

NEURAL CREST STEM CELL POPULATION IN CRANIOMAXILLOFACIAL DEVELOPMENT AND TISSUE REPAIR

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Abstract

Neural crest cells, delaminating from the neural tube during migration, undergo an epithelial-mesenchymal transition and differentiate into several cell types strongly reinforcing the mesoderm of the craniofacial body area – giving rise to bone, cartilage and other tissues and cells of this human body area. Recent studies on craniomaxillofacial neural crest-derived cells have provided evidence for the tremendous plasticity of these cells. Actually, neural crest cells can respond and adapt to the environment in which they migrate and the cranial mesoderm plays an important role toward patterning the identity of the migrating neural crest cells. In our experience, neural crest-derived stem cells, such as dental pulp stem cells, can actively proliferate, repair bone and give rise to other tissues and cytotypes, including blood vessels, smooth muscle, adipocytes and melanocytes, highlighting that their use in tissue engineering is successful. In this review, we provide an overview of the main pathways involved in neural crest formation, delamination, migration and differentiation; and, in particular, we concentrate our attention on the translatability of the latest scientific progress. Here we try to suggest new ideas and strategies that are needed to fully develop the clinical use of these cells. This effort should involve both researchers/clinicians and improvements in good manufacturing practice procedures. It is important to address studies towards clinical application or take into consideration that studies must have an effective therapeutic prospect for humans. New approaches and ideas must be concentrated also toward stem cell recruitment and activation within the human body, overcoming the classical grafting.

Keywords: Neural crest, CMF, development, differentiation, tissue engineering, translational medicine.

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Introduction

The neural crest, a distinctive transient embryonic cell population, was initially identified by the Swiss embryologist Wilhelm His in 1868 as “the intermediate cord” (*Zwischenstrang*) because of its origin between the neural plate and non-neural ectoderm in the neurula-stage chick embryo. In a paper on the development of the cranial nerves in chick embryos, Marshall (1879) used the term *neural ridge* for the cells that give rise to cranial and spinal nerve ganglia. Realising that this term was less descriptive than was desirable, a year later he swapped *neural ridge* with *neural crest*. The neural crest has been called the “fourth germ layer”, because of its importance. Indeed, it is a multipotent tissue that gives rise to various differentiated cell types in the adult organism, and is unique to vertebrate embryos (Thorogood, 1989).

The induction of the neural crest begins during neurulation, when the borders of the neural plate, also known as the neural folds, converge at the dorsal midline to form the neural tube. This, subsequently, becomes covered by future epidermal ectoderm. Neural crest cells are positioned on the top (dorsal) edges of the forming neural folds. Once the neural tube has closed, the neural crest cells form a distinct population of cells resting on top of the neural tube. The interaction between the neural plate and the epidermal ectoderm causes the induction of the neural crest cells, which migrate from the dorsal neural tube – along defined pathways – to a number of sites at which they stop migrating. During migration, cells undergo an epithelial-mesenchymal transition and differentiate into a very wide range of derivatives. These include skin pigment cells (melanocytes and other pigments), neurons of the dorsal root ganglia of spinal nerves, the autonomic nervous system (both sympathetic and parasympathetic ganglia), Schwann cells responsible for myelination of peripheral nerves, pericytes and smooth muscle cells of the vascular system, including the major vessels of the heart, the adrenal medulla and leptomeninges (Smith and Schoenwolf, 1989; Keller *et al.*, 1992). Neural crest cells are also important in the pharyngeal arches and head, forming some bones and cartilage in the lower jaw as well as portions of the eyes, ears and teeth.

Given the wide variety of differentiation fates and the capacity for self-renewal, neural crest cells are often considered to be a stem-cell-like population. In fact, the neural crest can generate a great variety of cell and tissue

types and for this reason represents a multipotent cell population. The “stemness” of neural crest cells is shown by experiments that revealed that, *in vivo* at least, some neural crest cells give rise to multiple cell types including neurons, smooth muscle and melanocytes (Bronner-Fraser and Fraser, 1989; Frank and Sanes, 1991). The term “NCSCs” (neural crest stem cells) was coined by Stemple and Anderson when showing that *in vitro* neural crest cells not only have the ability to give rise to autonomic neurons, glia and smooth muscle, but also to self-renew, a unique characteristic of stem cells (Stemple and Anderson, 1992).

Neural Crest Formation: Induction, Delamination and Migration

The complete induction of neural crest cells is a complex process that involves the activity of extracellular factors produced by the inductive tissues that activate a genetic program in the ectodermal cells. Once this genetic program is activated in the neural crest cells, a second set of genes, the proper neural crest genes, are transcribed. One of the important functions of this second set of genes is to allow survival (or maintenance) of the recently induced neural crest cells. Finally, the two set of genes work together to control delamination, migration and differentiation of the neural crest cells (Morales *et al.*, 2005).

Induction

The induction of neural crest is a multistep process, starting at the early gastrula stage and continuing until neural tube closure. Several studies have concentrated on the identification of the signalling molecules involved in the induction of the neural crest. The signals that activate neural crest cells are released from the neural plate, the epidermis and the lateral mesoderm. At least four distinct signalling pathways have been implicated in this process in different species. These include bone morphogenetic proteins (BMP), Wnts, fibroblast growth factors (FGF), and more recently the Notch/Delta signalling pathways (Huang and Saint-Jeannet, 2004; Klymkowsky *et al.*, 2010).

The two members of the BMP family involved in the inductive process are BMP-4 and BMP-7 and are expressed by the epidermal ectoderm. They elicit the formation and migration of neural crest cells from the neural plate. Findings led to the proposal of a model in which the different fates of the ectoderm derivatives are specified by a gradient in BMP activity (Graham, 2003). The upstream factors that control BMP-4 expression and restrict the generation of neural crest cells to the neural and non-neural ectoderm boundary have recently been identified and are involved in the Delta/Notch pathway. Moreover, several studies suggest that other signals are required, in addition to BMPs, in order to induce the neural crest. A much more robust induction occurred when inhibition of BMP signalling was accompanied by exposure to Wnts or FGFs (Sasaki *et al.*, 2006; Monsoro-Burq *et al.*, 2003). Several lines of evidence suggest that members of the Wnt family of secreted glycoproteins can act as neural crest inducers (Mayor *et al.*, 1997; LaBonne and Bronner-Fraser, 1998).

Delamination and migration

Once specified, neural crest cells must delaminate from the neural tube to migrate to their destinations and undergo a transition from the epithelial to the mesenchymal type (Nieto, 2001). During the entire process of neural crest development, neural crest cells undergo a series of transformations that include the modification of their cell-cell and cell-substrate adhesion properties, improving their migration capability (Duband, 2006).

At the beginning of neural tube formation, nascent neural crest cells remain integrated at the dorsal side of the neural tube and are morphologically indistinguishable from other neural epithelial cells (Achilleos and Trainor, 2012). At the end of the delamination step, crest cells are clearly distinct from neural tube cells and can be recognised both molecularly and morphologically. Indeed, neural crest cells are regular, elongated and radially oriented, and become first more rounded and irregular in shape, and then, when they are completely separated from the neural tube, they flatten over the surface of the neural tube and extend tangentially to it. In addition, alterations in cellular adhesion also occur: E-cadherin is replaced by N-cadherin and cadherin-6B, until onset of migration. Subsequently, after their complete exclusion from the neural epithelium, neural crest cells lose N-cadherin and cadherin-6B expression. Finally, migrating neural crest cells start expressing cadherin-7 and/or cadherin-11, two types of cadherin expressed by fibroblasts (Duband, 2006).

Changes in the interaction with the extracellular matrix also occur, favouring the release of these cells from the neural tube. Several *in vitro* studies have indicated that Rho-B, a member of the rho family GTP-binding proteins that control cell adhesion and motility through dynamic regulation of the actin cytoskeleton, plays a specific role in the control of neural crest delamination (Tondeleir *et al.*, 2014).

There are opposing views about the role of Slug in neural crest delamination: in fact, some studies affirm that it plays a fundamental role, while others state that, at certain axial levels, it is neither necessary nor sufficient. More recently, using an inducible inhibitory mutant, Slug was found to be involved in the formation of neural crest precursors and in neural crest migration (LaBonne and Bronner-Fraser, 2000). Since one of the first events for an epithelial cell to become migratory is the loss of cell-to-cell adhesion molecules, it was suggested that targets of Slug could be molecules such as cadherins. It was demonstrated that an overexpression of a neuroepithelial cadherin or a crest cadherin in the neural tube impaired neural crest emigration (Theveneau and Mayor, 2012). This indicated that fine regulation of cadherin expression is fundamental for the emigration of the neural crest from the neural tube (Nieto, 2001).

Upon migrating from the dorsal surface of the neural tube, neural crest cells enter a cell-free zone that is rich in extracellular matrix molecules. Although they are readily detectable in this cell-free zone, they soon become morphologically indistinguishable from tissues through which they migrate. The migration begins cranially and gradually extends caudally. The overall migration can

be summarised by three pathways: a ventral pathway, a lateral pathway and a dorsal route. Cells that go around the notochord and neural tube characterise the ventral pathway (Cerny *et al.*, 2004).

Cessation of migration is not a simple process. Some cessation processes involve increasing the production of chondroitin sulphate and decreasing the amount of collagen and other substrates. The chondroitin sulphate and aggrecan-rich extracellular matrix encountered by deeply migrating neural crest cells inhibits the formation of cell-to-cell attachments so that neural crest cells migrate as single cells. Consequently, chondroitin sulphate and other glycoconjugates provide barriers to crest cell migration. Other parts of the embryo (notochord) secrete substances, such as trypsin- and chondroitinase-labile molecules, that make migration difficult. Migrating neural crest cells influence the patterning of other cell types.

Neural Crest Domains

Neural crest cells originating from different positions along the anterior-posterior axis develop into various tissues. The regions of the neural crest can be divided into four main functional domains, which include the craniomaxillofacial (CMF) neural crest, trunk neural crest, vagal and sacral neural crest, and cardiac neural crest. Each domain responds to specific migration and differentiation signals to generate the appropriate cells and tissues (Taneyhill, 2008). In this review we will concentrate on CMF neural crest-derived cells.

Craniomaxillofacial neural crest

The vertebrate head is a complex assemblage of cranial specialisations, including the central and peripheral nervous systems, viscer- and neuro-cranium, musculature and connective tissue. The primary differences that exist between vertebrates and other chordates relate to their craniofacial organisation. Therefore, the evolution of the head is considered fundamental to the origins of vertebrates (Gans and Northcutt, 1983).

In the 1890s, Julia Platt claimed that the cartilage of the craniofacial area, the pharyngeal arch skeletons and the dentine-forming cells (odontoblasts) of the teeth of the mudpuppy, *Necturus maculosus*, arose from the ectoderm adjacent to the neural tube (Platt, 1893, 1894, 1897). Platt's results were extremely controversial as they ran counter to the prevailing germ layer theory of the day, and her hypothesis of the cranial skeletogenic origin of the neural crest only truly gained acceptance 50 years later, primarily through the seminal work of Sven Horstadius (Horstadius, 1950).

The cranial neural crest (CNC) consists of several populations that differ in their migratory pathways, patterns of gene expression, and types of derivatives. For functional purposes, CNC cells can be subdivided into regions designated here as caudal forebrain, midbrain, rostral hindbrain and caudal hindbrain (which overlaps with rostral vagal) neural crest cells. Each population has a somewhat different pattern of migration and prospective derivatives. At the midbrain level, CNC cells emerge and

migrate primarily as a broad, unsegmented sheet of cells under the ectoderm. These cells contribute to a wide range of derivatives that include the periocular skeleton, connective tissue of the eye, membrane bones of the face, ciliary ganglion, part of the trigeminal ganglion and Schwann cells (Le Douarin and Kalcheim, 1999).

The hindbrain is a unique region of the neural tube because it is the only portion of the developing brain that is overtly segmented. It is subdivided into eight segments or rhombomeres. Rhombomeres represent compartments of lineage restriction such that clones of cells do not cross rhombomere boundaries (Fraser *et al.*, 1990). Hindbrain neural crest cells migrate in a segmental fashion as three broad streams emanating laterally adjacent to rhombomeres (r) r1, r2, r4 and r6, with no CNC cells apparent at the level of r3 or r5. The first of these streams populates the trigeminal ganglion and mandibular arch; the second populates the hyoid arch, as well as the geniculate and vestibular ganglia; the third stream populates the third and fourth branchial arches and associated peripheral ganglia. The DiI-labelling experiments of Lumsden and colleagues suggested that r3 and r5 do not form neural crest cells, consistent with the possibility that the segmental migratory pattern reflects a patterned origin within the neural tube (Noden, 1975; Lumsden *et al.*, 1991).

Because of the difficulties in culturing embryos, microsurgical manipulation of embryonic tissue and maintaining the dye throughout the long period of embryogenesis, it was not possible to obtain a systematic lineage analysis of neural crest cell derivatives in mammals. The biological function of these CNC-derived cells has been studied in a variety of animal models and two possible theories have been put forth: first, these neural crest cells may carry certain pre-acquired molecular signals as they migrate into the branchial arch so they can induce epithelium to form various craniofacial structures (Woo and Fraser, 1998; Artinger *et al.*, 1999); second, CNC cells acquire positional identity at the time they reach their final destination and contribute to the formation of various craniofacial structures. Certain growth and transcription factors have been implicated as important regulators for the critical epithelial-mesenchymal interactions. These factors stimulate multipotent neural crest-derived cells to form neural crest derivatives and to differentiate in specific tissue cells (Thesleff and Sharpe, 1997; Saldivar *et al.*, 1997; Tucker *et al.*, 1999).

Molecular Basis and Gene Involvement

Some understanding of the molecular basis of hindbrain and neural crest specification has come from analyses of the expression and function of *Hox* homeobox genes, homologues of the homeotic genes of *Drosophila*. Each *Hox* gene is expressed in an overlapping domain along the anterior-posterior axis of the embryo, but each gene has a characteristic segmental limit of expression at its anterior boundary. In the developing head, this spatially restricted expression pattern is seen in the hindbrain, with the anterior limits of *Hox* gene expression corresponding to rhombomere boundaries at two-segment intervals. As the

neural crest migrates from the rhombomeres into specific branchial arches, it retains the particular combination or code of *Hox* gene expression that is characteristic of the rhombomeres from which it originated. Thus, the neural crest from each axial level conveys a unique combinatorial *Hox* code. This code can be considered to specify form and pattern for the different branchial arch-derived regions of the head and neck (Hunt *et al.*, 1991a).

Another gene that may be involved in segmental patterning, both in the hindbrain and neural crest, is the zinc finger gene *Krox-20*. *Krox-20* is expressed in alternating domains in the neural plate that later correspond to r3 and r5, where it acts as a transcriptional regulator of *HoxB2* expression (Sham *et al.*, 1993). In addition to its expression in r3 and r5, *Krox-20* is detectable in neural crest precursors in the dorsal midline of the neural tube extending from caudal r5 into r6. These *Krox-20*-expressing cells leave the neural tube at the 13-somite stage, emerging near the r5/6 border and migrating caudally and ventrally. By combining Dil cell marking techniques with *in situ* hybridisation (Izpisua-Belmonte *et al.*, 1993), it was possible to determine the rhombomeric origin of some neural crest cells that carry the *Krox-20* transcription factor (Nieto *et al.*, 1991). The results showed that both r5 and r6 contributed to the *Krox-20*-expressing neural crest cells that migrate caudally around the otic vesicle. *Krox-20*-expressing cells first migrate from mid-to-caudal r6 and shortly thereafter from rostral r6 and r5. Its expression is transient, becoming undetectable in the neural crest by the 23-somite stage (Bronner-Fraser, 1994).

Gooseoid is another homeobox-containing transcription factor, originally isolated in *Xenopus* from a dorsal blastopore lip cDNA library. The dorsal blastopore lip has long been known to be ultimately responsible for organisation of the complete body axis in the early embryo. However, when *gooseoid* was knocked out in transgenic mice, the body axis was formed normally but the embryos exhibited a number of craniofacial defects (Rivera-Pérez *et al.*, 1995; Yamada *et al.*, 1995). In wild type mice, *gooseoid* transcripts were detected at later stages of development in the osteogenic mesenchyme of the developing mandible, tongue and middle ear. In the mutants, the mandible was hypoplastic and lacked coronoid and angular processes, whilst there were defects in several bones, including the maxillary, palatine and pterygoid. As a homeobox-containing transcription factor, it would appear that *gooseoid* is involved in essential inductive tissue interactions during formation of the head, but has a redundant function in the mouse gastrula organiser.

Another gene that has produced an even more perplexing phenotype is *endothelin-1* (*ET-1*). *Endothelin-1* encodes a vasoactive peptide expressed in vascular endothelial cells and is thought to play a role in the regulation of blood pressure. Mice with targeted disruption of *ET-1* had no abnormalities in their cardiovascular system but did have a marked reduction in tongue size, micrognathia and cleft palate (Kurihara *et al.*, 1994). Components of the *ET* pathway are now known to be involved in development of the cephalic neural crest. One of the two G protein-coupled endothelin receptors, *ET-A*, is expressed in the neural crest-derived ectomesenchyme of the branchial

arches, whilst its primary ligand, *ET-1*, is expressed in arch epithelium, pharyngeal pouch endothelium and arch core paraxial mesoderm. The *ET-A/ET-1* pathway appears to be important for proper patterning of the caudal regions of the first arch (Tucker *et al.*, 1999). Targeted disruption of *ET-A* or *ET-1* in mice produced craniofacial defects that resembled a human condition called CATCH-22, which is characterised by abnormal facies and cardiovascular defects (Wilson *et al.*, 1993). It has recently been shown that the craniofacial defects in the *ET-A*^{-/-} mice are, in part, due to an absence of the *gooseoid* transcription factor (Clouthier *et al.*, 1998).

Another important pathway involved in craniofacial development is kinin-kallikrein signalling. This is active in a particular region highly conserved of embryos of *Xenopus* and zebrafish, defined extreme anterior domain (EAD) including mouth. Deregulation of this pathway, leads to the failure of forming the mouth and to abnormal development and migration of the neural crest (Jacox *et al.*, 2014).

The classical view of craniofacial development and neural crest patterning, was that regional diversity in the vertebrate head was a consequence of patterning information provided by the neural crest (Noden, 1983; Hunt *et al.*, 1991b).

Craniofacial Patterning Advanced Studies

Recently, there have been significant advances in our understanding of craniofacial patterning through neural crest cell transpositions within the hindbrains of mice (Golding *et al.*, 2000; Trainor and Krumlauf, 2000) and zebrafish embryos (Schilling *et al.*, 2001). In contrast to the classic analyses performed in avian embryos, these new studies uncovered a consistently high degree of CNC cell plasticity. In heterotopic transplantations of neural crest cells within mouse and zebrafish hindbrains, graft-derived neural crest cells migrated into the nearest arch without any evidence of path finding; concomitantly, there was inappropriately downregulated *Hox* gene expression in these cells (Trainor and Krumlauf, 2000). In zebrafish embryos, the transplanted cells were followed for longer periods, such that the cells activated new gene expression programs and they differentiated and contributed to pharyngeal cartilage appropriate to their new axial location (Schilling, 2001). These results demonstrated that the axial character of cranial neural crest cells are neither fixed nor passively transferred from the hindbrain to the branchial arches and periphery of the head. The plasticity observed in neural crest cell patterning correlated with molecular analyses that identified distinct *cis*-regulatory elements controlling *Hox* gene expression in different tissues, such as the neural tube and neural crest (Maconochie *et al.*, 1999). Consequently, these results supported a new model called the “neural crest plasticity and independent gene regulation model” as a mechanism for describing neural crest and craniofacial development. This argues that neural crest cells can respond and adapt to the environment in which they migrate. Furthermore, it is suggested that the cranial mesoderm plays an important role in patterning the

identity of the migrating neural crest cells (Golding *et al.*, 2000; Trainor and Krumlauf, 2000).

The observations described above, demonstrating that neural crest cells are plastic and that gene expression is independently regulated in different tissues of the head, provides a mechanism for how neural crest cells can be modified or evolve in response to the environment through which they migrate, independently of the neural tube from which they are derived. Although the hindbrain exerts a profound influence in establishing the foundations of vertebrate head development, a rigid neural crest pre-patterning model in which the program for head morphogenesis is set in the neural tube would offer very restricted opportunities for diversifying head structures. In contrast, the neural crest plasticity and independent gene regulation model could provide the flexibility and adaptability that facilitates diversity, and we can speculate that it might be one reason for the successful radiation of vertebrates into new environments. This is because neural crest plasticity and independent gene regulation offers the potential for generating substantially distinct cranial phenotypes by subtle, minor changes of the primordial pattern. Successful adaptation and developmental flexibility is particularly evident in the bird beak, which has undergone extensive diversification and modification during evolution. Bird beaks are used for numerous diverse functions, including eating, defence, scratching, courting and preening. Even though beak morphology is remarkably variable between species, and even within species, each beak is derived from comparable tissues during development. The lower beak is bipartite and is formed by the fusion of the paired mandibular cartilages; in contrast, the tripartite upper beak is derived from the fusion of the frontonasal mass to the paired lateral nasal and maxillary cartilages. All of the beak's cartilage and connective tissue is derived from neural crest cells (Helms and Schneider, 2003). The outer cornified layers of the beak come from the ectoderm, while the mastication/articulation muscles come from the mesoderm (Noden, 1983). Evolutionary modifications in beak morphology could be driven by any, if not all, of these tissue components. Recently, an elegant series of interspecies neural crest cell transplantations revealed that neural crest cells direct beak morphology in a species-dependent and autonomous manner (Helms and Schneider, 2003). When quail neural crest cells, which normally give rise to narrow, short beaks, were transplanted into duck hosts, the resulting chimeras exhibited beak morphologies characteristic of the quail. Conversely, when duck neural crest cells, which normally produce longer, flatter bills, were transplanted into quail hosts, the resulting chimera developed beaks typical of ducks. Interestingly, the donor neural crest cells also transformed host-derived structures. The morphology of the beak epidermis and egg tooth, and the positioning of the external nasal openings in the chimeras, were all characteristic of the species from which the neural crest cells were derived. This is despite the fact that the egg tooth and epidermis are host tissue-derived structures. Most significant, however, was the observation in the quail/duck chimeras that the spatiotemporal patterns of

gene expression in the facial mesenchyme (donor derived) and the ectoderm (host derived) were always characteristic of the donor. Hence, when donor quail neural crest cells were transplanted into ducks, the chimeras exhibited accelerated gene expression programs characteristic of the quail even in tissues derived from the duck host. This demonstrated that neural crest cells pattern beak development in a species-specific manner by maintaining their own molecular programs and by regulating the patterns of gene expression and differentiation in adjacent tissues. Therefore, neural crest cells can regulate the spatial patterns of cellular differentiation around them during craniofacial development. Morphometric measurements of the beaks obtained in quail/duck chimeras revealed only minor size and shape changes. The beaks that developed in the chimeras were more characteristic of the species origin of the donor neural crest cells rather than of the host into which they were transplanted. The implication of this observation is that during evolution, the ectodermal tissues surrounding the neural crest-derived beak may influence neural crest cell differentiation only to a minor extent. This may not be that surprising given that neural crest-derived cartilage and bone, to which soft tissues such as the muscles and connective tissue must adhere, provide the scaffolding for the vertebrate head.

Role of Neural-Crest-Derived Stem Cells: First Evidence in CMF Repair

The use of human pluripotent stem cells (hPSCs) in regenerative medicine is becoming increasingly diffuse, particularly in the reconstruction of the craniomaxillofacial complex of patients with both hard and soft tissue deficits due to trauma, tumour or congenital disease. The craniomaxillofacial complex is of fundamental importance for many different functions, some of which are vital, such as breathing and eating, while others are non-vital but still important for social relationships, such as aesthetics and the delivery of senses such as sight, smell and sound. For these reasons, loss of craniomaxillofacial tissue has serious psychological consequences, so it is important to seek solutions to optimise the life of people with maxillofacial trauma or head and neck cancer (Teo and Vallier, 2010).

After neurulation, the cells of the neural crest migrate and give rise to various cell populations of the adult body. They also have the ability to self-renew. Therefore, they may be considered stem cells (Pierret *et al.*, 2006). In the adult organism, several different tissues contain cells with a self-renewal capacity and differentiation potential resembling that of neural crest cells during embryonic development. Neural crest stem cells (NCSCs) have been described in dorsal root ganglion (DRG), gut, cornea, heart and skin (Achilleos and Trainor, 2012).

A study by Nagoshi *et al.* (2008) recently showed the existence of NCSCs (*i.e.*, cells with mesenchymal and neural potential) in the bone marrow. Although their frequency drastically decreases with age, multipotent NCSCs in adult bone marrow could provide an attractive source for future cell replacement therapies. In particular,

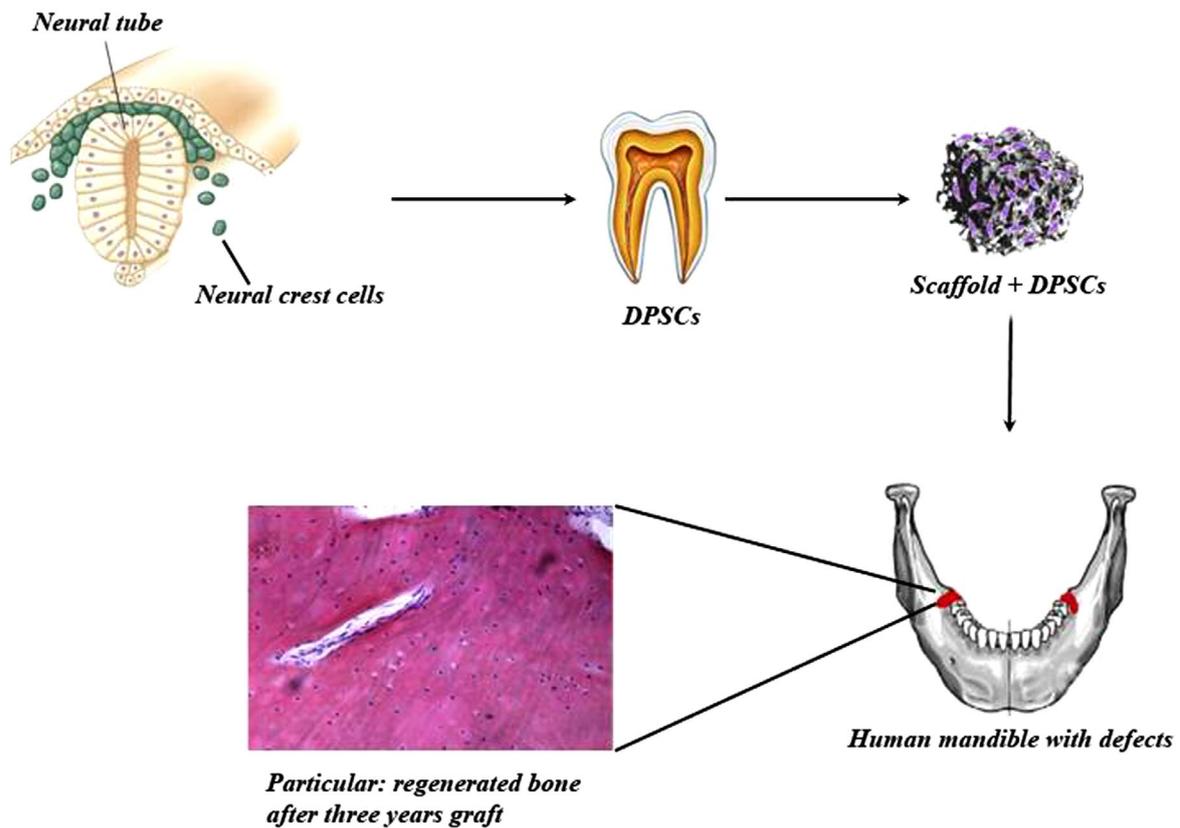


Fig. 1. Representation of human mandible defect repair using DPSCs seeded on a scaffold.

bone marrow stromal cells (BMSCs) are capable of differentiating into osteoblasts, chondrocytes, adipocytes and myoblasts (Prockop, 1997). Several studies have also shown their ability to give rise to neurons and glia *in vitro* and, after transplantation, in the central nervous system (Hofstetter *et al.*, 2002; Arnhold *et al.*, 2006). More interesting is the recent discovery that neural crest-related stem cells can be isolated from mammalian craniofacial tissues, such as dental pulp (Sasaki *et al.*, 2008), periodontal ligaments (Widera *et al.*, 2007; Techawattanawisal *et al.*, 2007) and dental follicles (d'Aquino *et al.*, 2011; Schiraldi *et al.*, 2012) or even salivary glands (Takahashi *et al.*, 2014). Overall, dental pulp stem cells (DPSCs) are more suitable than bone marrow mesenchymal stem cells (BM-MSCs) for mineralised tissue regeneration (Alge *et al.*, 2010).

In vivo, when transplanted into immunocompromised mice, DPSCs generated dentin-like structures (Gronthos *et al.*, 2000) or bone (Laino *et al.*, 2005; d'Aquino *et al.*, 2007). *In vitro*, DPSCs generated adipocytes, smooth muscle cells and differentiated into functionally active neurons, as assessed by electrophysiological analysis (Arthur *et al.*, 2008). Moreover, it was recently demonstrated that DPSCs express proteins involved in melanogenesis at different points during their differentiation *in vitro*, even when they were not stimulated by a selective differentiating medium for melanocytes, demonstrating for the first time that DPSCs are capable of spontaneously differentiating

into mature melanocytes (Paino *et al.*, 2010). In addition, DPSCs were able to form capillary-like structures when cultured with VEGF (Marchionni *et al.*, 2009).

In a clinical study, d'Aquino *et al.* (2009) demonstrated that a DPSC/collagen sponge bio-complex completely restored human mandible bone defects. After three years, a follow-up study (Giuliani *et al.*, 2013) demonstrated that the regenerated bone was uniformly vascularised and qualitatively compact. This result demonstrated that DPSCs successfully repair bone. In this context (Fig. 1), compact bone regenerated after DPSC engraftments in the mandible could be considered of fundamental importance to limit pathologic fracture and to guarantee better quality of life in oral cancer patients (La Noce *et al.*, 2014). These results demonstrated that DPSCs successfully repair bone, although the portion of the reconstructed mandible was made of compact bone instead of the expected physiological spongy bone. The latter highlights a major issue and burning question in stem cell use and grafting into patients: do the stem cells exactly reconstruct the damaged tissue and site where they are transplanted or can they divert their fate giving rise to other cells and tissues? In the reported cases, the different quality of bone was more an advantage than a disadvantage, but the issue is of paramount importance, mostly for bigger reconstructions. A further issue, which correlates with the different bone quality, is linked to the quality and quantity of stem cells needed for each grafting.

Conclusions and Perspectives

Stem cells represent an important source in regenerative medicine. Their use in recent years has allowed us to ameliorate tissue reconstruction techniques considerably with the purpose of improving human healthcare. Damage to the craniomaxillofacial tissue resulting into scars and deformities, besides causing physical ailments, including disorders to the different sensory systems and respiratory tract, may have significant psychological consequences for the patient. The craniofacial NCSCs, owing to their ability to regenerate and support new tissues, their easy isolation and their ongoing presence in the adult organism, are good candidates for regenerative medicine in craniomaxillofacial damage.

New ideas and strategies are needed to fully develop the potential for clinical applications of these cells. This effort should involve both researchers/clinicians and improvements in good manufacturing practice (GMP) procedures/rules. It is important to address studies towards clinical application or, at least, keep in consideration that all studies must have an effective therapeutic prospect for humans. The GMP procedures that must be followed by researchers/clinicians, even for the engraftment of autologous cells, are often difficult to follow and need to be simplified by the use of new technologies. The latter will probably reduce the gap existing between stem cell research, which has expanded at an exponential rate, and its therapeutic applications that have progressed much more slowly.

In the next few years, the targets of cell therapy will be to identify and understand cell differentiation mechanisms, so that optimal cell populations and culture conditions can be used in a more efficient treatment of disease; it is also important to evaluate the role of specific and new molecules that recruit endogenous stem cells for tissue regeneration. This strategy is hopefully an easier way towards a clinically acceptable procedure. Therefore, we expect that increased application of stem cells will strengthen the translatability to supporting clinical trials.

From this point of view, it would be of great interest to study neural crest-derived stem cells and their fate, particularly in the oromaxillofacial region. Future strategies include: (i) studies devoted to understand both the quality and quantity of stem cells needed for each grafting, as it is more important to transplant less stem cells of good quality than a great number of them, which then quickly die; and (ii) studies of specific patterns that become, day by day, of major interest. In addition, it is also of importance to stimulate endogenous stem cells rather than grafting them into the patient. In this regards progress has been made by scientists and this seems to be another important future strategy to obtain tissue regeneration inside the patient's body (Arany *et al.*, 2014).

Moreover, great importance has been given in recent years to epigenetic studies, since it is known that epigenetic regulation of gene expression can affect both the maintenance and the fate of stem cell state and the commitment to a differentiated profile (Paino *et al.*, 2014). The use of neural crest-derived stem cells, supported by drugs that affect DNA indirectly by modifying epigenetic

factors, may represent the final step in the improvement of regenerative medicine.

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