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COMMENTARY

Ras-ERK signalling in endochondral ossification: insights from neurofibromin

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Neurofibromatosis type 1 (NF1) is an autosomal dominant genetic disorder with an incidence of 1:3000–3500. Neurofibromatosis derives its name from the small cutaneous and subcutaneous neurofibromas (nerve-related tumours) and larger plexiform neurofibromas that are characteristic of the disease. However, NF1 is also associated with a range of other distinguishing features including specific neurocognitive deficiencies and musculoskeletal manifestations that affect individuals with variable penetrance.[1] Skeletal defects such as dystrophic scoliosis and congenital tibial dysplasia remain extremely challenging to manage and have a high associated morbidity.[2]

Over the past 10 years, the underlying molecular mechanisms for NF1 affecting bone have been progressively elucidated by a number of key studies. The *NF1* gene product, neurofibromin, has RAS-GTPase activating proteins (GAP) activity that negatively regulates RAS activity. Moreover, a point mutation that specifically abrogates this RAS-GAP function has been associated with skeletal defects in one patient family.[3] Cell culture experiments have demonstrated that that the loss of NF1 increases RAS activity in a genotype- and cell type-dependent manner.[4] This may be particularly relevant to the study of focal bone lesions, which have been reported to be associated with a localised double inactivation of NF1.[5] However, even without loss of the second allele, bone complications in NF1 and other RASopathies (diseases featuring increased RAS activity) have been reported to be associated with reduced bone mineral density and increased bone resorption.[6]

The signalling pathways downstream of RAS are numerous and their relative importance is likely to be contextual to the cell/tissue type, to rely on crosstalk from other signalling pathways and to be dependent on the specific activating ligand(s). RAS-mitogen-activated protein kinase kinase (MEK)-extracellular signal-regulated kinase (ERK) signalling is a canonical pathway that typically increases cell proliferation, impedes progenitor differentiation and ameliorates apoptosis.[7] However, other pathways can be activated by RAS signalling including phosphoinositide 3-kinase (PI3K), c-Jun N-terminal kinases (JNK), mammalian target of rapamycin (mTOR), p38 kinase and cyclic adenosine monophosphate/protein kinase A. In the context of bone, RAS-PI3K has been linked to increased osteoclastogenesis in NF1.[8] Although other RAS effector pathways including ERK were upregulated in NF1 osteoclasts, a PI3K inhibitor was able to provide a functional rescue, and was more effective than a MEK inhibitor in effecting osteoclast migration.[8] In osteoblasts, both ERK- and JNK-dependent signals have been shown to be upregulated.[9] MEK inhibition has been recently suggested as a strategy for the treatment of *Nf1*-deficient bone repair.[10]

The control of ERK1/2 by RAS-dependent and RAS-independent pathways has a potential to affect not only bone but also cartilage. In cartilage, ERK signalling has been reported to promote chondrocyte maturation and not proliferation,[11] which contrasts its reported role in a majority of other tissues. ERK signalling may be important not only for cartilage formation but also for cartilage and joint disease. In a mouse model of rheumatoid arthritis, phospho-ERK levels were found to be increased and clinical arthritis scores were decreased following treatment with the specific MEK inhibitor PD184352.[12] Furthermore, in osteoarthritis the signalling from chondrocytes to the subchondral bone is dependent on an ERK signalling pathway.[13] These findings are not unexpected as pro-inflammatory stimuli classically increase ERK activity.

In the recent study by Ono *et al.*,[14] the researchers used an $Nf1_{col2}^{-/-}$ knockout mouse model to examine the role of RAS-MEK-ERK signalling in cartilage and endochondral repair. It was hypothesised that a loss of neurofibromin in *Col2a1*-cre-expressing chondrocytes would increase RAS-ERK signalling and effect cartilage maturation. The authors reported a number of key findings:

1. In the growth plate, neurofibromin expression was localised to the pre-hypertrophic chondrocytes, and was absent from proliferating and hypertrophic chondrocytes. It has been previously reported that ERK activity remains elevated in hypertrophic chondrocytes,[11] suggesting that RAS-ERK activity can be regulated in cells lacking neurofibromin expression by an alternate pathway(s).

2. In cultured chondrocytes, double inactivation of NF1 in cultured chondrocytes resulted in decreased *matrix metalloprotease-9* and *-13* expression as well as increased *Rankl/Opg* ratio. In coculture, $Nf1_{col2}^{-/-}$ chondrocytes produced increased TRAP+ cells, particularly when supplemented with Vitamin D. This implicates NF1 in the regulation of cartilage breakdown in addition to its potential role in chondrocyte maturation.
3. Double inactivation of neurofibromin in *Col2a1*-expressing osteochondral progenitors resulted in growth plate disruption. No change was seen at birth, but by weaning the hypertrophic zone was significantly reduced. On the basis of the normal expression of neurofibromin, it can be concluded that decreased RAS-ERK activity in the pre-hypertrophic chondrocytes that normally express neurofibromin leads to reduced chondrocyte maturity. This was consistent with previous reports in which cultured embryonic limbs were treated with MEK inhibitor U0126 and showed reduced hypertrophic chondrocytes.[15] However, the lack of a skeletal phenotype in $Nf1_{col2}^{-/-}$ neonatal mice suggests regulation of RAS-ERK signalling by alternate RAS-GAP factors during development.
4. The growth plate disruptions seen in $Nf1_{col2}^{-/-}$ mice were hypothesised to be analogous to achondrodysplasia (dwarfism) and further speculated to involve a fibroblast growth factor (FGF)–ERK signalling axis. Consistent with this concept, sustained ERK1/2 activation was seen in cultured $Nf1_{col2}^{-/-}$ chondrocytes treated with basic FGF2, but not those treated with epidermal growth factor (EGF).
5. C-type natriuretic peptide (CNP) has previously been used in preclinical models of achondrodysplasia to antagonise FGF receptor-3–ERK signalling. A heterozygous mutant *Fgfr3 (Y367C/+)* mouse model treated with a CNP analog was shown to inhibit FGF-mediated mitogen-activated protein kinase activation and improve bone growth.[16] Similarly, the authors used a stabilised CNP–Fc fusion (NC-2) that showed superior and sustained activity *in vivo* to treat the $Nf1_{col2}^{-/-}$ knockout mice. This leads to rescue of the phenotype including significant improvements in the columnar arrangement of chondrocytes, in the lengths of the proliferating/hypertrophic zones and in the overall size of the $Nf1_{col2}^{-/-}$ mice. It must be noted that CNP has been reported to affect other signalling pathways including the JNK pathway;[17] JNK has been also found to be upregulated and a potential point of intervention in NF1.[9,18] Although a decrease in pERK1/2 expression was demonstrated with NC-2, it has not been conclusively shown that this is the only pathway that mediates the $Nf1_{col2}^{-/-}$ knockout phenotype.

Interestingly, although short stature has been reported in NF1, this is not a consistent feature of the condition and it is not associated with achondrodysplasia/dwarfism or growth plate disruption. In individuals with NF1 it is possible that short stature could be caused by *NF1* haploin sufficiency;[2] these individuals do not display significant cartilage defects comparable with those reported by Ono *et al.* in the $Nf1_{col2}^{-/-}$ mice. Nevertheless, these mice have been a powerful tool for demonstrating the importance of RAS signalling in maintenance of the growth plate and may have an impact on the management of other conditions.

1. This work shows great promise for FGF–ERK antagonists (including NC-2) as treatments for achondrodysplasia and for other genetic conditions in which growth plate defects are associated with ERK dysregulation.
2. In the context of osteoarthritis, which also features ERK upregulation, NC-2 may be an effective intervention. Furthermore, a recent report has suggested beneficial outcomes when using a combination of hyaluronic acid and ERK inhibitor for osteoarthritis.[19]

Conflict of Interest

The authors declare no conflict of interest.

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