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Costello syndrome H-Ras alleles regulate cortical development

Annie Paquin [a,e], Christian Hordo [a,b,c], David R. Kaplan [b,c,e], Freda D. Miller [a,c,d,e,*]

[a] Stem Cell Biology Program, Hospital for Sick Children, Toronto, Canada
[b] Cell Biology Program, Hospital for Sick Children, Toronto, Canada
[c] Department of Molecular Genetics, University of Toronto, Toronto, Canada
[d] Department of Physiology, University of Toronto, Toronto, Canada
[e] Institute of Medical Science, University of Toronto, Toronto, Canada

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ABSTRACT

Genetic mutations in H-Ras cause Costello syndrome (CS), a complex developmental disorder associated with cortical abnormalities and profound mental retardation. Here, we have asked whether there are perturbations in precursor cell proliferation, differentiation, or survival as a consequence of expressing CS H-Ras alleles that could explain the cognitive deficits seen in this disorder. Two different H-Ras alleles encoding mutations present in CS patients, H-RasG12V and H-RasG12S were expressed in cortical progenitors in culture and in vivo by in utero electroporation and their effects on cortical precursor cell fate examined. Expression of both mutants in cultured precursors inhibited neurogenesis and promoted proliferation and astrogenesis. In vivo, expression of either form of CS H-Ras promoted cell proliferation and inhibited neurogenesis. Moreover, these H-Ras mutants promoted premature gliogenesis, causing formation of astrocytes at a time when normal gliogenesis has not yet begun, ultimately leading to an increase in the number of astrocytes postnatally. Thus, aberrant H-Ras activation enhances neural precursor cell proliferation, and perturbs the normal genesis of neurons and glial cells, effects that likely contribute to the cortical abnormalities and cognitive dysfunction seen in CS.

Introduction

During mammalian development, the cerebral cortex is generated through a heterogeneous pool of precursor cells that can produce neurons and glial cells. Emerging evidence suggests that growth factors determine the precise timing and extent of the genesis of these two cell types (Miller and Gauthier, 2007). In vivo, cortical neurogenesis starts early in development at embryonic day 12 (E12) and ends at around E18, at which point astrogenesis starts and continues through the first weeks of life. These sequential waves of differentiation are regulated by both intrinsic mechanisms and growth factors in the neural environment. Many of these growth factors converge onto the Ras signaling pathway, which has been shown to play a central role in regulating neural precursor cell proliferation, survival, and cell fate decisions (Samuels et al., 2009). With specific regard to the developing cortex, aberrant H-Ras activation in postmitotic neurons causes neuronal cell bodies to hypertrophy and subsequent ventricular enlargement (Heumann et al., 2000; Hennekam, 2003). Moreover, components of the Ras signaling cascade, including SHP-2, MEK, ERK2 and C/EBP have been shown to be play important roles in cortical precursor cell neurogenesis (Gauthier et al., 2007; Ménard et al., 2002; Paquin et al., 2005; Samuels et al., 2008, 2009). In contrast, activation of the gp130-JAK-STAT pathway by cardiotrophin-1 has been shown to be required for astrocyte formation (Bonni et al., 1997; Barnabé-Heider et al., 2005).

Recent evidence suggests that germline mutations in Ras pathway proteins are the cause of the neuro-cardio-facial-cutaneous (NCFC) syndromes (Bentires-Alj et al., 2006). These syndromes include Noonan syndrome, Costello syndrome (CS), neurofibromatosis-1 (NF1), LEOPARD syndrome, and cardio-facio-cutaneous syndrome. Individuals with these disorders present with a combination of facial abnormalities and heart defects. Importantly, mental retardation and cognitive dysfunction are also common. In this regard, we recently asked whether aberrant development of neural precursors might contribute to the cortical abnormalities seen in this family of disorders, and focused upon Noonan syndrome, where the SHP2 protein tyrosine phosphatase is aberrantly activated (Gauthier et al., 2007). These studies demonstrated that SHP2-mediated dysregulation of the MEK-ERK and gp130-JAK-STAT pathways caused aberrant genesis of neurons and glial cells in the embryonic cortex, and provided support for the idea that neural precursor dysfunction might

* Corresponding author. Stem Cell and Developmental Biology, Hospital for Sick Children, MaRS Centre, 12-313 TMDT East Tower, 101 College St., Toronto, Ontario, Canada M5G 1L7. Fax: +1 416 813 2212.
E-mail address: fredam@sickkids.ca (F.D. Miller).

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be an underlying cause of perturbed neural development and the resultant cognitive deficits in this family of disorders.

Costello syndrome is one member of the NCFC family of syndromes where all of the patients show mental retardation (Costello 1971, 1977). Moreover, imaging studies of CS patients show that close to 50% show neuroanatomical perturbations including corpus collosum agenesis, enlarged ventricles, frontal and cerebellar atrophy, cerebellar malformations, hypodense white matter, and hydrocephalus (Delrue et al., 2003; Hennekam, 2003; Gripp et al., 2002; Gripp, 2005; Zampino et al., 2007). Recently, point mutations in H-Ras have been identified as responsible for causing this disorder (Aoki et al., 2005). In about 80% of cases, the mutation is situated at position 12 in the guanine nucleotide binding site. These mutations result in decreased GTP hydrolysis, causing increased and sustained Ras activity, and thus act as gain of function mutations. These findings, together with previous work suggesting that components of the Ras pathway regulate cortical development, suggest that the cortical abnormalities seen in CS may well be a consequence of perturbed cortical cell genesis. Further support for this idea comes from work on the *NF1* gene, which encodes a tumor suppressor that, amongst several activities, is a negative regulator of Ras (Hegedus et al, 2007). There is a high incidence of cognitive dysfunction in NF1 patients (Hyman et al., 2005), and mice where NF1 is genetically ablated in CNS precursors exhibit a variety of deficits in glial cells, including globally reactive astrogliosis and increased proliferation of glial precursor cells (Zhu et al., 2005; Hegedus et al., 2007). On the basis of these findings, we have hypothesized that aberrant H-Ras activation causes cognitive dysfunction by perturbing the proliferation and the differentiation of neural precursor cells and their progeny.

Here, we provide evidence in support of this hypothesis, demonstrating that expression of two Costello syndrome H-Ras alleles in cortical precursors enhances cell proliferation and inhibits neurogenesis in vivo. We also demonstrate that they cause premature gliogenesis, which ultimately leads to an increase in astrocytes postnatally. These findings suggest that aberrant H-Ras activation disrupts normal cortical development and this may explain the cortical abnormalities seen in CS.

Materials and methods

Culture of cortical precursor cells

Cortical precursor cells were cultured as previously described (Barnabé-Heider and Miller, 2003; Paquin et al., 2005; Gauthier et al., 2007). Briefly, cortices were dissected from embryonic day 12 (E12) to E13 CD1 mouse embryos in ice-cold HBSS (Invitrogen, Gaithersburg, MD) and transferred to neurobasal medium (Invitrogen) containing 500 μm L-glutamine (Cambrex Biosciences, Hopkinton, MA), 2% B27 supplement (Invitrogen), and 1% penicillin-streptomycin (Invitrogen). The medium was supplemented with 40 ng/ml FGF2 (Promega, Madison, WI). The tissue was mechanically triturated with a plastic pipette into single cells and cells were plated on two-well chamber slides (Nunc, Naperville, IL). Chamber slides were previously coated with 2% laminin and 1% poly-D-lysine (BD Biosciences, Bedford, MA) and cell density was 250,000 cells/well.

Transfection and CNTF stimulation of cortical precursor cells

For transfections, 1 to 2 h after plating, 2 μg of DNA and 3 μl of Fugene 6 (Roche, Welwyn Garden City, UK) mixed with 200 μl of Opti-MEM (Invitrogen) were incubated at room temperature for 45 min and then added to each well. The H-RasG12V and H-RasG12S plasmids consist of point mutations at codon 12 (GGC) where glycine is mutated to a valine (GTC) or a glycine (AGC) respectively. H-RasG12S was generated by site-directed mutagenesis from the H-RasG12V plasmid (Clontech, cat#631924). An empty vector was used as a control (pEF-GM), and a plasmid encoding EGFP under the CMV-promoter (pEGFP) was used as a marker for cotransfected precursor cells (Paquin et al., 2005; Gauthier et al., 2007). The day following plating, 50 ng/ml ciliary neurotrophic factor (CNTF; Peprotech, Rocky Hill, NJ) was added in some experiments (as specified), by changing one half of the medium.

In utero electroporation

In utero electroporation was performed as previously described (Paquin et al., 2005; Bartkowska et al., 2007; Gauthier et al., 2007). Briefly, E13/E14 CD1 pregnant mice were anesthesized with isoflurane, and a midline incision was performed to access the embryos. A total of 4 μg of DNA was injected in the lateral ventricle of each embryo with 0.05% trypan blue as a tracer. We used a nuclear EGFP expression plasmid driven from the EF1-promoter (pEF-GFP). This pEF-GFP was coelectroporated with pEF-GM (empty vector), H-RasG12V, or H-RasG12S. After injection, electroporation was performed using a square electroporator CUY21 EDIT (TR Tech, Japan), delivering five 50 ms pulses of 40 V with 950 ms intervals per embryo. Embryos were then put back in utero and left to further develop for 3–8 days. For analysis, brains were fixed in 4% paraformaldehyde (PFA) (Electron Microscopy Sciences, Hatfield, PA) at 4 °C overnight, cryoprotected in 30% sucrose at 4 °C overnight, and embedded in OCT compound (Sakura Finetek, Torrance, CA). The brains were kept at −80 °C until cryosectioned (16 μm) and immunostained.

Immunocytochemistry

Immunocytochemistry of cultured cells and tissue sections was performed as described (Paquin et al., 2005; Bartkowska et al., 2007; Gauthier et al., 2007). For immunocytochemistry of cultured cells, cells were washed with HEPES-buffered saline (HBS) and fixed with 4% PFA for 15 min, permeabilized with 0.2% NP-40 (USB Corporation, Cleaveland, OH) in HBS, and blocked with buffer containing 6% normal goat serum (NGS) (Jackson ImmunoResearch, West Grove, PA) and 0.5% bovine serum albumin (BSA) (Jackson ImmunoResearch) for 1–2 h at room temperature. Cells were then incubated with primary antibodies in HBS containing 3% NGS and 0.25% BSA at 4 °C overnight. After washing with HBS, cells were incubated with secondary antibodies prepared in HBS containing 3% NGS and 0.25% BSA at room temperature for 1 h. Samples were then washed with HBS, counterstained with Hoechst 33258 (1:1000; Sigma, St-Louis, MO) for 2 min, and mounted with GelTol (Fisher Scientific, Houston, TX). For immunocytochemistry of tissue sections, sections were dried at 37 °C for 15 min, washed in phosphate buffer solution (PBS) (Hyclone, Logan, UT), and postfixed with 4% PFA for 10–15 min. They were then blocked and permeabilized with 10% BSA and 0.3% Triton X-100 (EMD Chemicals Inc., Gibbstown, NJ) for 1 h. The M.O.M. blocking kit (Vector Laboratories, Burlingame, CA) was then used according to the manufacturer's protocol. Sections were incubated with primary antibodies at 4 °C overnight, washed with PBS, and incubated with secondary antibodies at room temperature for 1 h. They were then counterstained with Hoechst 33258 for 2 min and mounted with GelTol. The primary antibodies used were mouse anti-GFP (1:1000; Invitrogen), rabbit anti-GFP (1:500; Chemicon, Temecula, CA), mouse anti-H-Ras (1:400; Calbiochem), rabbit anti-phosphoErk (1:500, Cell Signaling Technology), mouse anti-Ki67 (1:200; BD Biosciences), mouse anti-HuD (1:200; Invitrogen), mouse anti-βIII-tubulin (1:800; Covance, Princeton, NJ), rabbit anti-GFAP (1:1000, Accurate Chemical and Scientific Corp., Westbury, NY), rabbit anti-cleaved caspase-3 (1:500; Cell Signaling Technology), and goat anti-doublecortin (1:100; Santa Cruz). The secondary antibodies used for immunocytochemistry were indocarbocyanine (Cy3)-conjugated goat anti-mouse and anti-rabbit IgG (1:400; Jackson ImmunoResearch), (Cy3)-conjugated donkey anti-goat IgG (1:1000; Jackson ImmunoResearch),