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The reduced osteogenic potential of *Nf1*-deficient osteoprogenitors is EGFR-independent

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ABSTRACT

Neurofibromatosis type 1 (NF1) is a common genetic disorder caused by mutations in the *NF1* gene. Recalcitrant bone healing following fracture (*i.e.* pseudarthrosis) is one of the most problematic skeletal complications associated with NF1. The etiology of this condition is still unclear; thus, pharmacological options for clinical management are limited. Multiple studies have shown the reduced osteogenic potential of *Nf1*-deficient osteoprogenitors. A recent transcriptome profiling investigation revealed that *EREG* and *EGFR*, encoding epiregulin and its receptor Epidermal Growth Factor Receptor 1, respectively, were among the top over-expressed genes in cells of the NF1 pseudarthrosis site. Because EGFR stimulation is known to inhibit osteogenic differentiation, we hypothesized that increased EREG and EGFR expression in *NF1*-deficient skeletal progenitors may contribute to their reduced osteogenic differentiation potential. In this study, we first confirmed *via* single-cell mRNA sequencing that *EREG* over-expression was associated with *NF1* second hit somatic mutations in human bone cells, whereas *Transforming Growth Factor beta 1* (*TGFβ1*) expression was unchanged. Second, using *ex-vivo* recombined *Nf1*-deficient mouse bone marrow stromal cells (mBMSCs), we show that this molecular signature is conserved between mice and humans, and that epiregulin generated by these cells is overexpressed and active, whereas soluble TGFβ1 expression and activity are not affected. However, blocking either epiregulin function or EGFR signaling by EGFR1 or pan EGFR inhibition (using AG-1478 and Poziotinib respectively) did not correct the differentiation defect of *Nf1*-deficient mBMSCs, as measured by the expression of *Alpl*, *Ibsp* and alkaline phosphatase activity. These results suggest that clinically available drugs aimed at inhibiting EGFR signaling are unlikely to have a significant benefit for the management of bone non-union in children with NF1 PA.

1. Introduction

Neurofibromatosis type 1 (NF1) is a common genetic disorder that occurs in 1 of 3500 live births [1,2]. It is a pleiotropic condition that affects various organs, including the skin, eyes, nervous system and skeleton [3,4]. In bone, complications include osteopenia, short stature, chest wall deformities, sphenoid wing dysplasia, dystrophic scoliosis and tibia bowing that progresses to fracture and pseudarthrosis (PA, *i.e.* recalcitrant bone healing/non-union) [2,5]. Patients with NF1 are heterozygous for inherited *NF1* mutations, and approximately half of all cases of NF1 occur from spontaneous *de novo* mutations of the *NF1* gene [6]. Second-hit somatic *NF1* mutations have been observed in cells from 75% of the NF1 PA biopsies analyzed [7,8]. In other words, one inactivating *NF1* variant can be inherited from a parent (or *de novo*) and the other allele subsequently acquires a somatic inactivating variant in cells of the tibia, leading to loss of *NF1* function. In mice, *Nf1* is expressed in bone cells, including osteoprogenitors [9], differentiated osteoblasts [10], chondrocytes [11,12] and osteoclasts [13]. Evidence from mouse models suggest that *NF1* loss of heterozygosity in humans occurs in skeletal progenitor cells of the tibia [10,14,15].

In contrast to most cases of fracture in children, which usually progress to bone union within weeks, 2–5% of children with NF1 present with recalcitrant bone healing despite multiple attempts with surgical stabilization. The condition starts in early childhood with an initial

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and unilateral bowing of the tibia that often progresses to fracture and non-union. It has the highest morbidity among other NF1 skeletal complications, with little clinical management options [16–18], and often leads to amputation of the affected limb [19]. Bone Morphogenic Proteins (BMPs) are currently used off-label with variable success, and under clinical investigation for efficacy [20–22], although BMP2 did not show a beneficial effect on its own in preclinical models [10,23]. Hence, finding new therapeutic options for the management of this condition is a significant clinical need.

NF1 encodes neurofibromin, a 280 KDa cytosolic multi-domain protein. The central GTPase-related domain (GRD) of neurofibromin facilitates the conversion of RAS-GTP (active form of RAS) to RAS-GDP (inactive form of RAS) and hence acts as a brake on RAS downstream signaling [24], including the MAPK pathways [11,25,26]. *In vitro* studies using bony biopsies from children with NF1 PA have shown that periosteal cells from the pseudartrotic site have a blunted response to osteogenic differentiation signals compared to cells from unaffected sites [27,28]. Studies based on the use of murine osteoprogenitor cells have provided further evidence that *Nf1* is necessary for proper osteogenic differentiation [10,29]. Bone mesenchymal cells deficient for *Nf1*, including chondrocytes and osteoblasts, are characterized by high RAS and ERK1/2 activation compared to WT controls [10,11,14,30]. Although this molecular signature was expected to contribute to the impaired differentiation of *Nf1*-deficient osteoprogenitor cells, two independent studies indicated that MEK blockade was unable to rescue the differentiation phenotype of these cells or to improve bone healing in two mouse models of NF1 PA [10,23]. The exact underlying mechanism of this differentiation phenotype thus remains not well understood, although Rhodes et al. reported that excess *Tgfβ1* expression in *Nf1*-deficient mouse osteoblasts might be involved [31]. However, a recent transcriptome analysis using RNA-sequencing of cells cultured from rare biopsies collected from the PA site of children with NF1 did not reveal a change in *TGFβ1* expression (Dr. Rios, unpublished data) compared to iliac crest-derived bone cell (herein referred to as $NF1^{+/-}$). Rather, this study identified a significant upregulation of *EGFR* and *EREG* [7]. Epiregulin, encoded by *EREG*, is one of the seven Epithelial Growth Factor (EGF) family members that preferentially binds to and activates EGFR1 and Erb-B4 forms among the four cloned EGFRs [32–34]. These findings sparked great interest because 1) increased EGFR signaling is known to inhibit osteoprogenitor cell differentiation [35–43]; 2) drugs are clinically available to block EGFR signaling, thus raising the possibility of rapidly repurposing EGFR inhibitors to promote the differentiation of *NF1*-deficient osteoprogenitors and potentially bone healing in cases of NF1 bone non-union, and 3) the beneficial impact of RAS [44], TGFβ [31] or β-catenin [45] inhibition on bone healing in preclinical models of NF1 PA is expected to take additional effort and time to translate to the clinic. Based on these observations, we hypothesized that sustained EGFR signaling in *Nf1*-deficient osteoprogenitors contributes to their differentiation defect.

2. Results

2.1. EREG expression is increased in human bone cells characterized by NF1 double hit mutations

Consistent with previously published data, single-cell sequencing confirmed highly significant upregulation of *EREG* in $NF1^{-/-}$ clonal cells that harbor a germline p.R461X variant and a somatic p.Asn510_Lys2333del large deletion, compared to patient-matched $NF1^{+/-}$ cells (Fig. 1A). *EGFR* expression was slightly, though not significantly, higher in the *NF1*-deficient clonal cell line (Fig. 1B). No significant differences in gene expression were observed for genes encoding *TGFβ* ligands nor *TGFβ* receptors (Fig. 1C–H), in contrast to a previous report using *Nf1*-deficient osteoblasts extracted from the $Col1_{2.3kb}$-cre;$Nf1^{f/f}$ mouse model [31].

These observations led us to assess the expression of *Tgfb1* in WT and *Nf1*-deficient mouse bone marrow stromal cells (mBMSCs). For this purpose, $Nf1^{f/f}$ mBMSCs were cultured and infected with a cre-expressing adenovirus (herein referred to as *Nf1*-deficient) or a GFP-expressing adenovirus (herein referred to as WT control). Loss of *Nf1* gene expression following cre-expressing adenovirus transduction was confirmed by a significant reduction (>90%) in *Nf1* gene expression by qRT-PCR compared to Ad-GFP control (Fig. 1I). No difference in *Tgfb1* expression was found in *Nf1*-deficient mBMSCs (Fig. 1J). *Tgfb1* was also expressed at similar levels in WT and *Nf1*-deficient mouse embryonic fibroblasts (MEFs) isolated from WT and $Nf1^{-/-}$ embryos, considered to be more immature mesenchymal progenitor cells than mBMSCs (Fig. 1K) and in WT and *Nf1*-deficient calvaria-derived cells that are considered more committed to the osteoblast lineage (Fig. 1L). No detectable difference in the amount of soluble total TGFβ1 (measured by ELISA, Fig. 1M) nor secreted active TGFβ1 (measured by Western Blot, Fig. 1N) was observed between the conditioned medium (CM) from WT and *Nf1*-deficient mBMSCs. Finally, the CM from WT and *Nf1*-deficient mBMSCs resulted in similar levels of activation of a sensitive SMAD-responsive luciferase reporter MDA231 cell line (limit of detection: 1 ng/ml, Supplementary Fig. 1A and O) [46], and to similar level of p-SMAD2 activation in CM-treated WT BMSCs (Fig. 1P). Collectively, these data strongly suggest that increased TGFβ1 production by *Nf1*-deficient osteoprogenitors is not the main cause of the impaired osteogenic potential of these cells.

2.2. Epiregulin is ectopically expressed and active in Nf1-deficient mBMSCs

Because single-cell sequencing confirmed that *NF1* deficiency in human bone cells was associated with *EREG* over-expression, we sought to determine whether this phenotype was conserved in mBMSCs. Using the same strategy of *ex vivo Nf1* ablation as indicated above, we found *Ereg* to be expressed in *Nf1*-deficient mBMSCs at three times the level of WT mBMSCs (Fig. 2A). This increase was confirmed at the protein level (Fig. 2B). In contrast, expression of *Egfr* was not altered in *Nf1*-deficient mBMSCs (Fig. 2C), though EGFR protein abundance was higher in these cells (Fig. 2D). A similar increase in *Ereg* expression was also detected in rib primary $Nf1^{f/f}$ chondrocytes infected with Ad-cre compared to Ad-GFP viruses (Supplementary Fig. 1B). The expression of other EGFR ligands, including *Betacellulin* (*Btc*), *Epidermal Growth Factor* (*Egf*), *Transforming Growth Factor a* (*Tgfa*) and *Amphiregulin* (*Areg*), was undetectable in both WT and *Nf1*-deficient mBMSCs (data not shown). These results suggest that neurofibromin signaling represses *Ereg* expression in both human and mouse BMSCs and that EGFR protein synthesis or stability is regulated by mechanisms that are neurofibromin-dependent and post-transcriptional.

Epiregulin is synthesized as a precursor membrane-bound protein that must be cleaved for biological activity and activation of EGFR [33]. To determine if *Nf1*-deficient mBMSCs generate higher amount of active epiregulin than WT mBMSCs, a cell line highly sensitive to EGFR ligands (A431 cells) [47] was treated with the CM from WT and *Nf1*-deficient mBMSCs. Both CMs led to EGFR activation (phosphorylation), but the CM from *Nf1*-deficient mBMSCs was three times more potent than the one from WT mBMSCs (Fig. 2E). In addition, EGFR activity following treatment with the *Nf1*-deficient CM was blocked following addition of an epiregulin-neutralizing antibody (Fig. 2E). These results suggest that the CM of *Nf1*-deficient mBMSCs contains higher amount of active epiregulin compared to WT mBMSCs.

2.3. Inhibition of EGFR signaling fails to rescue the osteogenic differentiation of Nf1-deficient mBMSCs

Because chronic activation of EGFR leads to inhibition of osteogenic differentiation (32–33,36–40,42) and *Nf1*-deficient mBMSCs overexpress both EGFR and its ligand epiregulin, we sought to block EGFR signaling to determine if excessive EGFR signaling contributed to the