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Cranial neural crest cells contribution to craniofacial bone development and regeneration

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Compliance with Ethical Standards

Conflict of Interest

Piera Smeriglio and Antoine Zalc declare no conflict of interest.

Human and Animal Rights and Informed Consent

This article does not contain any studies with human or animal subjects performed by any of the authors.

Introduction

1 The craniofacial skeleton is a crucial component of vertebrate development. It is the structure
2 that protects the brain, and it is essential for respiration, food intake and communication.
3 Additionally, the craniofacial skeleton shapes our face, which is one major definition of our very
4 self. Given its essential functions, congenital craniofacial syndromes – which represent a third
5 of all congenital malformations within the human population (Gilbert-Barnes, 2010) – or
6 traumatic injuries to the head skeleton – can have a profound impact on our health and quality
7 of life. When available, treatments of such syndromes or trauma require heavy maxillo-facial
8 surgeries and reconstruction. Regenerative medicine has made tremendous progress in
9 developing treatments and procedures to enhance craniofacial tissue repair in patients. Most
10 commonly used procedures include autologous bone transplantation (Ho-Shui-Ling et al.,
11 2018; Neovius and Engstrand, 2010), bone tissue engineering techniques (Aghali, 2017; Dang
12 et al., 2018) including bone distraction – whereby new bone is generated by applying stress
13 (stretching) to the endogenous bone tissue (McCarthy et al., 2001) – and more recently stem
14 cell-based therapies (Dupont et al., 2010; Jeon et al., 2016). However, these techniques
15 present the risk of generating unsuitable structures (with ectopic bone formation), relatively
16 poor integration of the new graft or cells within the existing bone and the surrounding soft
17 tissues and they are limited by the size of tissue to replace. Stem cell-based therapy bears an
18 additional risk of genetic and epigenetic mutations which can promote tumor formation
19 (Glaeser et al., 2021; Luo et al., 2014; Zhang et al., 2013).

20 The repair of severely damaged or missing bones should ideally occur through the induction
21 of an endogenous regenerative response, alleviating the need to harvest tissue from the
22 patient or a donor, and avoiding additional issues such as rejection of the tissue transplant.
23 Moreover, endogenous regeneration results in the formation of a structure (i) similar in pattern
24 to the original anatomy and (ii) better integrated within the native tissues including the
25 surrounding muscles, nerves, and vasculature. Data from regenerative species show that
26 controlled cell dedifferentiation is an essential determinant to insure an adequate endogenous
27 regenerative response (Gerber et al., 2018; McCusker et al., 2015; Vieira and McCusker,
28 2018). Understanding how cell plasticity is regulated is then crucial to enhance tissue resident
29 stem cells mobilization and expansion, reduce the tumorigenic risks and altogether promote
30 an efficient endogenous regeneration.

31 The majority of craniofacial bones derive from cranial neural crest cells (CNCC) – a transient
32 stem cell-like population arising in the most rostral part of the embryo soon after gastrulation
33 (Le Douarin and Kalcheim, 1999; Noden and Trainor, 2005). Within the ectoderm lineage, at
34 the border between the neural plate and the surface ectoderm, CNCC are induced as an
35 epithelial cell type (Simões-Costa and Bronner, 2015; Theveneau and Mayor, 2012), that
36 subsequently undergoes an epithelial-to-mesenchymal transition (EMT). CNCC then
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1 delaminate from the dorsal epithelium and migrate dorso-ventrally through the embryo to
2 populate various locations in the craniofacial complex where they differentiate into diverse cell
3 types (Simões-Costa and Bronner, 2015; Soldatov et al., 2019). CNCC present an
4 extraordinary differentiation potential since they generate not only ectoderm derivatives, such
5 as neurons, glia and melanocytes, but also give rise to cells canonically associated with the
6 mesoderm such as bones, cartilage and smooth muscles – also referred to as
7 ectomesenchyme (Le Douarin et al., 2004; Simões-Costa and Bronner, 2015). Thus, CNCC
8 “break” the rules set during gastrulation as they generate derivatives that extend beyond the
9 potential of their germ layer of origin (Perera and Kerosuo, 2021). This unique differentiation
10 potential can be explained by the fact that CNCC express pluripotency programs at the onset
11 of their development (Buitrago-Delgado et al., 2015; Lignell et al., 2017). Furthermore, it was
12 recently shown that CNCC are able to reactivate *Oct4* and the associated pluripotency
13 programs (Scerbo and Monsoro-Burq, 2020; Zalc et al., 2021) during their formation. Together,
14 these studies suggest that a deeper understanding of how CNCC regulate the expression of
15 pluripotency programs could unveil new strategies to stimulate cell plasticity *in vivo* during
16 post-natal tissue repair. Future regenerative therapies will need to recapitulate these
17 processes to enhance endogenous regeneration and ameliorate craniofacial tissue repair.

18 In this review we will briefly summarize how CNCC contribute to craniofacial bone development
19 and highlight the newest findings regarding gene regulation of ossification. We will focus on
20 the recent discovery on the origin of CNCC remarkable plasticity and finally, we will question
21 how this plasticity could be used to enhance craniofacial bone regeneration and discuss on
22 the latest procedures enhancing craniofacial bone healing.

23 Given the limitation of words, we will only focus on the cranial neural crest, even though
24 accumulating evidence suggest that the trunk neural crest could also have a skeletogenic
25 capacity *in vivo* (reviewed in Rodrigues-Da-Silva et al., 2022).

26 **Neural crest contribution to the craniofacial skeleton**

27 During embryogenesis bone can either form via the endochondral ossification process, where
28 mesenchymal progenitors form a cartilaginous template that is gradually replaced by bone
29 tissue, or intramembranous ossification, during which mesenchymal cells directly differentiate
30 into osteoblasts, with no cartilaginous intermediate. Intramembranous bones are predominant
31 in the head forming the cranial vault together with most bones of the face. The
32 intramembranous ossification process starts *in utero* and ends at different postnatal times
33 depending on the type of bone. For example, the clavicles are not fully ossified at birth allowing
34 the postnatal growth and development of the brain. Although most of the bone originates from
35 mesodermal precursor, some facial bones, as well as the endocranium, are derived from
36 CNCC (Noden and Trainor, 2005). Development of the craniofacial skeleton requires the
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1 precise differentiation of CNCC into osteoblasts or chondrocytes. Following CNCC migration
2 and colonization of the facial prominences and branchial arches, CNCC aggregate, condense,
3 and differentiate into a common osteochondral progenitor and then into more differentiated
4 chondrocytes or osteoblasts (Bhatt et al., 2013). The molecular regulations orchestrating
5 craniofacial ossification were recently reviewed in great details (Dash and Trainor, 2020).
6 Harmonious craniofacial ossification requires the precise action of CNCC intrinsic transcription
7 factors such as SOX9, RUNX2 and MSX1/2 in association with extrinsic inputs that include
8 fibroblast growth factor (FGF), Wingless-related integration site (WNT) and Transforming
9 growth factor/Bone morphogenetic protein (TGF β /BMP) signaling pathways. Thus, gene
10 expression and signaling pathways must be specifically activated and terminated in the correct
11 location at the proper developmental time to ensure a *bona fide* craniofacial development.
12 Recent studies further exemplified that inaccurate regulation of gene expression in CNCC
13 leads to severe craniofacial defect. A mouse model constitutively activating the activin A
14 receptor type I (ACVR1) to enhance BMP signaling in CNCC results in ectopic cartilage
15 formation in the craniofacial region (Yang et al., 2021). The study further showed that the
16 increased BMP signaling inhibits autophagy via the mTORC1 pathway and blocks the
17 autophagic degradation of β -catenin, causing CNCC to adopt a chondrogenic identity. This
18 phenotype was then rescued by inhibiting mTORC1 signaling to reactivate the Wnt/ β -catenin
19 signaling pathway (Yang et al., 2021). mTORC1 was also shown to mediate the function of the
20 acetyltransferase GCN5 – a highly conserved enzyme and potent activator of chondrocyte
21 maturation – during craniofacial development (Pezoa et al., 2020). Interestingly in this context,
22 GCN5 is not acting as an epigenetic regulator but probably via direct activation of mTORC1
23 pathway (Pezoa et al., 2020). Epigenetic regulation also plays a role in the CNCC ossification.
24 In fact, inhibition of KMT2D function – a histone methylase which mutations are associated
25 with Kabuki syndrome congenital craniofacial disorder – in the neural crest lineage alters
26 osteochondral progenitor differentiation and results in craniofacial hypoplasia (Shpargel et al.,
27 2020). We have also demonstrated a link between the epigenetic modulator Ten eleven
28 translocation enzyme 1 (TET1) and chondrogenic differentiation (Smeriglio et al., 2020). Loss
29 of TET1 expression impairs chondrogenesis via tissue-specific changes in 5-
30 hydroxymethylcytosine (5hmC) landscape and reduces the expression of cartilage markers. It
31 remains to be established if this mechanism has a direct impact on CNCC. A recent
32 breakthrough study found that in the neural crest lineage, mutation of the tumor suppressor
33 *Brca1* resulted in neonatal death of the mutant animals which presented with a cleft palate and
34 reduced skull due to the reduction in size of craniofacial bones. The reduction in bones size
35 was not due to osteogenic differentiation but by a strong defect in osteogenic proliferation and
36 survival due to an increased DNA damage in skeletogenic precursor cells as demonstrated by
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1 the inhibition of p53 which is sufficient to rescue the *Brca1* mutant phenotype *in vivo* (Kitami et
2 al., 2018).

3 Balance between osteogenesis and chondrogenesis is essential for correct development of
4 the craniofacial skeleton. Using mice deficient for Yap and Taz in the neural crest lineage it
5 was demonstrated this pathway promotes osteogenic genes expression while repressing
6 chondrogenic fate via the action of the Wnt/ β -catenin pathway. The Yap/Taz signaling pathway
7 is thus involved in regulating this equilibrium and resulting mutants presented with cranial bone
8 defects and ectopic cartilage formation (Zhao et al., 2022). Gene regulatory networks
9 orchestrating bone and cartilage formation and differentiation have been and are still being
10 dissected and characterized in great details (Liao et al., 2022) which represent a great resource
11 to find potential new strategies to stimulate osteo- and chondrogenesis during bone repair.
12 Nevertheless, the mechanisms conferring CNCC its remarkable plasticity – with their capacity
13 to generate cell types that extend beyond their ectoderm germ layer origin – was only recently
14 uncovered and needs to be explored in more depth.
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25 **Origin of CNCC cellular plasticity**

26 CNCC have a much broader differentiation potential than their ectodermal lineage of origin and
27 have been challenging the three-germ layer theory for almost a century (history of neural crest
28 biology has recently been reviewed in Kelsh et al., 2021). Several pieces of evidence have
29 demonstrated and confirmed the contribution of CNCC in the formation of the cranial cartilage
30 and bone, but many key questions are still open, primarily concerning the mechanisms through
31 which these cells reach their final skeletogenic fate.
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36 Pioneer studies using fluorescent intracellular dye to label single pre-migratory neural crest
37 cells to follow their fate after migration in early embryos demonstrated CNCC plasticity *in vivo*
38 (Bronner-Fraser and Fraser, 1988; Collazo et al., 1993; Serbedzija et al., 1992, 1994). These
39 experiments also revealed that pre-migratory neural crest cells are composed of a mixture of
40 multipotent and more restricted subpopulations. More recently, studies performed in avian and
41 *Xenopus* embryos showed a subpopulation of pre-migratory CNCC expresses pluripotency
42 factor genes such as *Nanog*, *Klf4*, and *Oct4* supporting the notion of CNCC exceptional
43 potency (Lignell et al., 2017). *In situ* hybridization performed in *Xenopus* embryos showed
44 neural crest specifier genes are co-expressed with pluripotency markers (Buitrago-Delgado
45 et al., 2015), suggesting pluripotency program is retained from the blastula stage into the
46 CNCC lineage. Moreover, when derived from blastula-stage embryos, animal pole-derived
47 explants could generate all three germ layers under defined culture conditions. Yet, this
48 potential was lost when explants were taken later during development as gastrula-stage cells
49 have already undergone lineage commitment. However, when converting gastrula-derived
50 explants to neural plate border identity (through the over-expression of *Pax3* and *Zic1*),
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1 explants reacquired the capacity to form ectoderm, mesoderm as well as endoderm – even
2 though neural crest cells do not endogenously form endodermal derivatives (Buitrago-Delgado
3 et al., 2015).

4 In contrast, a single-cell RNA-sequencing (scRNA-seq) study investigating 136,966 single-cell
5 transcriptomes obtained from 10 early *Xenopus* developmental stages failed to uncover a
6 cluster of cells with enriched expression of pluripotency markers (Briggs et al., 2018). Though
7 one can argue that the sequencing technique used for the experiment was not sensitive
8 enough to detect the retention of a pluripotency programs in neural plate border cells at low
9 transcriptional levels. Alternatively, this approach does not detect non-transcriptional
10 regulation, such as epigenetic modifications of enhancers regulating the expression of genes
11 responsible for the increase in CNCC differentiation potential. Along the same line, a recent
12 study identified miR-302 as a post-transcriptional regulator of CNCC plasticity. This miRNA
13 appears to maintain chromatin accessibility, to directly target *Sox9* and expand the period of
14 ectomesenchyme specification and enlarge CNCC developmental potential (Keuls et al.,
15 2023). Recent data obtained in *Xenopus* and mouse embryos showed pluripotency programs
16 are in fact reactivated during CNCC formation (Scerbo and Monsoro-Burq, 2020; Zalc et al.,
17 2021). Careful analysis of *Oct4* spatiotemporal expression in mouse embryos revealed that –
18 in late neurula embryo – *Oct4* is not expressed in the developing head fold. Yet, it is reactivated
19 later, in the most anterior part of the embryo following somitogenesis, demonstrating that rather
20 than being maintained from the epiblast, pluripotency programs are transiently reactivated in
21 the prospective CNCC following head-folds formation. Moreover, this transient re-expression
22 of pluripotency programs was shown to be essential for CNCC to expand their differentiation
23 potential as inhibition of *Oct4* reactivation at the onset on CNCC induction severely impairs
24 facial ectomesenchyme specification and survival, directly linking the reactivation of
25 pluripotency programs with CNCC cellular potential expansion (Zalc et al., 2021). In addition,
26 analysis of *Oct4*⁺ CNCC open chromatin landscape confirmed that regulatory elements
27 controlling expression of mesenchymal genes such as *Pdgfra* or *Mef2c* are already accessible
28 in pre-migratory CNCC – 8 to 12 hours before any transcripts coding for these mesenchymal
29 specification genes are being detected in migratory CNCC – confirming previous epigenetics
30 profiling experiments that identified regulatory elements contribute to neural crest cell fate
31 decisions (Rada-Iglesias et al., 2011; Minoux et al., 2017; Williams et al., 2019; Zalc et al.,
32 2021). Furthermore, the transcription factor TFAP2 α was shown to physically interacts with the
33 OCT4-SOX2 dimer to modify its chromatin binding from pluripotency to CNCC enhancers and
34 thus regulate developmental potential of this population (Hovland et al., 2022). Together, these
35 studies suggest that CNCC differentiation programs are already primed before EMT, allowing
36 CNCC to adapt to future environmental cues they may encounter during and after their
37 migration to issue a correct craniofacial development.

Neural crest cells and bone regeneration

In mammals, bone tissue has an excellent repair capacity, however its ability to heal large defects remains limited (Kiernan et al., 2018). Thus, stimulating endogenous regeneration is necessary to treat severe craniofacial tissue injuries to alleviate the need of tissue transplantation from the patient or a donor and avoid additional complications such as transplant or scaffold rejection.

Skeletal stem cells (SSC) are the common tissue-resident progenitor cells giving rise to bone, cartilage, and stromal elements during bone regeneration (Robey et al., 2007; Chan et al., 2015, 2018). Accumulating evidence suggest that bone regeneration relies on SSC recapitulating developmental programs to ensure the repair process. For example, following femoral fracture, SSC are mobilized and display increased proliferation, viability, and enhanced osteogenic function. Moreover, a recent report shows that enriched 3D-hydrogel transplantation induces expansion of the *Msx1*⁺ skeletal stem cells and enhanced bone regeneration in a model of calvaria injury (Zhang et al., 2022). Transcriptome analysis of the injury-responsive mouse SSC showed a striking overlap between molecular programs active during long bone development and regeneration, such as *BMP* and Hedgehog signaling (Marecic et al., 2015). However, one can argue these signals are pivotal hubs that are used in various tissue and contexts. Similarly, SSC were shown to play a significant role in mandibular repair during distraction osteogenesis – a procedure consisting in cutting and separating bone, to allow bone repair process to fill in the gap (Fang et al., 2004). Moreover, it has been shown that, during the repair process, SSC reactivate neural crest transcriptional programs which enhances bone formation and tissue repair (Ransom et al., 2018). While both long and craniofacial bone regeneration rely on the reactivation of developmental programs for efficient repair, CNCC-derived bones regenerate better compared to mesoderm-derived long bones (Leucht et al., 2008; Wang et al., 2009). However, it is still unclear whether this is due to the lack of expression of the *Hox* genes in anterior craniofacial bones (Leucht et al., 2008; Wang et al., 2009) or to the ability of the craniofacial SSC to more efficiently reactivate developmental programs than long bone SSC is still unclear.

Deeper understanding of the molecular mechanisms mobilizing SSC and stimulating cell potency could then be translated to ameliorate craniofacial endogenous regenerative responses, tissue repair and healing. Up to date, the ability of adjuvant therapies to enhance endogenous bone repair has been studied using various animal models. During mandibular distraction, treatment with deferoxamine was shown to accelerate bone consolidation in rats (Donneys et al., 2013) by chelating iron, which results in the stimulation of the hypoxia inducible factor 1- α (HIF-1 α) pathway – a master regulator of cellular response to hypoxia (Wang et al., 1995; Iyer et al., 1998). Using a rat model of mandibular distraction osteogenesis, another

1 study demonstrated that activating the stromal cell–derived factor-1 (SDF1)/chemokine
2 receptor-4 (CXCR4) pathway promoted migration of endogenous mesenchymal stem cells to
3 the distraction site (Cao et al., 2013). However, this study did not determine the contribution of
4 the recruited mesenchymal stem cells to the distraction regeneration but still represent a
5 promising avenue to explore since the SDF1 signaling is also involved in CNCC migration
6 (Theveneau et al., 2010) during embryogenesis.
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9 Homologous and heterologous bone transplantation are one of the most common surgical
10 procedures utilized for damaged bone repair. However, many limitations and challenging post-
11 operative complications can occur with this procedure, such as site infection or immunologic
12 reaction. Thus, alternative treatments for repair and regeneration need to be explored. For
13 example, chondrocytes from other sources could be harvested and expanded *in vitro*
14 (Smeriglio et al., 2015a) alone or in combination with bioengineering tools such as biomimetic
15 hydrogels (Smeriglio et al., 2015b). These cells can be then grafted on the site of bone
16 regeneration to contribute to bone repair. Another possible strategy focuses on nasal cartilage
17 biopsies that can be harvested under local anesthesia, with minimal donor site morbidity (Lan
18 et al., 2017). Such biopsies have been shown to be a good source of nasal chondrocytes that
19 display a better proliferation and chondrogenic capacity than articular chondrocytes *ex vitro*
20 and *in vivo* (Rotter et al., 2002; Wolf et al., 2008) and have a superior ability to integrate the
21 surrounding tissue when implanted to repair cartilage defects (reviewed in Li et al., 2020).
22 These represent a source of easily accessible material in relatively abundant quantity and are
23 promising avenue to further explore in the future.
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36 **Conclusion**

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38 The craniofacial skeleton represents one major derivative of the cranial neural crest (Jiang
39 et al., 2002; Noden and Trainor, 2005). Because of the crucial functions of this structure,
40 any defects, either injury or disease-associated, have an enormous impact on quality of
41 life. While bone is a tissue with very efficient regenerative capacities, about 10% of bone
42 fractures are unable to self-repair (Sheen and Garla, 2022) and will require transplantation
43 or stem-cell therapies. Regenerative medicine has made tremendous progresses in
44 developing treatments and procedures to increase tissue repair in patients. Nevertheless,
45 it is essential to find new ways to stimulate endogenous regeneration to overcome the
46 limitations of autologous and heterologous transplantations, including graft rejection.
47 Stimulating the endogenous repair also results in the formation of a better integrated
48 structure within surrounding tissues and similar in pattern to the original. Several studies
49 of SSC contribution to bone repair demonstrated the importance of recapitulating
50 developmental processes in post-natal bone repair processes. Characterizing the gene
51 regulatory networks governing bone development and the mechanisms controlling SSC
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potential within their niche represent fundamental goals in the fields of developmental biology, stem cell research and regenerative medicine. Furthermore, understanding the molecular regulations of cell plasticity during development will be essential to enhance SSC expansion, stimulate tissue resident stem cells and reduce the tumorigenic risks of stem cell transplantation. This knowledge will be essential to establish prototype procedures aiming at enhancing endogenous regenerative responses during tissue repair. It will likely lead to protocols increasing the viability and adaptability of stem-cell or tissue transplants, which will ameliorate autograft integration and overall repair process. Translating this knowledge will allow to engineer better regenerative therapies for humans suffering traumatic injuries or congenital syndromes.

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Cranial neural crest cells contribution to craniofacial bone development and regeneration

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Compliance with Ethical Standards

Conflict of Interest

Piera Smeriglio and Antoine Zalc declare no conflict of interest.

Human and Animal Rights and Informed Consent

This article does not contain any studies with human or animal subjects performed by any of the authors.

Abstract

1 **Purpose of Review** We aim to summarize (i) the latest evidence on cranial neural crest cells
2 (CNCC) contribution to craniofacial development and ossification; (ii) the recent discoveries on
3 the mechanisms responsible for their plasticity; and (iii) the newest developed procedures to
4 ameliorate maxillofacial tissue repair.
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8 **Recent Findings** CNCC display a remarkable differentiation potential – that exceeds the
9 capacity of their germ layer of origin. Recent studies identified novel molecular regulations of
10 craniofacial development within the neural crest lineage and also discovered how CNCC
11 naturally expand their plasticity.
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14 **Summary** Traumatic craniofacial injuries or congenital syndromes can be life-threatening,
15 require invasive maxillofacial surgery and can leave deep sequels on our health or quality of
16 life. With accumulating evidence showing CNCC-derived stem cells potential to ameliorate
17 craniofacial reconstruction and tissue repair, we believe a deeper understanding of how CNCC
18 regulate their plasticity is essential to ameliorate endogenous regeneration and improve tissue
19 repair therapies.
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Introduction

1 The craniofacial skeleton is a crucial component of vertebrate development. It is the structure
2 that protects the brain, and it is essential for respiration, food intake and communication.
3 Additionally, the craniofacial skeleton shapes our face, which is one major definition of our very
4 self. Given its essential functions, congenital craniofacial syndromes – which represent a third
5 of all congenital malformations within the human population [1] – or traumatic injuries to the
6 head skeleton – can have a profound impact on our health and quality of life. When available,
7 treatments of such syndromes or trauma require heavy maxillo-facial surgeries and
8 reconstruction. Regenerative medicine has made tremendous progress in developing
9 treatments and procedures to enhance craniofacial tissue repair in patients. Most commonly
10 used procedures include autologous bone transplantation [2], [3], bone tissue engineering
11 techniques [4], [5] including bone distraction – whereby new bone is generated by applying
12 stress (stretching) to the endogenous bone tissue [6] – and more recently stem cell-based
13 therapies [7], [8]. However, these techniques present the risk of generating unsuitable
14 structures (with ectopic bone formation), relatively poor integration of the new graft or cells
15 within the existing bone and the surrounding soft tissues and they are limited by the size of
16 tissue to replace. Stem cell-based therapy bears an additional risk of genetic and epigenetic
17 mutations which can promote tumor formation [9••]–[11].

18 The repair of severely damaged or missing bones should ideally occur through the induction
19 of an endogenous regenerative response, alleviating the need to harvest tissue from the
20 patient or a donor, and avoiding additional issues such as rejection of the tissue transplant.
21 Moreover, endogenous regeneration results in the formation of a structure (i) similar in pattern
22 to the original anatomy and (ii) better integrated within the native tissues including the
23 surrounding muscles, nerves, and vasculature. Data from regenerative species show that
24 controlled cell dedifferentiation is an essential determinant to insure an adequate endogenous
25 regenerative response [12]–[14]. Understanding how cell plasticity is regulated is then crucial
26 to enhance tissue resident stem cells mobilization and expansion, reduce the tumorigenic risks
27 and altogether promote an efficient endogenous regeneration.

28 The majority of craniofacial bones derive from cranial neural crest cells (CNCC) – a transient
29 stem cell-like population arising in the most rostral part of the embryo soon after gastrulation
30 [15], [16]. Within the ectoderm lineage, at the border between the neural plate and the surface
31 ectoderm, CNCC are induced as an epithelial cell type [17], [18], that subsequently undergoes
32 an epithelial-to-mesenchymal transition (EMT). CNCC then delaminate from the dorsal
33 epithelium and migrate dorso-ventrally through the embryo to populate various locations in the
34 craniofacial complex where they differentiate into diverse cell types [17], [19]. CNCC present
35 an extraordinary differentiation potential since they generate not only ectoderm derivatives,
36 such as neurons, glia and melanocytes, but also give rise to cells canonically associated with
37

1 the mesoderm such as bones, cartilage and smooth muscles – also referred to as
2 ectomesenchyme [17], [20]. Thus, CNCC “break” the rules set during gastrulation as they
3 generate derivatives that extend beyond the potential of their germ layer of origin [21]. This
4 unique differentiation potential can be explained by the fact that CNCC express pluripotency
5 programs at the onset of their development [22], [23]. Furthermore, it was recently shown that
6 CNCC are able to reactivate *Oct4* and the associated pluripotency programs [24•], [25••]
7 during their formation. Together, these studies suggest that a deeper understanding of how
8 CNCC regulate the expression of pluripotency programs could unveil new strategies to
9 stimulate cell plasticity *in vivo* during post-natal tissue repair. Future regenerative therapies will
10 need to recapitulate these processes to enhance endogenous regeneration and ameliorate
11 craniofacial tissue repair.

12 In this review we will briefly summarize how CNCC contribute to craniofacial bone development
13 and highlight the newest findings regarding gene regulation of ossification. We will focus on
14 the recent discovery on the origin of CNCC remarkable plasticity and finally, we will question
15 how this plasticity could be used to enhance craniofacial bone regeneration and discuss on
16 the latest procedures enhancing craniofacial bone healing.

17 Given the limitation of words, we will only focus on the cranial neural crest, even though
18 accumulating evidence suggest that the trunk neural crest could also have a skeletogenic
19 capacity *in vivo* [26].

30 **Neural crest contribution to the craniofacial skeleton**

31 During embryogenesis bone can either form via the endochondral ossification process, where
32 mesenchymal progenitors form a cartilaginous template that is gradually replaced by bone
33 tissue, or intramembranous ossification, during which mesenchymal cells directly differentiate
34 into osteoblasts, with no cartilaginous intermediate. Intramembranous bones are predominant
35 in the head forming the cranial vault together with most bones of the face. The
36 intramembranous ossification process starts *in utero* and ends at different postnatal times
37 depending on the type of bone. For example, the clavicles are not fully ossified at birth allowing
38 the postnatal growth and development of the brain. Although most of the bone originates from
39 mesodermal precursor, some facial bones, as well as the endocranium, are derived from
40 CNCC [16]. Development of the craniofacial skeleton requires the precise differentiation of
41 CNCC into osteoblasts or chondrocytes. Following CNCC migration and colonization of the
42 facial prominences and branchial arches, CNCC aggregate, condense, and differentiate into a
43 common osteochondral progenitor and then into more differentiated chondrocytes or
44 osteoblasts [27]. The molecular regulations orchestrating craniofacial ossification were
45 recently reviewed in great details [28]. Harmonious craniofacial ossification requires the
46 precise action of CNCC intrinsic transcription factors such as SOX9, RUNX2 and MSX1/2 in
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1 association with extrinsic inputs that include fibroblast growth factor (FGF), Wingless-related
2 integration site (WNT) and Transforming growth factor/Bone morphogenetic protein
3 (TGF β /BMP) signaling pathways. Thus, gene expression and signaling pathways must be
4 specifically activated and terminated in the correct location at the proper developmental time
5 to ensure a *bona fide* craniofacial development. Recent studies further exemplified that
6 inaccurate regulation of gene expression in CNCC leads to severe craniofacial defect. A mouse
7 model constitutively activating the activin A receptor type I (ACVR1) to enhance BMP signaling
8 in CNCC results in ectopic cartilage formation in the craniofacial region [29]. The study further
9 showed that the increased BMP signaling inhibits autophagy via the mTORC1 pathway and
10 blocks the autophagic degradation of β -catenin, causing CNCC to adopt a chondrogenic
11 identity. This phenotype was then rescued by inhibiting mTORC1 signaling to reactivate the
12 Wnt/ β -catenin signaling pathway [29]. mTORC1 was also shown to mediate the function of
13 the acetyltransferase GCN5 – a highly conserved enzyme and potent activator of chondrocyte
14 maturation – during craniofacial development [30]. Interestingly in this context, GCN5 is not
15 acting as an epigenetic regulator but probably via direct activation of mTORC1 pathway [30].
16 Epigenetic regulation also plays a role in the CNCC ossification. In fact, inhibition of KMT2D
17 function – a histone methylase which mutations are associated with Kabuki syndrome
18 congenital craniofacial disorder – in the neural crest lineage alters osteochondral progenitor
19 differentiation and results in craniofacial hypoplasia [31]. We have also demonstrated a link
20 between the epigenetic modulator Ten eleven translocation enzyme 1 (TET1) and
21 chondrogenic differentiation [32]. Loss of TET1 expression impairs chondrogenesis via tissue-
22 specific changes in 5-hydroxymethylcytosine (5hmC) landscape and reduces the expression
23 of cartilage markers. It remains to be established if this mechanism has a direct impact on
24 CNCC. A recent breakthrough study found that in the neural crest lineage, mutation of the
25 tumor suppressor *Brca1* resulted in neonatal death of the mutant animals which presented with
26 a cleft palate and reduced skull due to the reduction in size of craniofacial bones. The reduction
27 in bones size was not due to osteogenic differentiation but by a strong defect in osteogenic
28 proliferation and survival due to an increased DNA damage in skeletogenic precursor cells as
29 demonstrated by the inhibition of p53 which is sufficient to rescue the *Brca1* mutant phenotype
30 *in vivo* [33].

31 Balance between osteogenesis and chondrogenesis is essential for correct development of
32 the craniofacial skeleton. Using mice deficient for Yap and Taz in the neural crest lineage it
33 was demonstrated this pathway promotes osteogenic genes expression while repressing
34 chondrogenic fate via the action of the Wnt/ β -catenin pathway. The Yap/Taz signaling pathway
35 is thus involved in regulating this equilibrium and resulting mutants presented with cranial bone
36 defects and ectopic cartilage formation [34]. Gene regulatory networks orchestrating bone
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1 and cartilage formation and differentiation have been and are still being dissected and
2 characterized in great details [35] which represent a great resource to find potential new
3 strategies to stimulate osteo- and chondrogenesis during bone repair. Nevertheless, the
4 mechanisms conferring CNCC its remarkable plasticity – with their capacity to generate cell
5 types that extend beyond their ectoderm germ layer origin – was only recently uncovered and
6 needs to be explored in more depth.
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10 **Origin of CNCC cellular plasticity**

11 CNCC have a much broader differentiation potential than their ectodermal lineage of origin and
12 have been challenging the three-germ layer theory for almost a century (history of neural crest
13 biology has recently been reviewed in [36]. Several pieces of evidence have demonstrated and
14 confirmed the contribution of CNCC in the formation of the cranial cartilage and bone, but many
15 key questions are still open, primarily concerning the mechanisms through which these cells
16 reach their final skeletogenic fate.
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23 Pioneer studies using fluorescent intracellular dye to label single pre-migratory neural crest
24 cells to follow their fate after migration in early embryos demonstrated CNCC plasticity *in vivo*
25 [37]–[40]. These experiments also revealed that pre-migratory neural crest cells are composed
26 of a mixture of multipotent and more restricted subpopulations. More recently, studies perform
27 in avian and *Xenopus* embryos showed a subpopulation of pre-migratory CNCC expresses
28 pluripotency factor genes such as *Nanog*, *Klf4*, and *Oct4* supporting the notion of CNCC
29 exceptional potency [23]. *In situ* hybridization performed in *Xenopus embryos* showed neural
30 crest specifiers genes are co-expressed with pluripotency markers [22], suggesting
31 pluripotency program is retained from the blastula stage into the CNCC lineage. Moreover,
32 when derived from blastula-stage embryos, animal pole-derived explants could generate all
33 three germ layers under defined culture conditions. Yet, this potential was lost when explants
34 were taken later during development as gastrula-stage cells have already undergone lineage
35 commitment. However, when converting gastrula-derived explants to neural plate border
36 identity (through the over-expression of *Pax3* and *Zic1*), explants reacquired the capacity to
37 form ectoderm, mesoderm as well as endoderm – even though neural crest cells do not
38 endogenously form endodermal derivatives [22].
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50 In contrast, a single-cell RNA-sequencing (scRNA-seq) study investigating 136,966 single-cell
51 transcriptomes obtained from 10 early *Xenopus* developmental stages failed to uncover a
52 cluster of cells with enriched expression of pluripotency markers [41]. Though one can argue
53 that the sequencing technique used for the experiment was not sensitive enough to detect the
54 retention of a pluripotency programs in neural plate border cells at low transcriptional levels.
55 Alternatively, this approach does not detect non-transcriptional regulation, such as epigenetic
56 modifications of enhancers regulating the expression of genes responsible for the increase in
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1 CNCC differentiation potential. Along the same line, a recent study identified miR-302 as a
2 post-transcriptional regulator of CNCC plasticity. This miRNA appears to maintain chromatin
3 accessibility, to directly target *Sox9* and expand the period of ectomesenchyme specification
4 and enlarge CNCC developmental potential [42•]. Recent data obtained in *Xenopus* and
5 mouse embryos showed pluripotency programs are in fact reactivated during CNCC formation
6 [24•], [25••]. Careful analysis of *Oct4* spatiotemporal expression in mouse embryos revealed
7 that – in late neurula embryo – *Oct4* is not expressed in the developing head fold. Yet, it is
8 reactivated later, in the most anterior part of the embryo following somitogenesis,
9 demonstrating that rather than being maintained from the epiblast, pluripotency programs are
10 transiently reactivated in the prospective CNCC following head-folds formation. Moreover, this
11 transient re-expression of pluripotency programs was shown to be essential for CNCC to
12 expand their differentiation potential as inhibition of *Oct4* reactivation at the onset on CNCC
13 induction severely impairs facial ectomesenchyme specification and survival, directly linking
14 the reactivation of pluripotency programs with CNCC cellular potential expansion [25••]. In
15 addition, analysis of *Oct4*⁺ CNCC open chromatin landscape confirmed that regulatory
16 elements controlling expression of mesenchymal genes such as *Pdgfra* or *Mef2c* are already
17 accessible in pre-migratory CNCC – 8 to 12 hours before any transcripts coding for these
18 mesenchymal specification genes are being detected in migratory CNCC – confirming previous
19 epigenetics profiling experiments that identified regulatory elements contribute to neural crest
20 cell fate decisions [43]–[45], [25••]. Furthermore, the transcription factor TFAP2 α was shown
21 to physically interacts with the OCT4-SOX2 dimer to modify its chromatin binding from
22 pluripotency to CNCC enhancers and thus regulate developmental potential of this population
23 [46]. Together, these studies suggest that CNCC differentiation programs are already primed
24 before EMT, allowing CNCC to adapt to future environmental cues they may encounter during
25 and after their migration to issue a correct craniofacial development.

44 **Neural crest cells and bone regeneration**

45 In mammals, bone tissue has an excellent repair capacity, however its ability to heal large
46 defects remains limited [47]. Thus, stimulating endogenous regeneration is necessary to treat
47 severe craniofacial tissue injuries to alleviate the need of tissue transplantation from the patient
48 or a donor and avoid additional complications such as transplant or scaffold rejection.

49 Skeletal stem cells (SSC) are the common tissue-resident progenitor cells giving rise to bone,
50 cartilage, and stromal elements during bone regeneration [48]–[50]. Accumulating evidence
51 suggest that bone regeneration relies on SSC recapitulating developmental programs to
52 ensure the repair process. For example, following femoral fracture, SSC are mobilized and
53 display increased proliferation, viability, and enhanced osteogenic function. Moreover, a recent
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1 report shows that enriched 3D-hydrogel transplantation induces expansion of the *Msx1*⁺
2 skeletal stem cells and enhanced bone regeneration in a model of calvaria injury [51].
3 Transcriptome analysis of the injury-responsive mouse SSC showed a striking overlap
4 between molecular programs active during long bone development and regeneration, such as
5 *BMP* and Hedgehog signaling [52]. However, one can argue these signals are pivotal hubs
6 that are used in various tissue and contexts. Similarly, SSC were shown to play a significant
7 role in mandibular repair during distraction osteogenesis – a procedure consisting in cutting
8 and separating bone, to allow bone repair process to fill in the gap [53]. Moreover, it has been
9 shown that, during the repair process, SSC reactivate neural crest transcriptional programs
10 which enhances bone formation and tissue repair [54]. While both long and craniofacial bone
11 regeneration rely on the reactivation of developmental programs for efficient repair, CNCC-
12 derived bones regenerate better compared to mesoderm-derived long bones [55], [56].
13 However, it is still unclear whether this is due to the lack of expression of the *Hox* genes in
14 anterior craniofacial bones [55], [56] or to the ability of the craniofacial SSC to more efficiently
15 reactivate developmental programs than long bone SSC is still unclear.

24 Deeper understanding of the molecular mechanisms mobilizing SSC and stimulating cell
25 potency could then be translated to ameliorate craniofacial endogenous regenerative
26 responses, tissue repair and healing. Up to date, the ability of adjuvant therapies to enhance
27 endogenous bone repair has been studied using various animal models. During mandibular
28 distraction, treatment with deferoxamine was shown to accelerate bone consolidation in rats
29 [57] by chelating iron, which results in the stimulation of the hypoxia inducible factor 1- α (HIF-
30 1 α) pathway – a master regulator of cellular response to hypoxia [58], [59]. Using a rat model
31 of mandibular distraction osteogenesis, another study demonstrated that activating the stromal
32 cell-derived factor-1 (SDF1)/chemokine receptor-4 (CXCR4) pathway promoted migration of
33 endogenous mesenchymal stem cells to the distraction site [60]. However, this study did not
34 determine the contribution of the recruited mesenchymal stem cells to the distraction
35 regeneration but still represent a promising avenue to explore since the SDF1 signaling is also
36 involved in CNCC migration [61] during embryogenesis.

46 Homologous and heterologous bone transplantation are one of the most common surgical
47 procedures utilized for damaged bone repair. However, many limitations and challenging post-
48 operative complications can occur with this procedure, such as site infection or immunologic
49 reaction. Thus, alternative treatments for repair and regeneration need to be explored. For
50 example, chondrocytes from other sources could be harvested and expanded *in vitro* [62] alone
51 or in combination with bioengineering tools such as biomimetic hydrogels [63]. These cells can
52 be then grafted on the site of bone regeneration to contribute to bone repair. Another possible
53 strategy focuses on nasal cartilage biopsies that can be harvested under local anesthesia, with
54 minimal donor site morbidity [64]. Such biopsies have been shown to be a good source of
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nasal chondrocytes that display a better proliferation and chondrogenic capacity than articular chondrocytes *ex vitro* and *in vivo* [65], [66] and have a superior ability to integrate the surrounding tissue when implanted to repair cartilage defects (reviewed in Li et al., 2020). These represent a source of easily accessible material in relatively abundant quantity and are promising avenue to further explore in the future.

Conclusion

The craniofacial skeleton represents one major derivative of the cranial neural crest [16], [68]. Because of the crucial functions of this structure, any defects, either injury or disease-associated, have an enormous impact on quality of life. While bone is a tissue with very efficient regenerative capacities, about 10% of bone fractures are unable to self-repair [69] and will require transplantation or stem-cell therapies. Regenerative medicine has made tremendous progresses in developing treatments and procedures to increase tissue repair in patients. Nevertheless, it is essential to find new ways to stimulate endogenous regeneration to overcome the limitations of autologous and heterologous transplantations, including graft rejection. Stimulating the endogenous repair also results in the formation of a better integrated structure within surrounding tissues and similar in pattern to the original. Several studies of SSC contribution to bone repair demonstrated the importance of recapitulating developmental processes in post-natal bone repair processes. Characterizing the gene regulatory networks governing bone development and the mechanisms controlling SSC potential within their niche represent fundamental goals in the fields of developmental biology, stem cell research and regenerative medicine. Furthermore, understanding the molecular regulations of cell plasticity during development will be essential to enhance SSC expansion, stimulate tissue resident stem cells and reduce the tumorigenic risks of stem cell transplantation. This knowledge will be essential to establish prototype procedures aiming at enhancing endogenous regenerative responses during tissue repair. It will likely lead to protocols increasing the viability and adaptability of stem-cell or tissue transplants, which will ameliorate autograft integration and overall repair process. Translating this knowledge will allow to engineer better regenerative therapies for humans suffering traumatic injuries or congenital syndromes.

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Cranial neural crest cells contribution to craniofacial bone development and regeneration

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Compliance with Ethical Standards

Conflict of Interest

Piera Smeriglio and Antoine Zalc declare no conflict of interest.

Human and Animal Rights and Informed Consent

This article does not contain any studies with human or animal subjects performed by any of the authors.

Abstract

1 **Purpose of Review** We aim to summarize (i) the latest evidence on cranial neural crest cells
2 (CNCC) contribution to craniofacial development and ossification; (ii) the recent discoveries on
3 the mechanisms responsible for their plasticity; and (iii) the newest developed procedures to
4 ameliorate maxillofacial tissue repair.
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8 **Recent Findings** CNCC display a remarkable differentiation potential – that exceeds the
9 capacity of their germ layer of origin. Recent studies identified novel molecular regulations of
10 craniofacial development within the neural crest lineage and also discovered how CNCC
11 naturally expand their plasticity.
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14 **Summary** Traumatic craniofacial injuries or congenital syndromes can be life-threatening,
15 require invasive maxillofacial surgery and can leave deep sequels on our health or quality of
16 life. With accumulating evidence showing CNCC-derived stem cells potential to ameliorate
17 craniofacial reconstruction and tissue repair, we believe a deeper understanding of how CNCC
18 regulate their plasticity is essential to ameliorate endogenous regeneration and improve tissue
19 repair therapies.
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Introduction

1 The craniofacial skeleton is a crucial component of vertebrate development. It is the structure
2 that protects the brain, and it is essential for respiration, food intake and communication.
3 Additionally, the craniofacial skeleton shapes our face, which is one major definition of our very
4 self. Given its essential functions, congenital craniofacial syndromes – which represent a third
5 of all congenital malformations within the human population [1] – or traumatic injuries to the
6 head skeleton – can have a profound impact on our health and quality of life. When available,
7 treatments of such syndromes or trauma require heavy maxillo-facial surgeries and
8 reconstruction. Regenerative medicine has made tremendous progress in developing
9 treatments and procedures to enhance craniofacial tissue repair in patients. Most commonly
10 used procedures include autologous bone transplantation [2], [3], bone tissue engineering
11 techniques [4], [5] including bone distraction – whereby new bone is generated by applying
12 stress (stretching) to the endogenous bone tissue [6] – and more recently stem cell-based
13 therapies [7], [8]. However, these techniques present the risk of generating unsuitable
14 structures (with ectopic bone formation), relatively poor integration of the new graft or cells
15 within the existing bone and the surrounding soft tissues and they are limited by the size of
16 tissue to replace. Stem cell-based therapy bears an additional risk of genetic and epigenetic
17 mutations which can promote tumor formation [9–11].

18 The repair of severely damaged or missing bones should ideally occur through the induction
19 of an endogenous regenerative response, alleviating the need to harvest tissue from the
20 patient or a donor, and avoiding additional issues such as rejection of the tissue transplant.
21 Moreover, endogenous regeneration results in the formation of a structure (i) similar in pattern
22 to the original anatomy and (ii) better integrated within the native tissues including the
23 surrounding muscles, nerves, and vasculature. Data from regenerative species show that
24 controlled cell dedifferentiation is an essential determinant to insure an adequate endogenous
25 regenerative response [12]–[14]. Understanding how cell plasticity is regulated is then crucial
26 to enhance tissue resident stem cells mobilization and expansion, reduce the tumorigenic risks
27 and altogether promote an efficient endogenous regeneration.

28 The majority of craniofacial bones derive from cranial neural crest cells (CNCC) – a transient
29 stem cell-like population arising in the most rostral part of the embryo soon after gastrulation
30 [15], [16]. Within the ectoderm lineage, at the border between the neural plate and the surface
31 ectoderm, CNCC are induced as an epithelial cell type [17], [18], that subsequently undergoes
32 an epithelial-to-mesenchymal transition (EMT). CNCC then delaminate from the dorsal
33 epithelium and migrate dorso-ventrally through the embryo to populate various locations in the
34 craniofacial complex where they differentiate into diverse cell types [17], [19]. CNCC present
35 an extraordinary differentiation potential since they generate not only ectoderm derivatives,
36 such as neurons, glia and melanocytes, but also give rise to cells canonically associated with

1 the mesoderm such as bones, cartilage and smooth muscles – also referred to as
2 ectomesenchyme [17], [20]. Thus, CNCC “break” the rules set during gastrulation as they
3 generate derivatives that extend beyond the potential of their germ layer of origin [21]. This
4 unique differentiation potential can be explained by the fact that CNCC express pluripotency
5 programs at the onset of their development [22], [23]. Furthermore, it was recently shown that
6 CNCC are able to reactivate *Oct4* and the associated pluripotency programs [24●], [25●●]
7 during their formation. Together, these studies suggest that a deeper understanding of how
8 CNCC regulate the expression of pluripotency programs could unveil new strategies to
9 stimulate cell plasticity *in vivo* during post-natal tissue repair. Future regenerative therapies will
10 need to recapitulate these processes to enhance endogenous regeneration and ameliorate
11 craniofacial tissue repair.

12 In this review we will briefly summarize how CNCC contribute to craniofacial bone development
13 and highlight the newest findings regarding gene regulation of ossification. We will focus on
14 the recent discovery on the origin of CNCC remarkable plasticity and finally, we will question
15 how this plasticity could be used to enhance craniofacial bone regeneration and discuss on
16 the latest procedures enhancing craniofacial bone healing.

17 Given the limitation of words, we will only focus on the cranial neural crest, even though
18 accumulating evidence suggest that the trunk neural crest could also have a skeletogenic
19 capacity *in vivo* [26].

30 **Neural crest contribution to the craniofacial skeleton**

31 During embryogenesis bone can either form via the endochondral ossification process, where
32 mesenchymal progenitors form a cartilaginous template that is gradually replaced by bone
33 tissue, or intramembranous ossification, during which mesenchymal cells directly differentiate
34 into osteoblasts, with no cartilaginous intermediate. Intramembranous bones are predominant
35 in the head forming the cranial vault together with most bones of the face. The
36 intramembranous ossification process starts *in utero* and ends at different postnatal times
37 depending on the type of bone. For example, the clavicles are not fully ossified at birth allowing
38 the postnatal growth and development of the brain. Although most of the bone originates from
39 mesodermal precursor, some facial bones, as well as the endocranium, are derived from
40 CNCC [16]. Development of the craniofacial skeleton requires the precise differentiation of
41 CNCC into osteoblasts or chondrocytes. Following CNCC migration and colonization of the
42 facial prominences and branchial arches, CNCC aggregate, condense, and differentiate into a
43 common osteochondral progenitor and then into more differentiated chondrocytes or
44 osteoblasts [27]. The molecular regulations orchestrating craniofacial ossification were
45 recently reviewed in great details [28]. Harmonious craniofacial ossification requires the
46 precise action of CNCC intrinsic transcription factors such as SOX9, RUNX2 and MSX1/2 in
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1 association with extrinsic inputs that include fibroblast growth factor (FGF), Wingless-related
2 integration site (WNT) and Transforming growth factor/Bone morphogenetic protein
3 (TGF β /BMP) signaling pathways. Thus, gene expression and signaling pathways must be
4 specifically activated and terminated in the correct location at the proper developmental time
5 to ensure a *bona fide* craniofacial development. Recent studies further exemplified that
6 inaccurate regulation of gene expression in CNCC leads to severe craniofacial defect. A mouse
7 model constitutively activating the activin A receptor type I (ACVR1) to enhance BMP signaling
8 in CNCC results in ectopic cartilage formation in the craniofacial region [29]. The study further
9 showed that the increased BMP signaling inhibits autophagy via the mTORC1 pathway and
10 blocks the autophagic degradation of β -catenin, causing CNCC to adopt a chondrogenic
11 identity. This phenotype was then rescued by inhibiting mTORC1 signaling to reactivate the
12 Wnt/ β -catenin signaling pathway [29]. mTORC1 was also shown to mediate the function of
13 the acetyltransferase GCN5 – a highly conserved enzyme and potent activator of chondrocyte
14 maturation – during craniofacial development [30]. Interestingly in this context, GCN5 is not
15 acting as an epigenetic regulator but probably via direct activation of mTORC1 pathway [30].
16 Epigenetic regulation also plays a role in the CNCC ossification. In fact, inhibition of KMT2D
17 function – a histone methylase which mutations are associated with Kabuki syndrome
18 congenital craniofacial disorder – in the neural crest lineage alters osteochondral progenitor
19 differentiation and results in craniofacial hypoplasia [31]. We have also demonstrated a link
20 between the epigenetic modulator Ten eleven translocation enzyme 1 (TET1) and
21 chondrogenic differentiation [32]. Loss of TET1 expression impairs chondrogenesis via tissue-
22 specific changes in 5-hydroxymethylcytosine (5hmC) landscape and reduces the expression
23 of cartilage markers. It remains to be established if this mechanism has a direct impact on
24 CNCC. A recent breakthrough study found that in the neural crest lineage, mutation of the
25 tumor suppressor *Brca1* resulted in neonatal death of the mutant animals which presented with
26 a cleft palate and reduced skull due to the reduction in size of craniofacial bones. The reduction
27 in bones size was not due to osteogenic differentiation but by a strong defect in osteogenic
28 proliferation and survival due to an increased DNA damage in skeletogenic precursor cells as
29 demonstrated by the inhibition of p53 which is sufficient to rescue the *Brca1* mutant phenotype
30 *in vivo* [33].

31 Balance between osteogenesis and chondrogenesis is essential for correct development of
32 the craniofacial skeleton. Using mice deficient for Yap and Taz in the neural crest lineage it
33 was demonstrated this pathway promotes osteogenic genes expression while repressing
34 chondrogenic fate via the action of the Wnt/ β -catenin pathway. The Yap/Taz signaling pathway
35 is thus involved in regulating this equilibrium and resulting mutants presented with cranial bone
36 defects and ectopic cartilage formation [34]. Gene regulatory networks orchestrating bone
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1 and cartilage formation and differentiation have been and are still being dissected and
2 characterized in great details [35] which represent a great resource to find potential new
3 strategies to stimulate osteo- and chondrogenesis during bone repair. Nevertheless, the
4 mechanisms conferring CNCC its remarkable plasticity – with their capacity to generate cell
5 types that extend beyond their ectoderm germ layer origin – was only recently uncovered and
6 needs to be explored in more depth.
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10 **Origin of CNCC cellular plasticity**

11 CNCC have a much broader differentiation potential than their ectodermal lineage of origin and
12 have been challenging the three-germ layer theory for almost a century (history of neural crest
13 biology has recently been reviewed in [36]. Several pieces of evidence have demonstrated and
14 confirmed the contribution of CNCC in the formation of the cranial cartilage and bone, but many
15 key questions are still open, primarily concerning the mechanisms through which these cells
16 reach their final skeletogenic fate.
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23 Pioneer studies using fluorescent intracellular dye to label single pre-migratory neural crest
24 cells to follow their fate after migration in early embryos demonstrated CNCC plasticity *in vivo*
25 [37]–[40]. These experiments also revealed that pre-migratory neural crest cells are composed
26 of a mixture of multipotent and more restricted subpopulations. More recently, studies perform
27 in avian and *Xenopus* embryos showed a subpopulation of pre-migratory CNCC expresses
28 pluripotency factor genes such as *Nanog*, *Klf4*, and *Oct4* supporting the notion of CNCC
29 exceptional potency [23]. *In situ* hybridization performed in *Xenopus embryos* showed neural
30 crest specifiers genes are co-expressed with pluripotency markers [22], suggesting
31 pluripotency program is retained from the blastula stage into the CNCC lineage. Moreover,
32 when derived from blastula-stage embryos, animal pole-derived explants could generate all
33 three germ layers under defined culture conditions. Yet, this potential was lost when explants
34 were taken later during development as gastrula-stage cells have already undergone lineage
35 commitment. However, when converting gastrula-derived explants to neural plate border
36 identity (through the over-expression of *Pax3* and *Zic1*), explants reacquired the capacity to
37 form ectoderm, mesoderm as well as endoderm – even though neural crest cells do not
38 endogenously form endodermal derivatives [22].
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50 In contrast, a single-cell RNA-sequencing (scRNA-seq) study investigating 136,966 single-cell
51 transcriptomes obtained from 10 early *Xenopus* developmental stages failed to uncover a
52 cluster of cells with enriched expression of pluripotency markers [41]. Though one can argue
53 that the sequencing technique used for the experiment was not sensitive enough to detect the
54 retention of a pluripotency programs in neural plate border cells at low transcriptional levels.
55 Alternatively, this approach does not detect non-transcriptional regulation, such as epigenetic
56 modifications of enhancers regulating the expression of genes responsible for the increase in
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1 CNCC differentiation potential. Along the same line, a recent study identified miR-302 as a
2 post-transcriptional regulator of CNCC plasticity. This miRNA appears to maintain chromatin
3 accessibility, to directly target *Sox9* and expand the period of ectomesenchyme specification
4 and enlarge CNCC developmental potential [42●]. Recent data obtained in *Xenopus* and
5 mouse embryos showed pluripotency programs are in fact reactivated during CNCC formation
6 [24●], [25●●]. Careful analysis of *Oct4* spatiotemporal expression in mouse embryos revealed
7 that – in late neurula embryo – *Oct4* is not expressed in the developing head fold. Yet, it is
8 reactivated later, in the most anterior part of the embryo following somitogenesis,
9 demonstrating that rather than being maintained from the epiblast, pluripotency programs are
10 transiently reactivated in the prospective CNCC following head-folds formation. Moreover, this
11 transient re-expression of pluripotency programs was shown to be essential for CNCC to
12 expand their differentiation potential as inhibition of *Oct4* reactivation at the onset on CNCC
13 induction severely impairs facial ectomesenchyme specification and survival, directly linking
14 the reactivation of pluripotency programs with CNCC cellular potential expansion [25●●]. In
15 addition, analysis of *Oct4*⁺ CNCC open chromatin landscape confirmed that regulatory
16 elements controlling expression of mesenchymal genes such as *Pdgfra* or *Mef2c* are already
17 accessible in pre-migratory CNCC – 8 to 12 hours before any transcripts coding for these
18 mesenchymal specification genes are being detected in migratory CNCC – confirming previous
19 epigenetics profiling experiments that identified regulatory elements contribute to neural crest
20 cell fate decisions [43]–[45], [25●●]. Furthermore, the transcription factor TFAP2 α was shown
21 to physically interacts with the OCT4-SOX2 dimer to modify its chromatin binding from
22 pluripotency to CNCC enhancers and thus regulate developmental potential of this population
23 [46]. Together, these studies suggest that CNCC differentiation programs are already primed
24 before EMT, allowing CNCC to adapt to future environmental cues they may encounter during
25 and after their migration to issue a correct craniofacial development.

44 **Neural crest cells and bone regeneration**

45 In mammals, bone tissue has an excellent repair capacity, however its ability to heal large
46 defects remains limited [47]. Thus, stimulating endogenous regeneration is necessary to treat
47 severe craniofacial tissue injuries to alleviate the need of tissue transplantation from the patient
48 or a donor and avoid additional complications such as transplant or scaffold rejection.

49 Skeletal stem cells (SSC) are the common tissue-resident progenitor cells giving rise to bone,
50 cartilage, and stromal elements during bone regeneration [48]–[50]. Accumulating evidence
51 suggest that bone regeneration relies on SSC recapitulating developmental programs to
52 ensure the repair process. For example, following femoral fracture, SSC are mobilized and
53 display increased proliferation, viability, and enhanced osteogenic function. Moreover, a recent
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1 report shows that enriched 3D-hydrogel transplantation induces expansion of the Msx1⁺
2 skeletal stem cells and enhanced bone regeneration in a model of calvaria injury [51].
3 Transcriptome analysis of the injury-responsive mouse SSC showed a striking overlap
4 between molecular programs active during long bone development and regeneration, such as
5 *BMP* and Hedgehog signaling [52]. However, one can argue these signals are pivotal hubs
6 that are used in various tissue and contexts. Similarly, SSC were shown to play a significant
7 role in mandibular repair during distraction osteogenesis – a procedure consisting in cutting
8 and separating bone, to allow bone repair process to fill in the gap [53]. Moreover, it has been
9 shown that, during the repair process, SSC reactivate neural crest transcriptional programs
10 which enhances bone formation and tissue repair [54]. While both long and craniofacial bone
11 regeneration rely on the reactivation of developmental programs for efficient repair, CNCC-
12 derived bones regenerate better compared to mesoderm-derived long bones [55], [56].
13 However, it is still unclear whether this is due to the lack of expression of the *Hox* genes in
14 anterior craniofacial bones [55], [56] or to the ability of the craniofacial SSC to more efficiently
15 reactivate developmental programs than long bone SSC is still unclear.

25 Deeper understanding of the molecular mechanisms mobilizing SSC and stimulating cell
26 potency could then be translated to ameliorate craniofacial endogenous regenerative
27 responses, tissue repair and healing. Up to date, the ability of adjuvant therapies to enhance
28 endogenous bone repair has been studied using various animal models. During mandibular
29 distraction, treatment with deferoxamine was shown to accelerate bone consolidation in rats
30 [57] by chelating iron, which results in the stimulation of the hypoxia inducible factor 1- α (HIF-
31 1 α) pathway – a master regulator of cellular response to hypoxia [58], [59]. Using a rat model
32 of mandibular distraction osteogenesis, another study demonstrated that activating the stromal
33 cell-derived factor-1 (SDF1)/chemokine receptor-4 (CXCR4) pathway promoted migration of
34 endogenous mesenchymal stem cells to the distraction site [60]. However, this study did not
35 determine the contribution of the recruited mesenchymal stem cells to the distraction
36 regeneration but still represent a promising avenue to explore since the SDF1 signaling is also
37 involved in CNCC migration [61] during embryogenesis.

46 Homologous and heterologous bone transplantation are one of the most common surgical
47 procedures utilized for damaged bone repair. However, many limitations and challenging post-
48 operative complications can occur with this procedure, such as site infection or immunologic
49 reaction. Thus, alternative treatments for repair and regeneration need to be explored. For
50 example, chondrocytes from other sources could be harvested and expanded *in vitro* [62] alone
51 or in combination with bioengineering tools such as biomimetic hydrogels [63]. These cells can
52 be then grafted on the site of bone regeneration to contribute to bone repair. Another possible
53 strategy focuses on nasal cartilage biopsies that can be harvested under local anesthesia, with
54 minimal donor site morbidity [64]. Such biopsies have been shown to be a good source of
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nasal chondrocytes that display a better proliferation and chondrogenic capacity than articular chondrocytes *ex vitro* and *in vivo* [65], [66] and have a superior ability to integrate the surrounding tissue when implanted to repair cartilage defects (reviewed in Li et al., 2020). These represent a source of easily accessible material in relatively abundant quantity and are promising avenue to further explore in the future.

Conclusion

The craniofacial skeleton represents one major derivative of the cranial neural crest [16], [68]. Because of the crucial functions of this structure, any defects, either injury or disease-associated, have an enormous impact on quality of life. While bone is a tissue with very efficient regenerative capacities, about 10% of bone fractures are unable to self-repair [69] and will require transplantation or stem-cell therapies. Regenerative medicine has made tremendous progresses in developing treatments and procedures to increase tissue repair in patients. Nevertheless, it is essential to find new ways to stimulate endogenous regeneration to overcome the limitations of autologous and heterologous transplantations, including graft rejection. Stimulating the endogenous repair also results in the formation of a better integrated structure within surrounding tissues and similar in pattern to the original. Several studies of SSC contribution to bone repair demonstrated the importance of recapitulating developmental processes in post-natal bone repair processes. Characterizing the gene regulatory networks governing bone development and the mechanisms controlling SSC potential within their niche represent fundamental goals in the fields of developmental biology, stem cell research and regenerative medicine. Furthermore, understanding the molecular regulations of cell plasticity during development will be essential to enhance SSC expansion, stimulate tissue resident stem cells and reduce the tumorigenic risks of stem cell transplantation. This knowledge will be essential to establish prototype procedures aiming at enhancing endogenous regenerative responses during tissue repair. It will likely lead to protocols increasing the viability and adaptability of stem-cell or tissue transplants, which will ameliorate autograft integration and overall repair process. Translating this knowledge will allow to engineer better regenerative therapies for humans suffering traumatic injuries or congenital syndromes.

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