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MAPK/ERK Signaling Pathway Analysis in Primary Osteoblasts from Patients with Non-Syndromic Sagittal Craniosynostosis

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Abstract

Objective—The MAPK/ERK signaling pathway has been implicated in several craniosynostosis syndromes and represents a plausible target for therapeutic management of craniosynostosis. The causes of sagittal non-syndromic craniosynostosis (sNSC) have not been well understood and the role that MAPK/ERK signaling cascade plays in this condition warrants an investigation. We hypothesized that MAPK-signaling is misregulated in calvarial osteoblasts derived from patients with sNSC.

Methods—In order to analyze if the MAPK/ERK pathway is perturbed in sNSC we established primary calvarial osteoblast cell lines from patients undergoing surgery for correction of this congenital anomaly. Appropriate negative and positive control cell lines were used for comparison and we examined the levels of phosphorylated ERK by immunoblotting.

Results—Primary osteoblasts from patients with sNSC showed no difference in ERK1/2 phosphorylation with or without FGF2 stimulation as compared with control osteoblasts.

Conclusion—Under the described test conditions, we did not observe convincing evidence that MAP/ERK signaling contributes to the development of sNSC.

Keywords

FGF2; bFGF; FGFRs; sagittal craniosynostosis; non-syndromic; nonsyndromic; MAPK; ERK

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Introduction

Craniosynostosis (CS), defined as the premature fusion of cranial sutures, is a birth defect that results in abnormal head shape and may compromise brain growth and function. The overall prevalence of CS has been estimated to be 1 out of 2,000 to 3,000 births (Boyadjiev, 2007; Kimonis et al., 2007). Syndromic craniosynostosis (SC) presents with extra-cranial anomalies and developmental delays and accounts for approximately 15–20% of all CS cases. SC follows Mendelian patterns of inheritance and is associated with mutations in at least eight genes (*FGFR1*, *FGFR2*, *FGFR3*, *TWIST1*, *EFNB1*, *POR*, *MSX2* and *RAB23*), most of which involve the coronal sutures (Passos-Bueno et al., 2008; Melville et al., 2010; Wilkie et al., 2010). The vast majority of SC causing mutations has been found in fibroblast growth factor receptors (FGFRs) (Wilkie, 1997). These mutations act in a gain-of-function manner, resulting in hypersensitive FGFRs that respond to lower concentrations of FGF ligands or are constitutively active in the absence of fibroblast growth factor (FGF) (Ornitz and Marie, 2002; Miraoui and Marie, 2010).

The remainder of CS cases occur as isolated anomalies, termed non-syndromic craniosynostosis (NSC), that are not associated with other major malformations (Cohen, 2000). A minor fraction of these cases harbor mutations in *FGFR2*, *TWIST1*, *FREMI*, *LRIT3*, *EFNA4*, *ALX4*, as well as *RUNX2* duplications (Johnson et al., 2000; Weber et al., 2001; Merrill et al., 2006; Seto et al., 2007; Wilkie et al., 2007; Mefford et al., 2010; Vissers et al., 2011; Kim et al., 2012; Yagnik et al., 2012). This suggests common etiological mechanisms with SC. NSC appears to occur sporadically, and is believed to be a multifactorial trait with genetic influences and environmental contributions (Boyadjiev, 2007; Kimonis et al., 2007).

Fibroblast growth factors (FGFs) are ubiquitous and versatile peptides that regulate cell proliferation, migration, cell survival and differentiation during development, tissue repair or tumor growth (Ornitz and Itoh, 2001). FGF binding to FGFRs causes the receptor dimerization and activation of protein tyrosine kinase domains, which triggers several downstream signaling cascades involving MAP/ERK, PLC γ , and mTOR/AKT. This MAPK/ERK signaling pathway plays critical roles in cell proliferation and differentiation. It has been well established that aberrant activation of MAPK/ERK signaling causes syndromic forms of CS (Slater et al., 2008; Miraoui et al., 2010). Importantly, small molecule suppression of the MAPK/ERK-signaling cascade rescues the phenotype for murine models of Crouzon and Apert syndromes, stressing the involvement of the MAPK/ERK signaling pathway in SC (Eswarakumar et al., 2006; Shukla et al., 2007). Thus, it is plausible that similar abnormal activation of MAPK/ERK signaling is implicated in NSC. Here, we test the hypothesis that aberrant MAPK/ERK signaling contributes to sagittal NSC

Materials and Methods

Human Subjects

Informed consents were obtained from all patients and/or their parents. This study was approved by the Institutional Review Boards of the participating institutions and was conducted in accordance with institutional guidelines. All patients with sNSC were clinically assessed and found to have non-syndromic craniosynostosis without associated extracranial congenital anomalies or developmental delays. The CS was confirmed by computerized tomography of the head and by surgical protocols.

Cell Culture

Osteoblasts were isolated from human bone fragments collected at the site of the suturectomy during surgery for correction of sNSC. The specimens were kept at room temperature in sterile growth media and plated for cell growth as described below. Genetic analysis excluded mutations associated with syndromic forms of craniosynostosis in the relevant exons of *FGFR1*, *FGFR2*, *FGFR3*, and *TWIST* genes as previously described (Lemmon and Schlessinger, 1994; Boyadjiev, 2007; Richardson et al., 2011). Osteoblasts to be used as negative controls were isolated from cranial bones of children without recognizable genetic disorders undergoing surgical intervention for head trauma. Additionally, three human osteoblasts cell lines with known mutations (FGFR3 Pro250Arg, FGFR2 Pro253Arg, and FGFR2 Cys278Phe) were used as positive control cells. Bone tissues were washed with DPBS twice and after removal of periosteum, were dissected and minced by surgical scissors into fragments of 1–2 mm in size and plated on a 30 mm Petri dish. Bone tissue particles were cultured in DMEM media containing 20% fetal bovine serum with antibiotics and maintained in a water-jacketed incubator at 37°C with 5% CO₂ enrichment (Boyadjiev, 2007; Bhat et al., 2011). Sub-cultured osteoblasts were maintained in DMEM media with 10% fetal bovine serum and split 1:5 weekly or when confluent. The osteoblast origin of the cells was confirmed by reverse-transcriptase PCR, documenting expression of the osteoblast markers osteocalcin and bone-specific alkaline phosphatase.

Antibodies

The following antibodies were used for immunoblotting: rabbit anti-beta-tubuline (Cell signaling Tech, USA, 1/1,000), rabbit anti-phospho-ERK (Cell signaling Tech, USA, 1/1,000), and rabbit anti-ERK (Cell signaling Tech, USA, 1/1,000).

Immunoblotting

Osteoblasts were examined at baseline and after 30 minutes of treatment with FGF2 at 5 ng/mL concentration. Cells were washed in cold PBS and lysed in radioimmunoprecipitation assay buffer (25 mM Tris-HCl, pH7.6, 150 mM NaCl, 1% NP-40, 0.1% Sodium dodecyl sulfate, 1% Sodium deoxycholate and 5 mM EDTA) containing protease inhibitors (Roche, USA). The proteins concentration of cell lysates was determined with a bicinchoninic acid protein according to the manufacturer's protocol (Pierce, USA). Protein lysates were resolved in SDS-PAGE and transferred to PVDF membrane (Millipore, USA). Non-specific binding sites were blocked with 10% nonfat milk for 1hr at room temperature and membranes coated with primary antibodies were incubated with anti-rabbit or mouse IgG conjugated with horse radish peroxidase (HRP) for visualization using ECL plus (GE Health science, USA). The band intensities of phosphorylated ERK1/2 were normalized to the value of the untreated control #1.

Statistical analysis

For quantification of the western data, the immunoreactive bands were quantified by densitometric analysis with Image J (NIH, USA). The fold changes relative to values in the control group were represented as the mean $\pm$ SEM for five independent experiments. Statistical analysis was performed by Student's *t*-test. A *p*-value of less than 0.05 was considered significant.

Results and Discussion

In order to monitor the activity of ERK in sNSC osteoblasts, we established primary osteoblasts cell lines from eight patients with sNSC, three patients with SC (FGFR3 Pro250Arg, FGFR2 Pro253Arg and FGFR2 Cys278Phe), and two unaffected individuals.

We measured the phosphorylation status of ERK1/2 (MAPK 1/3) by immunoblotting (Fig. 1). The signal intensity of phosphorylated ERK2 was not significantly changed in response to FGF2, most likely due to saturation. The levels of phosphorylated ERK1 varied significantly even in control osteoblasts. Notably, we did not detect hyperphosphorylation of ERK1/2 in sNSC osteoblasts. Interestingly, we observed a significant hypophosphorylation of ERK1/2 in sNSC sample #8 (Fig. 1). These results suggest that there is no hyperactivation of the MAPK/ERK signaling, as measured by the level of ERK1/2 phosphorylation in response FGF2 in the sNSC cases we tested.

We observed a marginal increase of phosphorylated ERK1 in the absence of FGF2 only in FGFR2 P253R osteoblasts from a patient with Apert syndrome (compared to control #2 without FGF2, $p = 0.054$). This result suggests that FGFR2 Pro253Arg is constitutively active and potentiates downstream effectors in the absence of FGF2. Consistent with this result, phosphorylated ERK1/2 was enhanced in cultured bone marrow cells derived from Fgfr2 P253R transgenic mice (Yin et al., 2008). However, we did not observe an increase in phosphorylation of ERK1/2 in the other SC osteoblasts.

The MAPK/ERK pathway is a major component of the FGF-FGFR signal transduction network, involved in osteoblast proliferation and differentiation. Indeed, Fgf2 stimulation of this pathway in mice induces osteopontin expression (a marker of differentiated osteoblasts) and accelerated cranial suture fusion, while ERK blocker molecules caused both processes to cease (Kim et al., 2003). Thus, it is logical to conclude that ERK1/2 signaling in the MAPK pathway is critically involved in osteoblast development and differentiation. Depletion of the osteoprogenitor population in the suture, suppressed proliferation and accelerated differentiation of osteoblasts are all logical factors for the development of premature sutural closure leading to CS. However, our results suggest that this pathway is not implicated in sNSC. Recent data, however, have shown that *ERF* (a transcription factor downstream of the MAPK/ERK pathway) is mutant in some CS cases (Twigg et al., 2012). Thus, the potential effect of genetic alteration in MAPK/ERK components that lie downstream of ERK1/2 may explain why we don't see ERK1/2 hyperphosphorylation.

CS development, however, need not be limited to the MAPK/ERK pathway. Alternate G-protein signaling pathways such as those triggered by PLC γ or mTOR/Akt may be involved (Guenou et al., 2006; Moenning et al., 2009). Other potential pathways include proteins that are activated by alternate forms of MAPKs, such as JNKs (MKK 4/7) and p38 (MKK 3/6) (Chang and Karin, 2001). Additionally, other osteoprogenitor genes have been implicated in CS through studies of mutant murine models and these may act through pathways other than MAPK/ERK. Our recent work has implicated the BMP2/SMAD pathway mediated signaling in sNSC (Justice et al., 2012). In addition, rescue of the craniosynostosis phenotype in *Gli3*^{-/-} mice through deletion of one *Runx2* allele was recently reported (Tanimoto et al., 2012). Both TGF β , BMP2 and FGFR pathways converge on *RUNX2* (Lee et al., 2000; Teplyuk et al., 2009), an osteoblast regulator, that interacts with many NSC candidate genes as among them *MSX2* (Antonopoulou et al., 2004), *Twist1* (Yang et al., 2011), *ALX4*, (Yagnik et al., 2012), and *Nell1* (Truong et al., 2007). *GLI3*, *MSX2* and *DLX5* are all transcription factors also known to be involved in calvarial development (Newberry et al., 1998; Dodig et al., 1999; Ohba et al., 2008) and these factors may regulate each other (Newberry et al., 1998).

Conclusions

By immunoblot analysis of primary calvarial osteoblasts from probands with sNSC, we show lack of constitutive or induced activation of the MAP/ERK signaling pathway. On the contrary, one sNSC osteoblast cell line showed very low ERK1/2 signaling. Given our

relatively small sample size, the involvement of MAP/ERK pathway cannot be entirely excluded for sNSC. Our findings, however, suggest that under these test conditions, MAPK/ERK signaling does not contribute to the pathogenesis of sNSC. The role of genes downstream from ERK1/2 remains to be explored.

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Figure 1. ERK1/2 immunoblot with phosphorylation-specific antibodies of osteoblasts from probands with sNSC with appropriate negative and positive controls. The error bars represent standard deviation: # - $p < 0.05$ (compared to control #1 without FGF2, $n > 5$); * - $p < 0.05$ (compared to control #1 with FGF2, $n > 5$).