation and astrogliogenesis in consistent with *NF1* studies (Bender et al., 2015).

Besides *KRAS*, mutations in *BRAF*, *MAP2K1* (*MEK1*), and *MAP2K2* (*MEK2*) also found in cardio-facio-cutaneous syndrome. The most frequently mutated gene is *BRAF*, accounting for 75% of mutation-positive cases (Rauen, 2013). Most of these mutations have shown to activate RAS pathway; however, some of them showed impaired kinase activity (Rodriguez-Viciana and Rauen, 2008). Also, *BRAF* mutations found in cardio-facio-cutaneous syndrome are frequently located in the cysteine-rich domain or protein kinase domain while mutations found in cancers are located in catalytic domain (Rauen, 2013; Sarkozy et al., 2009). Like other RAS pathway components, *BRAF* plays important roles in neurodevelopmental processes. Firstly, *BRAF* is essential for maintenance of neural progenitor pool and proliferation of neural stem cells (Camarero et al., 2006). Patient-derived neural stem cells carrying *BRAF* p.Q257R mutation showed premature neural differentiation resulting rapid depletion of neural progenitor pool (Yeh et al., 2018). Since many subtypes of cells are derived from neural progenitor cells in highly sophisticated spatiotemporal order (Hanashima and Toma, 2015; Paridaen and Huttner, 2014) (Fig. 1A), depletion of neural progenitor pools can lead to the imbalance of neuronal and glial cells. *BRAF* is also involved in the neuronal development such as survival, migration, differentiation and maturation. *BRAF* promotes neuronal survival by reducing cAMP-mediated *Raf-1* (*C-raf*) inhibition and activating MEK (Dugan et al., 1999). Also, it promotes the survival of motor neuron and sensory neuron (Wiese et al., 2001). Unlike other RAF proteins, *BRAF* activity is critical for neuronal migration. When *B-raf* is substituted by *A-raf*, cortical upper layer neurons cannot migrate to cortical plate. Similarly, constitutive activation of *BRAF* leads to abnormal cortical lamination (Koh et al., 2018). Studies with knockout mice have shown that RAF activity, especially *RAF1* and *BRAF*, is essential for neuronal maturation and axon projection (Zhong et al., 2007). It has been speculated that RAF activity over a certain level is required for normal neuronal development. Besides promoting gliogenesis, *BRAF* signaling function as a positive regulator of astrocyte proliferation. Using strong gain-of-function mutation of *BRAF* (human V600E, mouse V637E), it has been shown that BRAF activation during embryonic stage increase proliferation of glial lineage cells in cortex and spinal cord (Koh et al., 2018; Li et al., 2014; Tien et al., 2012). *BRAF* activation at the adult stage also showed hyper-proliferation of astrocyte leading to astrocytoma (Gronych et al., 2011). Similarly, *RAF1* also promotes astrocyte proliferation in an autocrine/paracrine manner *in vitro* (Rhee et al., 2016).

BRAF also stimulates the oligodendrocyte maturation and differentiation. When *BRAF* is ablated, oligodendrocyte maturation is impaired (Galabova-Kovacs et al., 2008). They suggested that *BRAF* induce the oligodendrocyte differentiation and myelination by forming a complex with downstream

MAPK-ERK components, and *BRAF* can act as a rate-limiting activator. This result is concordant with previous findings that MAPK-ERK pathway plays important roles for oligodendrocyte maturation (Fyffe-Maricich et al., 2011; Ishii et al., 2012). Developmental defects in oligodendrocyte associated with BRAF mutations may explain some clinical manifestation such as thin corpus callosum and reduction of white matter volume (Yoon et al., 2007). A strong gain-of-function mutation of *BRAF* may associated with epilepsy (Koh et al., 2018; Prabowo et al., 2014; Urosevic et al., 2011). Koh et al. (2018) reported that constitutive activation of *BRAF* can lead to a morphological change of neuron and epileptogenesis. Yeh et al. (2018) also reported that neuronal precursor cells derived from cardio-facio-cutaneous syndrome patients with *BRAF* p.Q257R produce the neurons with high intrinsic excitability. *BRAF* participates in the various step of neurodevelopment processes such as maintenance of neural progenitor pool, fate specification, gliogenesis, and oligodendrocyte differentiation (Fig. 1B). However, most studies have focused on neurodevelopmental defects caused by ablation of *BRAF* or strong activation found in human cancers. Thus, how these findings are related to cardio-facio-cutaneous syndrome should be addressed more carefully.

CONCLUSION REMARK

The shared neurodevelopmental aspect of RAS-MAPK pathway activation is the enhanced proliferation of neural stem cell leading to hyper-proliferation and expansion of glial lineage cells. The imbalance of neuro-glial cellular subtypes during the brain development explains some neurological manifestations seen in RASopathies. Developmental defects in brain may associated with neurocognitive deficit as well as structural brain defects. As evident in oligodendrocyte development, cell type- and brain region-specific functions of RAS pathway components may explain diversity of clinical spectrum among RASopathies, especially clinical features related to myelination, white matter volume, and corpus callosum. Other developmental defects such as neuronal migration and neuronal morphology defect may also explain the structural brain defects and disrupted circuit formation affecting neurocognitive functions. Further studies would find detailed cellular and molecular mechanisms underlying clinical manifestations and its roots during neurodevelopment. Revealing these mechanisms would help to treat or to delay the progression of RASopathies at the earliest point as possible.

Disclosure

The authors have no potential conflicts of interest to disclose.

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