Myeloid-to-mesenchymal NGF-p75 signaling coordinates skeletal cell migration during repair

Running Title: NGF-p75 signaling in bone repair

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Abstract

Tissue repair relies on the coordination of mesenchymal precursor cells (MPCs) which migrate into the injury site, along with the invasion of blood vessels and sensory nerves. Our prior observations found that the neurotrophin Nerve growth factor (NGF) regulates sensory nerve ingrowth to skeletal repair sites via its high-affinity receptor. A body of work in cancer biology suggest that neurotrophins also engage their low-affinity receptor p75 to mediate cellular migration. Here, we observed conditional deletion of *p75* in MPCs or osteoblasts to disrupt bone repair independent of neurovascular ingrowth. Single cell sequencing identified defects in migration and wound healing among MPC populations. Deletion of *Ngf* among myeloid cells phenocopied *p75* conditional deletion animals. *In vitro* studies confirmed a myeloid-to-mesenchymal NGF-p75 axis which operates to induce cellular migration. Together, our data suggest a direct effect of myeloid-derived NGF on progenitor cells, in parallel to sensory nerve recruitment, required for injury repair.

Introduction

The regeneration of craniofacial bones of the mammalian skeleton requires the action of both intrinsic and extrinsic inductive factors from multiple cell types. Unlike the appendicular skeleton, intramembranous cranial bones form and are healed without a cartilaginous template by condensations of mesenchymal progenitor cells (1). Recent studies from our laboratory have demonstrated an essential role for skeletal sensory nerves in healing of experimental injuries in adult mouse bone, including calvarial bones (2, 3). Nerve growth factor (NGF) via its high-affinity receptor tropomyosin receptor kinase A (TrkA) regulates skeletal re-innervation, which is essential for later elements of bone repair, including revascularization and bone matrix deposition (2-4). However, emerging evidence suggests that neurotrophin signaling may have more pleiotropic effects in bone repair than previously considered.

In addition to induction of nerve ingrowth and promotion of nerve survival, pro-NGF binds to its low-affinity receptor p75 (encoded by *Ngfr*), which is present in a variety of mesenchymal cells (5). p75 expression has been correlated to cell migration and invasion in the fields of developmental and cancer biology. For example, Schwann cell migration from the dorsal root ganglia is significantly impaired in p75 null embryos (6). Likewise, p75-dependent signaling has an essential role in the migration of epidermal Langerhans cells (7). Similar observations have been made in neoplastic cells, in which stable knockdown of p75 in melanoma cells reduced cell migration and expression of major regulatory gene networks for cell migration (8, 9). NGF binding to the p75 receptor stimulates migration and initiates recruitment of various adaptors, which activate nuclear factor kappa B (NF- κ B), Ras homolog family member a (RhoA), and c-Jun N-terminal kinase (JNK) signaling (10, 11).

In this study, we have identified an alternate role for NGF signaling in the bone reparative process, one independent of skeletal sensory nerves. Depletion of *p75* in platelet-derived growth factor receptor α (PDGFR α)⁺ mesenchymal cells or osteocalcin (OC)⁺ osteoblasts impaired cellular migration into an osseous wound. Transcriptomics identified new mesenchymal subpopulations with potential roles in cell migration and immune response, altered in the context of *p75* deletion. Further, we identified an immune-to-skeletal cell signaling axis involving myeloid/macrophage-derived NGF which acts to directly stimulate skeletal cell migration during early bone repair.

Results

p75 is critical for stromal cell migration and calvarial regeneration

p75-expressing cells in the uninjured skull and injured site were identified using a previously validated PDGFR α -CreER^{T2};mT/mG (PDGFR α ^{mT/mG}) reporter animal (12) (**Fig. 1A,B**). Lineage tracing of PDGFR α -expressing cells was based on tamoxifen (TM) administration to promote the activity of inducible Cre, starting at 8 weeks of age. A circular bone defect was created in the non-suture associated frontal bone 14 days after the last TM administration. Within the frontal bone, *p75* protein was detected at low levels in the overlying periosteum and coincided with PDGFR α reporter activity in bone lining cells (**Fig. 1B**). Following injury, a notable expansion of the *p75*⁺Pdgfr α ⁺ cell population was detected along the outer edge of the injury defect site (**Fig. 1B**). To assess the requirement for *p75* in calvarial defect repair, *p75*^{fl/fl} animals were crossed with PDGFR α ^{mT/mG} lines to yield *p75*^{fl/fl};PDGFR α ^{mT/mG} (*p75*^{PDGFR α}) animals. *p75*^{fl/fl};mT/mG animals (*p75*^{fl/fl}) were used as

controls. Validation for *p75* deletion was performed using *p75* immunohistochemistry on calvarial injury sites within the *p75^{fl/fl}* and *p75^{PDGFR α}* mice (**Fig. 1C**). *p75* immunohistochemical staining was significantly reduced in *p75^{PDGFR α}* defect sites, in comparison to *p75^{fl/fl}* control defects. Next, frontal bone healing was assessed following *p75* deletion in *PDGFR α* -expressing cells over a 4-week period (**Fig. 1D-J**). Results demonstrated impaired bone healing among *p75^{PDGFR α}* animals, relative to *p75^{fl/fl}* controls. Micro-CT reconstructions and cross-sectional images demonstrated impaired re-ossification among *p75^{PDGFR α}* mice (**Fig. 1D**). Quantitative micro-CT metrics of bone healing were reduced among *p75^{PDGFR α}* mice, including Bone Volume (BV, **Fig. 1E**, 68.3% reduction), fractional Bone Volume (BV/TV, **Fig. 1F**, 68.3% reduction), mean diameter of the bone defect area (**Fig. 1G**, 40.9% increase), Bone fractional area (BFA, **Fig. 1H**, 54.8% reduction), and bone healing score – a previously validated semi-quantitative 4 point scoring system (13) (**Fig. 1I**, 30.0% reduction in comparison to *p75^{fl/fl}*). H&E staining confirmed a significant impairment of defect healing between bony fronts in *p75* mutant mice (**Fig. 1J**, black arrowheads).

To determine potential mechanisms driving this impaired bone healing, Ki67 immunostaining was first conducted to interrogate potential differences in proliferation (**Fig. 1K**). These results failed to show any significant differences in cell expansion, suggesting the impaired healing observed in *p75^{PDGFR α}* mice was not the result of reduced cell proliferation. Another potential mechanism through which defect repair could be inhibited in *p75* mutant mice is the failed recruitment of regenerating mesenchymal cells to the defect site. To assess progenitor cell migration into the defect, a lineage tracing strategy was employed using *PDGFR α ^{mT/mG}* animals. *PDGFR α* reporter activity highlights stromal cells which are recruited to populate the bone injury

site. Deletion of $p75$ in $PDGFR\alpha^+$ stromal progenitors resulted in a prominent decrease in stromal cell migration into the central defect region (**Fig. 1L**). This impaired recruitment of $PDGFR\alpha^+$ progenitors led to a reduced number of mature osteoblasts among $p75^{PDGFR\alpha}$ animals, as assessed by immunohistochemical staining for Osteocalcin (**Fig. 1M**). Our previous reports have demonstrated that specific impairment of the NGF-TrkA signaling axis leads to an impaired invasion of both the vasculature and sensory nerves into the cranial defect region (3). To determine whether a similar phenomenon may also be contributing to our impaired healing deficiencies, vascularity and innervation within the defect site were next evaluated (**Fig. 1N,O**). CD31 immunostaining of endothelium and TUBB3 immunostaining of axons suggested no difference in defect site revascularization or re-innervation among $p75^{fl/fl}$ and $p75^{PDGFR\alpha}$ mice. In aggregate, inhibition of $p75$ signaling in $PDGFR\alpha^+$ stromal cells led to significant reductions in injury-associated cell migration and a significant delay in bone defect healing, independent of changes in neurovasculature in bone.

Depletion of $p75$ in osteoblasts impairs osteoblast migration and calvarial healing

To further delineate the cell stage-specific expression of $p75$, uninjured calvarial samples were immunostained for $p75$ and the mature osteoblast marker OCN (**Fig. 2A**). These studies confirmed that $p75$ is indeed also expressed in mature osteogenic cells lining the periosteal bone surface. To assess the cell-specific requirements of $p75$ in osteoblasts, $p75^{fl/fl}$ animals were crossed with the Osteocalcin-Cre line to yield $p75^{fl/fl}; Osteocalcin-Cre$ ($p75^{OC}$) animals. $p75^{OC}$ animals had normal size and weight (**Fig. S1A,B**). Validation for $p75$ deletion was next performed using $p75$ immunohistochemistry on calvarial injury sites within the $p75^{fl/fl}$ and $p75^{OC}$ mice (**Fig. 2B,C**). Micro-CT analysis of uninjured frontal bones showed a slightly decreased BV and increased bone mineral density among $p75^{OC}$ animals (**Fig. S1C-G**). To

determine the role of osteoblast-specific p75 in injury repair, $p75^{\text{fl/fl}}$ and $p75^{\text{OC}}$ mice were analyzed 4 weeks after frontal bone defect creation (**Fig. 2D-I**). Micro-CT imaging demonstrated a reduction in defect re-ossification among $p75^{\text{OC}}$ animals (**Fig. 2D**), confirmed by quantitative micro-CT metrics of bone formation which demonstrated a significant reduction in all parameters among $p75^{\text{OC}}$ animals, including BV (**Fig. 2E**, 48.5% reduction), BV/TV (**Fig. 2F**, 48.5% reduction), an increase in remaining non-ossified diameter of the defect (**Fig. 2G**, 20.0% increase), BFA (**Fig. 2H**, 35.7% reduction), and semi-quantitative bone healing score (**Fig. 2I**, 21.6% reduction in comparison to $p75^{\text{fl/fl}}$).

In a mechanism similar to our above reported $p75^{\text{PDGFR}\alpha}$ mice, deletion of p75 in OCN^+ osteoblasts also led to a prominent decrease in progenitor cell migration into the defect site, as assessed by flow cytometry for PDGFR α among CD31 $^-$ CD45 $^-$ Ter119 $^-$ cells (**Fig. 2J,K**). Likewise, a similar 59.6% reduction in OCN^+ osteoblasts was observed among $p75^{\text{OC}}$ animals (**Fig. 2L**). Similar to $p75^{\text{PDGFR}\alpha}$ mice, vascularization (**Fig. 2M**) and innervation (**Fig. 2N**) within the defect site were unaffected in $p75^{\text{OC}}$ mice. In sum, p75 in OC-expressing osteoblasts is essential for osseous repair.

Single cell transcriptomic profiling of total cells and mesenchymal lineage subpopulation

To further understand the mechanisms driving impaired defect repair in p75-deficient mice, we employed single-cell RNA sequencing (scRNA-seq) of cells derived from the defect site of $p75^{\text{fl/fl}}$ and $p75^{\text{PDGFR}\alpha}$ mice at 7 days post-injury (**Fig. 3A**). Unsupervised clustering identified 8 groups (**Fig. 3B**), identified using expression patterns of known marker genes, including 2 groups of mesenchymal lineage cells (expressing *Paired related homeobox 1* (*Prrx1*), *Platelet*

derived growth factor receptor alpha (*Pdgfra*), *Msh homeobox 1* (*Msx1*), *Twist family BHLH transcription factor 1* (*Twist1*), and *Runt-related transcription factor 2* (*Runx2*)), 5 groups of hematopoietic cells (expressing *Protein tyrosine phosphatase receptor type C* (*Ptprc*) encoding CD45), and 1 group of endothelial cells/pericytes (expressing *Regulator of G-protein signaling 5* (*Rgs5*), and *Endomucin* (*Emcn*)) (**Fig. 3C**). While each cluster was represented by cells from both *p75^{fl/fl}* and *p75^{PDGFRα}* mice, some shifts in cluster proportions were observed, including enrichment of mesenchymal cluster cells derived from *p75^{fl/fl}* mice (**Fig. S2A,B**). Specificity of Cre recombination was confirmed in sequenced data in which EGFP expression was restricted to the PDGFRα-expressing mesenchymal clusters (**Fig. S2C**) and almost exclusively expressed in *p75^{PDGFRα}*-derived cells (**Fig. S2D**). Pathway analysis of differentially expressed genes (DEGs) revealed GO term enrichment in cell adhesion, positive regulation of cell migration, osteoblast differentiation, and wound healing in *p75^{fl/fl}* mice (**Fig. 3D**). In contrast, immune system process, inflammatory response, and regulation of cell shape were enriched in cells from *p75^{PDGFRα}* mice, implying altered cell shape, differentiation and matrix interactions (**Fig. 3D**). Focused analysis of mesenchymal lineage cells yielded 8 subpopulations (**Fig. 3E**). Early stem markers *Cd34*, *Thy1* were predominantly expressed in cluster 1 and 2, while multi-lineage progenitor markers Actin alpha 2, smooth muscle (*Acta2*) and Scleraxis (*Scx*) showed enriched expression in cluster 3/4 (**Fig. 3F**). Cluster 7 was classified as being likely derived from the dura mater owing to the selective expression of *Forkhead box protein C2* (*Foxc2*), while cluster 8 cells was found to express mature osteoblast markers *Bone gamma-carboxyglutamate protein 2* (*Bglap2*) and *Secreted phosphoprotein 1* (*Spp1*) (**Fig. 3F**). Deletion of *p75* in mesenchymal cells was further validated by downstream signaling-related gene expression, including genes of Rho, NF-κB and mitogen-activated protein kinase (MAPK) signaling pathways (**Fig. S3**). Pathway analysis of the

total mesenchymal cell populations showed that osteogenesis-related signaling pathways were inhibited among $p75^{\text{PDGFR}\alpha}$ cells, including Notch, Bone morphogenetic protein (BMP), Transforming growth factor beta (TGF β), Fibroblast growth factor (FGF), and Hippo signaling (**Fig. 3G**). Interestingly, pathway analysis of cluster-specific DEGs showed specific enrichment in GO terms such as cell adhesion, regulation of actin cytoskeleton organization, and cell migration, in cluster 5 cells (**Fig. 3H**). Owing to our previous results suggesting an impaired migration/recruitment of progenitor cells into the defect area, we further analyzed gene lists linked to biological functions needed for cell migration (**Fig. 3I**). Consistent with our cluster DEG assessment and in vivo results, cluster 5 showed significant enrichment for genes linked to receptor-mediated extracellular matrix interaction and cytokine receptor-mediated signaling, both of which were reduced in $p75^{\text{PDGFR}\alpha}$ cells (**Fig. 3I**). This data further suggested that p75 signaling within mesenchymal lineage cells is essential for cellular migration and osteogenic differentiation during bone injury repair.

p75 signaling plays a critical role in cell migration and osteogenic differentiation in vitro

To further determine the effect of p75 on stromal cell activity, primary neonatal mouse calvarial cells (NMCCs) from $p75^{\text{fl/fl}}$ animals were isolated and treated with adenovirus encoding the Cre recombinase (Ad-Cre) or control (Ad-GFP). The efficiency of $p75$ deletion was confirmed by qPCR (**Fig. 4A**) and validated by downstream signaling-related gene expression of RhoA, NF- κ B and MAPK signaling pathways (**Fig. S4**). Consistent with our *in vivo* analysis, NMCCs showed no change in cell proliferation rates among Ad-Cre- and Ad-GFP-treated groups (**Fig. 4B**). We did not observe an increase in apoptosis after Ad-Cre treatment by TUNEL assay (**Fig.**

4C). However, *p75* deletion reduced cell migration in wound-healing and transwell assays (Fig. 4D,E) as well as impaired osteogenic differentiation (Fig. 4F,G).

Next, the changes in the transcriptome of Ad-GFP- and Ad-Cre-treated NMCCs were examined using bulk RNA sequencing (RNA-seq). 14,668 protein-coding genes were expressed across all samples and had functional annotations. 577 transcripts, 3.93% of the total, showed a >2fold change (FC) increase among Ad-Cre-treated cells (red dots; 381 transcripts showed a significant increase ($p < 0.05$)), while 399 transcripts, 2.72% of the total, showed a >2FC increase among Ad-GFP-treated cells (green dots; 219 transcripts showed a significant decrease ($p < 0.05$)) (Fig. 4H). Quality control by principal component analysis (Fig. 4I) and hierarchical cluster of genes showing high sample deviation (Fig. 4J) confirmed strong reproducibility of sample replicas. Pathway analysis showed enrichment for GO terms linked to innate immune response, mesodermal cell differentiation, and cell-cell adhesion in Ad-GFP-treated cells, whereas neutrophil chemotaxis, extracellular matrix organization, and negative regulation of immune response were enriched in Ad-Cre-treated cells (Fig. 4K). Ingenuity Pathway Analysis (IPA) showed that the activated pathways in Ad-GFP-treated NMCCs are associated with the positive regulation of migration and osteogenesis, including IL-3 signaling, JAK/STAT signaling, and Erythropoietin signaling pathway (Z scores -2.236, -2, and -1.633; Fig. 4L) (14-19). Conversely, upregulated signaling pathways in Ad-Cre-treated NMCCs were associated with the negative regulation of cell migration and ossification, including IL-17 signaling as well as PTEN signaling (Z scores 1.89 and 1; Fig. 4L) (20-22). These data further confirmed functional differences previously observed among *p75*^{fl/fl} and *p75*^{PDGFR α} mice, including the low migration and osteogenic potential of *p75*-deficient calvarial stromal cells.

Macrophage-derived NGF regulates NMCC migration via p75 signaling

Previously, we have shown that *Ngf* derived from LysM-expressing monocytes/macrophages is essential for calvarial bone defect repair (3). To confirm this observation, *Ngf^{LysM}* mice were again subjected to frontal bone injuries and compared to *Ngf^{fl/fl}* control mice (**Fig. 5A**). Micro-CT reconstructions and cross-sectional images within the defect site demonstrated impaired re-ossification among *Ngf^{LysM}* mice in comparison to *Ngf^{fl/fl}* mice (**Fig. 5B**). In order to investigate a potential link between macrophage-derived NGF and stromal cell p75 signaling to enhance bone regeneration, migration was assessed *in vitro* and *in vivo* using *Ngf^{LysM}* or *Ngf^{fl/fl}* animals (**Fig. 5C-K**). Deletion of *Ngf* in LysM⁺ monocytes/macrophages led to a prominent decrease in stromal cell migration into the defect site, as assessed by PDGFR α immunostaining (**Fig. 5C**) and flow cytometry for PDGFR α of the microdissected bone defect site (**Fig. 5D,E**). To directly address the paracrine effects of macrophages on calvarial stromal cells, conditioned medium (CM) experiments were performed, harvesting CM from either *Ngf^{LysM}* or *Ngf^{fl/fl}* macrophages (**Fig. 5F**). Results demonstrated that CM from macrophages derived from *Ngf^{fl/fl}* mice promoted cell migration assessed by scratch wound healing assay, but this pro-migratory effect was not seen in NGF-depleted CM from *Ngf^{LysM}* macrophages (**Fig. 5G**). A similar result was observed with invasion assays, in which *Ngf^{fl/fl}* but not *Ngf^{LysM}* macrophage CM induced transwell invasion (**Fig. 5H**). Next, the effects of macrophage CM were assayed on NMCCs derived from *p75^{PDGFR α}* animals (**Fig. 5I,J**). After *in vitro* exposure to 4-Hydroxytamoxifen, a significant reduction in *p75* expression was observed among *p75^{PDGFR α}* NMCCs (**Fig. 5I**). Among control cells without 4-Hydroxytamoxifen treatment, macrophage CM again induced cellular migration. Notably, induction of cellular migration by macrophage CM was not seen among *p75* knockout

calvarial cells (**Fig. 5J**). In sum, macrophage-derived NGF appears to promote mesenchymal calvarial cell migration and bone healing via its low-affinity receptor p75.

Mesenchymal subpopulation changes macrophage phenotype and function through p75 signaling

In addition to terms linked to migration and wound healing, pathway analysis of scRNA-seq and bulk RNA-seq revealed a recurring theme of altered immune processes in the context of *p75* deletion (**Fig. 6A**). To investigate how mesenchymal *p75* deletion may affect immune cell function, we examined the characteristics of mesenchymal subpopulations and found that mesenchymal cluster 6 demonstrated DEGs enriched in several terms linked to immune cell recruitment and function (**Fig. 6B**). This mesenchymal cluster 6 was found to express high levels of inflammatory regulators, such as *Il1a*, *Il10*, and *Tnf*, which were found to have reduced expression in cells derived from *p75^{Pdgfra}* mice (**Fig. 6C**).

To determine whether a concomitant decrease was observed in macrophage signaling resulting from this reduced mesenchymal expression of inflammatory markers, we next investigated changes in macrophages within our scRNA sequencing of *p75^{fl/fl}* and *p75^{Pdgfra}* bone defects (**Fig. 6D-F**). While only a minor shift in overall population distribution was observed (**Fig. 6D, Fig. S2B**), expression of markers linked to both M1 and M2 macrophage activation were inhibited among *p75^{Pdgfra}* mice in comparison to *p75^{fl/fl}* mice (**Fig. 6E**). This was consistent with reductions in inflammatory pathways among macrophages within *p75^{Pdgfra}* defect sites, such as TNF α , IL1, IL6, the complement pathway, and Toll-like receptor signaling (**Fig. 6F**). In order to confirm this scRNA-seq data, macrophage number and activity were assessed by

immunohistochemistry within $p75^{\text{fl/fl}}$ and $p75^{\text{Pdgfra}}$ bone defect sections. First, a clear reduction in F4/80⁺ (**Fig. 6G**) or CD68⁺ (**Fig. 6H**) macrophages was observed within $p75^{\text{Pdgfra}}$ mice. TNF- α immunohistochemical staining was next performed, and confirmed a significant reduction across the $p75^{\text{Pdgfra}}$ defect site (**Fig. 6I**). This diminished inflammatory setting was also associated with a reduction of NGF immunoreactive cells with the $p75^{\text{Pdgfra}}$ defect site (**Fig. 6J**). In aggregate, depletion of $p75$ in PDGFR α -expressing mesenchymal cells significantly inhibits injury-associated macrophage activation and NGF expression, associated with a delay in bone defect repair.

p75 regulates human calvarial osteoblasts migration and osteogenesis

Finally, the role of $p75$ in human cells and tissue samples was examined (**Fig. 7**). To investigate the role of $p75$ in human calvarial osteoblasts, siRNA-mediated knockdown of $p75$ was performed (**Fig. 7A**). As in mouse NMCCs, knockdown of $p75$ inhibited cell migration in wound-healing and transwell assays (**Fig. 7B,C**) as well as impaired osteogenic differentiation (**Fig. 7D,E**). Next, detection of $p75$ expression was observed across human fracture calluses (**Fig. 7F**). In similarity to findings in mouse calvaria, $p75$ was abundantly expressed in bone lining cells of the fracture callus, especially in Osteocalcin⁺ bone lining osteoblasts (**Fig. 7G,H**). Thus, $p75$ in osteoblasts and their cellular precursors is essential for cell migration and bone repair – a finding conserved across mouse and human tissues.

Discussion

In this study, we sought to define the cellular and molecular events that accompany bone repair in genetic mouse models that enabled specific disruption of NGF- $p75$ signaling components in

bone. We show that NGF derived from myelomonocytic cells is acutely upregulated following bone injury, triggering a migration of p75-expressing mesenchymal cells into the damaged area. When this signaling pathway is disrupted, defective cellular migration, ossification, and a secondary blunting of the immune response are the result. Given these observations, it is not clear as to why experimental work with neutralizing antibodies to NGF have not shown a significant delay in bone healing (23, 24). It is probable that the analgesic effects of anti-NGF are observed at a lower dose than those needed to impede tissue repair. Nevertheless, when taken together with our past work, these data illustrate the multimodal and critical effects of NGF in bone repair, including coordinating proper mesenchymal cell migration, re-innervation and vascularization, and ossification – cellular functions which are regulated in concert via both NGF receptors.

Our studies focused on the role of p75 in mesenchymal progenitors and osteoblasts by using available transgenic animals. However, p75 expression is of course present in other cell types, including pericytes and immune cells. For example, proNGF-p75 signaling in the vasculature, including pericytes and endothelial cells, has been shown to mediate vascular response to injury of either the myocardium or injured limb in ischemia-reperfusion models (25-27). Our observations on unchanged vascularity in the context of *p75* conditional gene deletion thus likely stems from the choice of Cre driver. Likewise, macrophages are also responsive to the chemotactic properties of proNGF via the low-affinity receptor p75 (28). Thus, it is possible that p75 has more functional roles in tissue repair than would be illustrated by our experimental design. Second, our studies were focused on a single neurotrophin given our laboratories' interests and past work in this area (2-4, 29). Nevertheless, other neurotrophins such as BDNF

(Brain derived neurotrophic factor) are also expressed in human fracture sites including in endothelial and osteoblastic cells during early repair phases (30). It is likely that several neurotrophins have functionally redundant roles in regulating p75-mediated tissue ingrowth after bone injury – a hypothesis that has not yet been analyzed experimentally.

The extent to which NGF signaling can be targeted therapeutically for its beneficial effects on bone and bone repair represents an evolving area of interest in the orthopaedic research community. Several recent papers suggest that positive regulation of NGF-TrkA signaling may have beneficial effects in long bone fracture repair. For example, local injection of NGF stimulated early fracture maturation in a closed tibial fracture mouse model (31). Systemic administration of the partial TrkA agonist Gambogic amide has been used to improve indices of fibular fracture healing in mice (32), and analogously to augment skeletal adaptation to mechanical load (33). Our present study demonstrates the multiple cell specific functions that NGF signaling plays in bone repair. In combination with past work (2, 3), we document clear and potentially synergistic roles for proNGF-p75 and NGF-TrkA signaling in osseous repair.

Materials and Methods

Mice

All animal experiments were performed according to approved protocols (MO16M226 & MO19M366) of the Animal Care and Use Committee (ACUC) at Johns Hopkins University (JHU). Pdgfr α -CreERTM animals were a kind gift from the Dwight Bergles laboratory (34) and are commercially available (The Jackson Laboratory, Stock No. 018280, Bar Harbor, ME). OC-Cre animals were generated by the Clemens laboratory and are commercially available (JAX

Stock No. 019509). $\text{Pdgfr}\alpha^{\text{mT/mG}}$ or $\text{OC}^{\text{mT/mG}}$ mice were obtained by crossing $\text{Pdgfr}\alpha\text{-CreER}^{\text{TM}}$ or OC-Cre with mT/mG mice (JAX Stock No. 007576). $p75^{\text{PDGFR}\alpha}$ or $p75^{\text{OC}}$ mice were obtained by crossing $\text{Pdgfr}\alpha^{\text{mT/mG}}$ or $\text{OC}^{\text{mT/mG}}$ mice with $p75^{\text{fl/fl}}$ mice (JAX Stock No. 031162). Tamoxifen (TM; Sigma-Aldrich, St. Louis, MO) was dissolved in sunflower seed oil (Sigma-Aldrich) and injected intraperitoneally according to previously validated protocols (TM: 150 mg/kg/d for 5 d) (12, 35). LysM-Cre mice were purchased from the Jackson Laboratory (JAX Stock No. 004781). In order to achieve cell specific deletion of *Ngf*, Ngf^{LysM} were obtained by crossing LysM-Cre animals with $\text{Ngf}^{\text{fl/fl}}$ animals. When feasible, littermate analysis was performed by investigators blinded to the mouse genotype. All these lines were backcrossed for at least 8 generations to the C57BL/6J background before used for the experiments reported here.

Calvarial defect procedures

Calvarial defects were performed based on our prior methods (3, 35, 36). In $\text{Pdgfr}\alpha^{\text{mT/mG}}$ or $p75^{\text{PDGFR}\alpha}$ mice, TM administration was performed 14 d prior to defect creation as previously validated (12, 35). Mice were provided isoflurane (2-3% inh) and Buprenorphine (1 mg/kg sc). Briefly, hair overlying the calvaria was clipped, and a 1 cm skin incision was made over the midline skull to expose the frontal bone. Next, a 1.8 mm diameter, full-thickness, circular frontal bone defect was created in the non-suture associated frontal bone (right side) using a micro surgical drill and a trephine drill bit. Meticulous care was taken to protect the neighboring sutures and the underlying dura mater. Finally, the skin was sutured and the animals were monitored per established postoperative protocols. Mice were euthanized after 7 d or 28 d for analysis.

Radiographic analyses

Skulls were fixed in 4% paraformaldehyde (PFA) for 24 h and evaluated using a high-resolution micro-CT imaging system (SkyScan 1275; Bruker, Kontich, Belgium). Scans were obtained at an image resolution of 12 μm and set as 1 mm of aluminum filter, X-ray voltage of 65 kVP, anode current of 153 μA , exposure time of 160-218 ms, frame averaging of 6, and rotation step of 0.3 degrees. Three-dimensional images were then reconstructed from the 2D X-ray projections using a commercial software NRecon software (v1.7.0.4, SkyScan). For 3D morphometric analyses of images, CTAn software (v1.16, SkyScan), CTVol (v2.0, SkyScan), and CTVox (v3.2, SkyScan) were used. For calvarial defect analysis, a cylindrical volume of interest centered around each defect site was defined as the 1.8 mm in diameter and 1.2 mm in height with a threshold value of 70-255. Bone volume (BV) and fractional bone volume (bone volume/tissue volume (BV/TV)) were calculated from binary x-ray images. Bone fractional area (BFA) and defect diameter were calculated by using CTVox to create a 3D rendering of calvarial defect and measuring by ImageJ software (Version 1.8.0; NIH, Bethesda, MD). Lastly, a bone healing score from 0 to 4 was assigned by three blinded observers according to previously published grading scales for calvarial defect healing (13).

Histology and immunohistochemistry

After radiographic imaging, samples were decalcified in 14% EDTA for 21 days, embedded in optimal cutting temperature compound (OCT; Sakura, Torrance, CA), and sectioned in a coronal plane at 20 or 50 μm thickness. H&E staining was performed on serial sections. For immunofluorescent staining, all sections were incubated with trypsin enzymatic antigen retrieval

solution (Abcam, Cambridge, MA, USA) for 3 min at room temperature (RT). When 50 μ m sections were used, sections were next permeabilized with 0.5% Triton-X for 30 min. All sections were blocked with 5% goat or donkey serum in PBS for 1 h at RT and incubated with the primary antibodies (see **Table S1** for a summary of antibodies used) at 37 °C for 3 h or overnight at 4 °C. Next, secondary antibodies (1:200) were used with incubation for 2 h at RT. Sections were counterstained with DAPI mounting medium (Vector laboratories, Burlingame, CA). All histological sections were examined under a Zeiss 800 confocal microscope (Zeiss, Thornwood, NY) or Leica DM6 microscope (Leica Microsystems Inc, Wetzlar, Germany). Staining without primary antibody was used to exclude non-specific staining. For visualization of reporter activity, a wildtype specimen prepared in the same fashion was used as a control to identify the intensity of autofluorescence. Images were quantified using ImageJ or Imaris (Bitplane, Belfast, UK) software. For the lineage tracing of cell migration, the percentage of PDGFR α ⁺ cells was calculated in the central defect region. For the TUBB3 staining, the volume of TUBB3⁺ nerve fibers was quantified with three different sites of the defect span and the average value was taken as the final value of the sample. For all other immunohistochemistry, the fluorescence intensity was quantified on the edges of bone in single microscopical fields.

Isolation and culture of neonatal mouse calvarial cells

Primary neonatal mouse calvarial cells (NMCCs) were collected from *p75^{fl/fl}* or *p75^{PDGFR α}* embryos at postnatal day 2. Frontal and parietal bones were minced and subjected to six sequential enzymatic digestions with a mixture containing 1 mg/mL collagenase type I (Worthington Biochemical Corporation, Lakewood, NJ; LS004197) and 1 mg/mL collagenase type II (Worthington Biochemical Corporation; LS004177) (37). Cell fractions (from sequential

digestions 3-6) were collected and cultured in α -MEM supplemented with 15% (vol/vol) FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin. In select experiments, $p75^{\text{fl/fl}}$ NMCCs were exposed to an adenovirus encoding either Cre Recombinase (Ad-Cre, multiplicity of infection=150; 1045-HT, Vector Biosystems, Malvern, PA) or GFP (Ad-GFP, multiplicity of infection=150; 1060-HT, Vector Biosystems). In additional select experiments, $p75^{\text{PDGFR}\alpha}$ NMCCs were treated with 4-Hydroxytamoxifen (2 μ M) or vehicle control (Sigma-Aldrich).

Proliferation

Proliferation assays were performed in 96 well plates (2×10^3 NMCCs/well) and assayed at 48 h using the CellTiter96® AQueous One Solution Cell Proliferation Assay kit (MTS; Promega, Madison, WI) (38). Briefly, 20 μ l of MTS solution was added to each well. After incubation for 1 h at 37 °C, the plate was measured using an Epoch microspectrophotometer (BioTek, Winooski, VT) by absorbance at 490 nm.

Isolation of mouse macrophages and macrophage conditioned medium

Macrophages were collected from $Ngf^{\text{fl/fl}}$ or Ngf^{LysM} mice (8 weeks old mixed gender animals). Bone marrow cells were flushed from the long bones (femur and tibia) with culture medium and resuspended in medium containing macrophage colony-stimulating factor (M-CSF; 15 ng/mL) (39). After 7 d in culture, adherent bone marrow-derived macrophages were incubated in growth medium without M-CSF for 2 d and the supernatant was collected for conditioned medium experiments.

Migration

Cell migration was measured with Ibidi inserts (Ibidi, Planegg/Martinsried, Germany), where 2×10^4 NMCCs were seeded and grown to confluency. Inserts were removed and cell migration into the empty area was monitored by brightfield microscopy at 0 or 14 h. The equilibrium width of the gap was calculated using the ImageJ software. Here, gap closure = (scratch area at hour 0 – scratch area at hr 14)/scratch area at hr 0 $\times 100\%$. For the transwell migration assays, 2.0×10^4 NMCCs were resuspended in 100 μL of α -MEM medium without FBS and were placed in the upper well of 8- μm pore-size Transwell 24-insert plates (Corning, NY). The migration assay was performed by adding to the bottom well 600 μL of α -MEM medium supplemented with 15% FBS or conditioned medium from cultured macrophages. After 4 h, cells on the bottom of the inserts were fixed and stained with 0.5% crystal violet (Sigma-Aldrich) for 15 min. Pictures were taken by using 100 $\times$ magnification (Leica Microsystems Inc). Crystal violet was eluted using 33% acetic acid, and the eluent quantified by absorbance at 570 nm.

Osteogenic differentiation

NMCCs were seeded in 24-well plates at a density of 1×10^5 cells/well. Osteogenic differentiation medium (ODM) consisted of α -MEM, 10% FBS, 1% penicillin/streptomycin with 100 nM dexamethasone, 50 μM ascorbic acid and 10 mM β -glycerophosphate (40-42). 24 h after cell seeding, growth medium was replaced with ODM, replenished every 3-4 d. For alizarin red (AR) staining, cells were fixed with 4% paraformaldehyde and stained with a 2% alizarin red solution at 14 d of differentiation. Pictures were taken using Olympus Epson scanner (Los Angeles, CA), followed by incubation with 0.1 N sodium hydroxide and quantified using an Epoch microspectrophotometer (BioTek) by absorbance at 548 nm.

Ribonucleic acid isolation and quantitative real-time polymerase chain reaction

Total RNA was extracted from the cultured cells using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. 1 µg of total RNA was used for reverse transcription with iScript™ cDNA synthesis kit (Bio-Rad, Hercules, CA) following manufacturer's instructions. Real-time PCR was performed using SYBR™ Green PCR Master Mix (ThermoFisher Scientific, Waltham, MA) according to the manufacturer's protocol. Relative gene expression was calculated using a $2^{-\Delta\Delta C_t}$ method by normalization with *GAPDH*. Primer sequences are presented in **Table S2**.

Transcriptomics

The RNA content of Ad-Cre and Ad-GFP treated NMCCs was detected by total RNA sequencing. Briefly, *p75^{fl/fl}* NMCCs were exposed to Ad-Cre or Ad-GFP (multiplicity of infection=150) for 72h, then total RNA was extracted from Ad-Cre and Ad-GFP treated *p75^{fl/fl}* NMCCs by Trizol (Life technologies corporation, Gaithersburg, MD). The RNA samples were sent to the JHMI Transcriptomics and Deep Sequencing Core and quantified by deep sequencing with the Illumina NextSeq 500 platform (Illumina, San Diego, CA). Data analyses were performed using software packages including Partek Genomics Suite, Spotfire DecisionSite for Functional Genomics, QIAGEN Ingenuity® Pathway Analysis, and DAVID bioinformatics resources (43).

Apoptosis detection

Ad-Cre and Ad-GFP treated *p75^{fl/fl}* NMCCs were seeded in Millicell EZ SLIDE (MilliporeSigma, Burlington, MA) and cultured for 24 h. The cells were fixed in 4% PFA for 15

min and permeabilized with 0.25% Triton X-100 for 20 min. TUNEL assay was carried out using the Click-iT Plus TUNEL Assay for in situ apoptosis detection with Alexa Fluor 647 dyes (ThermoFisher Scientific). Pictures were taken using a Leica DM6 microscope.

Flow cytometry analysis

In order to detect cell migration *in vivo*, skull defects were microdissected 7 days after injury. The cells in the defect site were obtained by collagenase Type I/II (1 mg/mL) digestion using the above method. Briefly, cell fractions (from sequential digestions 1-6) were collected and resuspended in red blood cell lysis buffer (37°C for 5 min). After centrifugation, cells were resuspended in Hanks' Balanced Salt Solution (HBSS) and 0.5% bovine serum albumin. The resulting cells were further processed for cell sorting, using a mixture of the following directly conjugated antibodies (**Table S1**): anti-CD31-allophycocyanin (1:30), anti-CD45-allophycocyanin (1:30), anti-Ter119-allophycocyanin (1:30), anti-PDGFR α -BV421 (1:50) were added separately and incubated at 4°C for 20 min. Cells were then washed with PBS and examined with a DakoCytomation MoFlo (Beckman, Indianapolis, IN). FlowJo software (Tree Star Inc., Ashland, OR) was used for the analysis of flow cytometry data. Gating and PDGFR α frequency were set in relation to matching isotype control.

Single-cell RNA sequencing

Skulls were microdissected 7 days after defect creation and digested with collagenase Type I/II (1 mg/mL) digestion using the above method. Cell fractions (from sequential digestions 1-6) were collected and resuspended in red blood cell lysis buffer (37°C for 5 min). Digestions were subsequently filtered through 40 μ m sterile strainers. Cells were then washed in PBS and

resuspended in HBSS at a concentration of ~1000 cells/ μ l. Cell viability was assessed with Trypan blue exclusion on a Countess II (ThermoFisher Scientific) automated counter and showed a >85% viability. Cells were sent to the JHMI Transcriptomics and Deep Sequencing Core. The library was generated using the 10X Genomics chromium controller following the manufacturers protocol. Cell suspensions were loaded onto a Chromium Single Cell A chip along with reverse transcription (RT) master mix and single cell 3' gel beads, aiming for 10,000 cells per channel. Following generation of single-cell gel bead-in-emulsions (GEMs), reverse transcription was performed and the resulting Post GEM-RT product was cleaned up using DynaBeads MyOne Silane beads. Beads are provided by 10X with the kit. The cDNA was amplified, SPRIselect (Beckman Coulter, Brea, CA) cleaned and quantified then enzymatically fragmented and size selected using SPRIselect beads to optimize the cDNA amplicon size prior to library construction. An additional round of double-sided SPRI bead cleanup is performed after end repair and A-tailing. Fragmentation, end repair and a-tailing are all one reaction, followed by the double sided cleanup. Another single-sided cleanup is done after adapter ligation. Indexes were added during PCR amplification and a final double-sided SPRI cleanup was performed. Libraries were quantified by Kapa qPCR for Illumina Adapters (Roche) and size was determined by Agilent Bioanalyzer 2100. Read 1 primer, read 2 primer, P5, P7, and sample indices were incorporated per standard GEM generation and library construction via end repair, A-tailing, adaptor ligation and PCR. Libraries were generated with unique sample indices (SI) for each sample. Libraries were sequenced on an Illumina NovaSeq SP 100 cycle (San Diego, CA). *CellRanger* was used to perform sample de-multiplexing, barcode processing, and single cell gene counting (Alignment, Barcoding and UMI Count) at the JHMI Transcriptomics and Deep Sequencing Core. Downstream analysis steps were performed using *Seurat*. Cells were

first filtered to have >500 and <8,000 detected genes, as well as less than 20% mitochondrial transcripts. *SCTransform*, including regression for cell cycle scores derived using the *CellCycleScoring* function, and dimensional reductions using uniform manifold approximation and projection (UMAP) was performed using *Seurat*. Pathway activation scores were generated using the *AddModuleScore* function of *Seurat* using validated gene lists from KEGG pathways.

Statistical analysis

Data are presented as the mean $\pm$ one standard deviation (SD). Statistical analyses were performed using GraphPad Prism (Version 7.0) or R package *ggpubr*. *In vitro* experiments were performed in biologic and experimental triplicate. The number of animals used in the *in vivo* experiments are shown in figure legends. For cell depletion experiments, our depletion efficiency resulted in effect sizes of 1.75 or higher. For these scenarios, with at least 4 mice per group, a two-sample t test would provide 80% power to detect effect sizes of at least 1.75, assuming a two-sided 0.05 level of significance. The Kolmogorov–Smirnov test was used to confirm normal distribution of the data. A two-tailed Student’s *t* test or Wilcoxon test was used for two group comparisons. One-way and two-way ANOVA tests were used for multiple groups, followed by Tukey’s multiple comparisons test. **P*<0.05, ***P*<0.01, and ****P*<0.001 were considered significant.

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Figures and Tables

Fig. 1. Deletion of *p75* in *Pdgfra*-expressing cells impairs stromal cell migration and calvarial bone defect repair. (A) Schematic of experiment, in which *p75^{fl/fl}* or *p75^{PDGFRα}* animals (male, 2 mo old) were administered tamoxifen (TM), followed by frontal bone injury (circular calvarial defect, 1.8 mm diameter) after 14 d, with analysis performed 7 or 28 d post-operatively. (B) Immunohistochemistry for p75, appearing red, on the uninjured or calvarial injury site (d 7 post-injury) within PDGFRα^{mGFP} reporter sections. White dashed lines indicate bone edges. Yellow arrowheads indicate p75 and PDGFRα colocalization. TdTomato not shown. (C) Validation of *p75* deletion by p75 immunohistochemistry and semi-quantitative analysis in either *p75^{fl/fl}* or *p75^{Pdgfra}* mice, d7 post-injury. (D) Micro-CT reconstructions of the defect site in a top down view (above) and coronal cross sectional images (below) among *p75^{fl/fl}* and *p75^{Pdgfra}* animals, 28 d post-injury. Margins of original defect are indicated by dashed red lines. (E-I) Micro-CT quantification of bone healing among *p75^{fl/fl}* and *p75^{Pdgfra}* mice (28 d post-injury), including (E) Bone volume (BV), (F) Bone volume/tissue volume (BV/TV), (G) residual defect diameter, (H) Bone formation area (BFA), and (I) Bone healing score. (J) H&E staining of coronal cross-section of the healing defect site from *p75^{fl/fl}* and *p75^{Pdgfra}* mice. Black arrowheads indicate healing bone edges. (K) Cellular proliferation at the bone defect edge, as assessed by Ki67 immunofluorescent staining. Dashed white lines indicate bone edge. (L) Mesenchymal cell migration, as assessed by *Pdgfra*-CreER; mT/mG cell lineage tracing. Tile scan (left) and high-magnification images of the central defect (right) demonstrate migration of GFP⁺ (PDGFRα^{mGFP}) progenitor cells into the defect site at d7 post-injury. Tdtomato is not shown. (M-O) Immunohistochemical staining and semi-quantitative analysis of Osteocalcin (OCN) (M), CD31 (N), and Beta III Tubulin (TUBB3) (O) within the calvarial defect, d28 post-injury. Dashed white lines indicate bone edge. In graphs, each dot represents a single animal. Red scale bar: 500 μm. White scale bar: 100 μm. N=3 mice per group for (B) and N=6-7 mice per group for (C-O). Data are represented as mean ± 1 SD. **P*<0.05, ***P*<0.01, and ****P*<0.001 as assessed using a two-tailed Student's *t*-test.

Fig. 2. Deletion of *p75* in Osteocalcin (OC)-expressing cells impairs osteoblast migration and calvarial defect healing. (A) Immunohistochemistry for p75 (red) and OCN (green) on the uninjured calvarial bone. Yellow arrowheads indicate p75 and OCN co-localization. (B) Schematic of experiment, in which *p75^{fl/fl}* or *p75^{OC}* animals (male, 2 mo old) were subjected to frontal bone injury (circular calvarial defect, 1.8 mm diameter), with analysis performed 7 or 28 d post-operatively. (C) Validation of *p75* deletion by p75 immunohistochemistry in either *p75^{fl/fl}* or *p75^{OC}* mice, d28 post-injury. White dashed lines indicate bone edges. (D) Micro-CT reconstructions of the defect site in a top down view (above) and coronal cross sectional images (below) among *p75^{fl/fl}* and *p75^{OC}* animals, 28 d post-injury. Margins of original defect are indicated by dashed red lines. (E-I) Micro-CT quantification of bone healing among *p75^{fl/fl}* and *p75^{OC}* mice (28 d post-injury), including (E) Bone volume (BV), (F) Bone volume/tissue volume (BV/TV), (G) residual defect diameter, (H) Bone formation area (BFA), and (I) Bone healing score.

score. **(J)** Schematic of experiment, in which calvarial defects from either $p75^{\text{fl/fl}}$ or $p75^{\text{OC}}$ animals were harvested at d7 post-injury, the original defect site was microdissected, tissue dissociated and analyzed by flow cytometry. **(K)** Percentage of PDGFR α^+ cells among freshly isolated CD31 $^-$ CD45 $^-$ Ter119 $^-$ cells in the defect area from $p75^{\text{fl/fl}}$ or $p75^{\text{OC}}$ animals. **(L-N)** Immunohistochemical staining and semi-quantitative analysis of Osteocalcin (OCN) **(L)**, CD31 **(M)**, and Beta III Tubulin (TUBB3) **(N)** within the calvarial defect, 28 d post-injury. Dashed white lines indicate bone edge. In graphs, each dot represents a single animal. White scale bar: 100 μm . N=3-4 mice per group for **(A,K)** and N=6-7 mice per group for **(C-I, L-N)**. Data are represented as mean $\pm$ 1 SD. * P <0.05 and ** P <0.01 as assessed using a two-tailed Student's t -test.

Fig. 3. Single cell transcriptomics highlights altered signaling pathway activation and impaired cellular migration with *p75* conditional gene deletion. (A) Schematic of experiment, in which *p75^{fl/fl}* or *p75^{PDGFR α}* animals (male, 2 mo old) were administered tamoxifen (TM), subjected to frontal bone injury after 14 d (circular calvarial defect, 1.8 mm diameter), with analysis performed 7 d post-operatively. The defect area was microdissected, dissociated and subjected to single-cell RNA sequencing. (B) UMAP plot of total cells isolated from calvarial defect site among *p75^{fl/fl}* and *p75^{PDGFR α}* animals. EC: endothelial cell; Peri: pericyte. (C) Violin plots of marker gene expression for mesenchymal, EC/Peri, and immune cell clusters. (D) GO term analyses of enriched biological processes among all 16,043 cells from *p75^{fl/fl}* and *p75^{Pdgfra}* mice. (E) UMAP plot of mesenchymal and neuroectodermal cells only (clusters 1 and 2 in subfigure B). MLP: Multilineage progenitor. (F) Violin plots of marker gene expression from mesenchymal subclusters. (G) Specific pathway analysis among mesenchymal cells from *p75^{Pdgfra}* mice in comparison to *p75^{fl/fl}* mice. (H) GO term analyses of enriched biological processes among mesenchymal cluster 5. (I) Receptor-mediated ECM interaction and cytokine receptor-mediated signaling among total mesenchymal clusters or cluster 5 from *p75^{fl/fl}* and *p75^{Pdgfra}* mice. N=5 mice per group were used to yield a single cell population. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ as assessed using a Wilcoxon test or two-way ANOVA with Tukey's multiple comparisons test.

Fig. 4. Deletion of *p75* in NMCCs inhibits cell migration and mineralization. Neonatal mouse calvarial cells (NMCCs) were isolated from *p75^{fl/fl}* mice and exposed to Adenoviral GFP (Ad-GFP) or Ad-Cre *in vitro*. **(A)** *Ngfr* expression by qRT-PCR among Ad-GFP- and Ad-Cre-treated cells after 72 hrs. **(B)** Cellular proliferation among Ad-GFP- and Ad-Cre-treated cells, by MTS assays at 72 h. **(C)** Apoptosis among Ad-GFP- and Ad-Cre-treated NMCCs by TUNEL assay. **(D,E)** Cellular migration assessed by **(D)** scratch wound healing assay at 14 h or **(E)** transwell assay at 4 h among Ad-GFP- and Ad-Cre-treated groups. Representative 100x images with percentage gap closure shown. **(F)** Osteogenic differentiation of Ad-GFP- and Ad-Cre-

treated NMCCs by Alizarin Red (AR) staining and quantification at 14 d. **(G)** Osteogenic gene expression among Ad-GFP- and Ad-Cre-treated NMCCs, including *Runt-related transcription factor 2 (Runx2)*, *Osteocalcin (Bglap)*, and *Collagen type I $\alpha 1$ (Colla1)* at 7 d of differentiation. **(H-L)** Bulk total RNA sequencing (Illumina NextSeq 500 platform) among Ad-GFP- and Ad-Cre-treated NMCCs. **(H)** Differentially expressed genes (DEGs) of all 14,668 transcripts among Ad-Cre-treated NMCCs. Y-axis represents Log2 fold change (FC) for each gene. The number of upregulated DEGs ($\log_2 \text{FC} \geq 1$, red dots) is 577, the number of downregulated DEGs ($\log_2 \text{fold change} \leq -1$, green dots) is 399. **(I)** Principal component analysis among Ad-GFP- and Ad-Cre-treated cells. **(J)** Unsupervised hierarchical clustering among Ad-GFP- and Ad-Cre-treated NMCCs. **(K)** DAVID functional GO analysis of biological processes enrichment among Ad-GFP- and Ad-Cre-treated NMCCs. **(L)** QIAGEN Ingenuity Pathway Analysis (IPA) identified representative pathways that were upregulated (Z-score > 0 ; red color) or downregulated (Z-score < 0 ; green color) in Ad-Cre compared to Ad-GFP-treated NMCCs. Graphs represent mean values, while error bars represent 1 SD. Dot plots represent an individual sample, while whisker plots indicate mean values and 1 SD. *In vitro* experiments were performed in biologic and experimental triplicate. Black scale bar: 100 μm . White scale bar: 10 μm . * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ as assessed using a two-tailed Student's *t*-test.

Fig. 5. Macrophage-derived NGF regulates NMCC migration by p75 signaling. (A) Schematic of experiment, in which *Ngf^{fl/fl}* or *Ngf^{LysM}* animals (male, 2 mo old) were subjected to frontal bone injury (circular calvarial defect, 1.8 mm diameter), with analysis performed 7 or 28 d post-operatively. (B) Micro-CT reconstructions of the defect site in a top down view among *Ngf^{fl/fl}* and *Ngf^{LysM}* animals. Margins of original defect are indicated by dashed red lines. (C) Stomal/osteoprogenitor cell migration among *Ngf^{fl/fl}* and *Ngf^{LysM}* animals, as assessed by PDGFRα immunofluorescent staining. Tile scan (left) and high-magnification images of the defect site (right) demonstrate migration of PDGFRα⁺ progenitor cells into the defect site at d7 post-injury. White dashed lines indicate bone edges. (D) Schematic of experiment, in which calvarial from *Ngf^{fl/fl}* or *Ngf^{LysM}* animals (male, 2 mo old) were harvested at d7 post-injury and only the defect area was left (black circle). After digestion, cells were incubated with antibodies

and analyzed by flow cytometry. **(E)** Percentage of PDGFR α ⁺ cells among freshly isolated CD31⁻CD45⁻Ter119⁻ cells in the defect area from *Ngf*^{fl/fl} or *Ngf*^{LysM} animals. **(F)** Validation of *Ngf* deletion in macrophages from *Ngf*^{LysM} mice in comparison to *Ngf*^{fl/fl} mice. Quantitative RT-PCR performed after isolation and expansion in M-CSF. **(G,H)** NMCC migration assessed by **(G)** scratch wound healing assay at 14 h or **(H)** transwell assay at 4 h with normal medium (Control) and conditional medium of cultured macrophages from *Ngf*^{fl/fl} or *Ngf*^{LysM} mice. Representative 100x images with percentage gap closure are shown. **(I)** Validation of *Ngfr* deletion in NMCCs from *p75*^{PDGFR α} mice after 4-Hydroxytamoxifen treatment. **(J)** Migration of NMCCs treated with or without 4-Hydroxytamoxifen assessed by scratch wound healing assay at 14 h with normal medium and conditional medium of cultured macrophages from *Ngf*^{fl/fl} mice. Representative 100x images with percentage gap closure are shown. Graphs represent mean values, while error bars represent 1 SD. Dot plots represent an individual sample or animal, while whisker plots indicate mean values and 1 SD. *In vitro* experiments were performed in biologic and experimental triplicate **(F-J)**. Red scale bar: 500 μ m. White scale bar: 100 μ m. N=4-5 per group for **(B-E)**. **P*<0.05, ***P*<0.01, and ****P*<0.001 as assessed using a two-tailed Student's *t*-test or one-way ANOVA with Tukey's multiple comparisons test.

Fig. 6. Deletion of p75 in PDGFR α -expressing mesenchyme results in secondary inflammatory changes in the bone defect microenvironment. (A) GO term analyses of enriched biological processes among mesenchymal cluster 6. **(B)** The expression of inflammatory factors among mesenchymal clusters from $p75^{fl/fl}$ and $p75^{PDGFR\alpha}$ mice. **(C)** Inflammatory pathways enriched in both NMCC bulk RNA-seq and mesenchymal clusters derived from scRNA-seq. **(D)** Distribution of macrophages from $p75^{fl/fl}$ and $p75^{PDGFR\alpha}$ mice in UMAP plot. **(E)** Heat map of marker gene expression of M1 and M2 macrophages across $p75^{fl/fl}$

and $p75^{\text{Pdgfra}}$ mice (Left). The analysis of macrophage activation among $p75^{\text{Pdgfra}}$ mice in comparison to $p75^{\text{fl/fl}}$ mice. (Right). Dashed black line indicates the same activity of macrophage polarization across $p75^{\text{fl/fl}}$ and $p75^{\text{Pdgfra}}$ genotypes. **(F)** The expression of inflammatory factors among macrophages from $p75^{\text{Pdgfra}}$ mice in comparison to $p75^{\text{fl/fl}}$ mice. Dashed black line indicates the same expression of inflammatory factors across $p75^{\text{fl/fl}}$ and $p75^{\text{Pdgfra}}$ genotypes. **(G-J)** Immunohistochemical staining and semi-quantitative analysis of F4/80 **(G)**, CD68 **(H)**, Tumor Necrosis Factor (TNF)- α **(I)**, and Nerve Growth Factor (NGF) **(J)** within the calvarial defect, d7 post-injury. Dashed white lines indicate bone edges. In graphs, each dot represents a single animal. White scale bar: 100 μm . N=5 mice per group for **(G-J)**. Data are represented as mean $\pm$ 1 SD. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ as assessed using a Wilcoxon test or two-tailed Student's t -test.

Fig. 7. Deletion of *p75* in human calvarial osteoblasts inhibits cell migration and mineralization. (A) *NGFR* expression by qRT-PCR after siRNA-mediated knockdown in human calvarial osteoblasts. NC, negative control (non-targeting siRNA). (B,C) Cellular migration assessed by (B) scratch wound healing assay at 8 h or (C) transwell assay at 4 h with or without *p75* siRNA. Representative 100x images with percentage gap closure shown. (D) Osteogenic differentiation after siRNA-mediated knockdown in human calvarial osteoblasts by Alizarin Red (AR) staining and quantification at 7 d. (E) Osteogenic gene expression with or without *p75* siRNA, including *Alkaline phosphatase (ALP)*, *Osterix (SP7)*, and *Osteocalcin (BGLAP)* at 7 d of differentiation. (F) Representative histologic appearance of a healing human fracture by H&E staining. (G) Immunohistochemistry for *p75* (red) and Osteocalcin (OCN, green) in healing human fracture sites (N=3). Nuclei, DAPI (blue). Dashed box indicates the location of H. (H) High magnification image for *p75* (red) and OCN (green) expression in the bone lining osteoblasts. Yellow arrowheads indicate *p75* and OCN co-localization. White dashed lines indicate bone edges. Graphs represent mean values, while error bars represent 1 SD. Dot

832 plots represent an individual sample, while whisker plots indicate mean values and 1 SD. *In vitro*
833 experiments were performed in biologic and experimental triplicate. Red scale bar: 500 μm .
834 Black/white scale bar: 100 μm . * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ as assessed using a two-
835 tailed Student's t -test.