REVIEW ARTICLE

Neural crest: The fourth germ layer

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ABSTRACT

The neural crest cells (NCCs), a transient group of cells that emerges from the dorsal aspect of the neural tube during early vertebrate development has been a fascinating group of cells because of its multipotency, long range migration through embryo and its capacity to generate a prodigious number of differentiated cell types. For these reasons, although derived from the ectoderm, the neural crest (NC) has been called the fourth germ layer. The non neural ectoderm, the neural plate and the underlying mesoderm are needed for the induction and formation of NC cells. Once formed, NC cells start migrating as a wave of cells, moving away from the neuroepithelium and quickly splitting into distinct streams. These migrating NCCs home in to different regions and give rise to plethora of tissues. Umpteen number of signaling molecules are essential for formation, epithelial mesenchymal transition, delamination, migration and localization of NCC. Authors believe that a clear understanding of steps and signals involved in NC formation, migration, etc., may help in understanding the pathogenesis behind cancer metastasis and many other diseases. Hence, we have taken this review to discuss the various aspects of the NC cells.

Key words: Delamination, epithelial mesenchymal transition, migration, neural crest cells

INTRODUCTION

Vertebrate head is a complex assembly of cranial specializations such as central and peripheral nervous systems, viscera- and neurocranium, musculature and connective tissue. The vertebrates differ from other chordates primarily in their craniofacial organization. The transition from invertebrate to vertebrate chordates was a multistep process, involving the formation and patterning of many new cell types and tissues. The evolution of early vertebrates was accompanied by the emergence of a specialized set of cells, called neural crest cells (NCC) which have long been the cells of great interest for developmental and evolutionary biologists due to their considerable influence on the complex development of the vertebrate head.[1]

The NCCs, a transient group of cells that emerges from the dorsal aspect of the neural tube during early vertebrate development has been a fascinating group of cells because of its multipotency, long range migration through embryo and its capacity to generate a prodigious number of differentiated cell types.[2]

For these reasons, although derived from the ectoderm, the neural crest (NC) has been called the fourth germ layer.[3] It has even been said, perhaps hyperbolically that "the only interesting thing about vertebrates is the NC" (quoted by Thorogood 1989).[3]

In view of considerable contribution from NCC, we have taken this review to discuss the various aspects of NC cells.

HISTORY OF NEURAL CREST

In 1868, a Swiss Embryologist, Wilhelm identified a unique transient embryonic cell population localized in between neural

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tube and the epidermis in the vertebrate embryo which[1,4] he named as Zwischenstrang-the intermediate cord.[1,4,5] Arthur Milnes Marshall (1878), appears to have named this intermediate cord as NC cells. Marshall used the term neural ridge for the cells that give rise to cranial and spinal ganglia, a year later, he replaced neural ridge with NC.[4] Although, initially NCCs were associated with the origins of neurons and ganglia, it was Julia Platt in 1890s, who demonstrated that the visceral cartilages of the head and dentine forming cells of the teeth also arise from the NC (Platt, 1897). This hypothesis of the cranial skeletogenic origins in the NC by Platt gained acceptance 50 years later as it ran counter to the prevailing germ layer theory of the day. Platt's theory was accepted primarily through the seminal work of Sven Horstadius (Horstadius, 1950).[4,5] who published a paper in 1950, 82 years after the discovery of the NC titled "The neural crest: Its properties and derivatives in the light of experimental research" which is stands a milestone on the road to understand the NC.[4]

In view of present evidence of NC as the fourth germ layer giving rise to astonishing number of cells and tissues of the body the following section of this review discusses the induction, epithelial mesenchymal transition (EMT), delamination, migration, regions, derivatives, role in tooth development, multipotency and stemness of NCCs and also to discuss its role in diseases.

NEURAL CREST INDUCTION

NCCs arise uniformly at the dorso-lateral edge of the closing neural folds, along almost the entire length of the vertebrate embryo neuraxis [Figure 1]. This region corresponds to the interface between the nonneural ectoderm (NNE) (presumptive epidermis or surface ectoderm) and the neural plate (neuroepithelium), commonly referred to as the neural plate border (NPB).[1,6] With the separation of the neural tube from the surface ectoderm, the cells lying along the dorsolateral sides of the neural tube undergo EMT to form NCCs.[1] It is evident that induction of the NC requires the presence of NNE, NPB and the mesoderm which is present below the ectoderm.[7]

At a molecular level it is the bone morphogenetic proteins (BMP), Wnt and fibroblast growth factor (FGF) pathway which will help to induce the formation of the NCCs.[8] The BMP and Wnt are produced by NPB and NNE while Wnt and FGF are derived from the mesoderm.[9] Moreover, Notch/Delta, retinoic acid (RA), Hedgehog and endothelin signaling also contribute to this process.[7]

NCC induction may be divided into two phases. In the first phase, FGF helps in induction either directly or through Wnt signaling. At this stage, BMP has to be inhibited and for this FGF serves as one of the antagonists. In the second phase, FGF is inhibited, thus, leading to activation of BMP which along with Wnt signaling converge to form a signaling pathway.[7] As a result of this, a set of transcription factors which specify the NPB (NPB specifiers) are formed. They include Msx1/2, Pax3/7, Zic1, Dlx3/5, Hairy2, Id3 and Ap2. A second set of transcription factors called the NC specifiers are then produced. These are Snail2, Snail FoxD3, Sox9/10, Twist, Id3cMyc and Ap2.[10] These NC specifiers are very important as they continue to help in the maintenance and ultimately control NC behavior from EMT to migration and differentiation.[7]

Once formed NCCs undergo EMT, start delaminating into separate cell populations and attain migratory qualities.

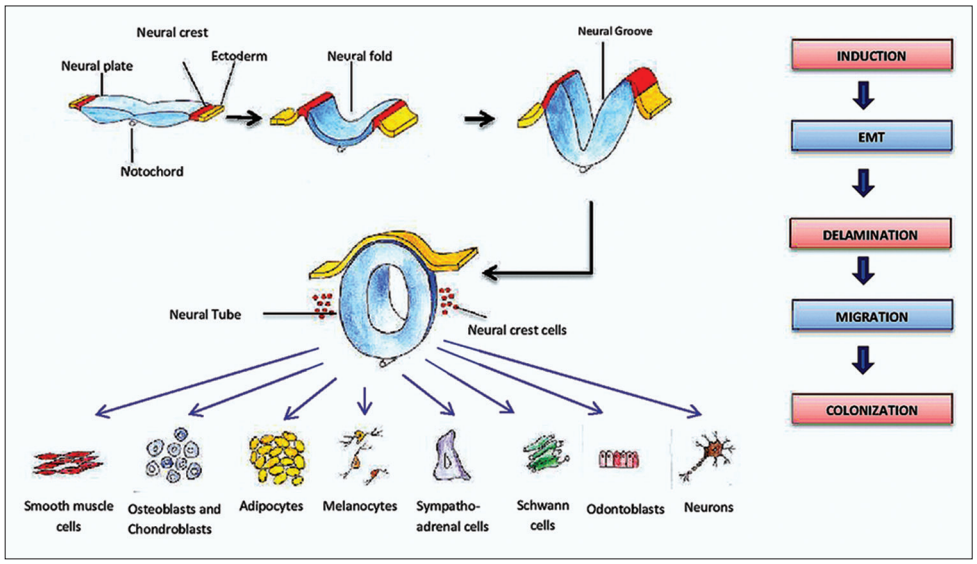

Figure 1: Neural crest cells, epithelial mesenchymal transition, delamination, migration. (concept modified and developed from Mayanil CS. Dev Neurosci 2013;35:361-72)

EPITHELIAL MESENCHYMAL TRANSITION AND DELAMINATION: MOLECULAR ORCHESTRATORS

Delamination defines the splitting of a tissue into separate populations regardless of the cellular mechanisms.[3] In contrast, EMT is a series of events at the molecular level bringing about a change from an epithelial to a mesenchymal phenotype.[11] All NC cells undergo EMT during their development [Figure 2].[12]

EMTs are marked by changes in cell adhesion and cell architecture. During delamination the main event that takes place in NCCs is down regulation of cell adhesion molecules.[12-15] This switch from strong cell adhesion promotes separation of NCC from the epithelium and allows onset of cell migration.[12]

NC cells start migrating as a wave of cells, moving away from the neuroepithelium and quickly splitting into distinct streams.[4,16,17] These migrating NCCs home into different regions and give rise to plethora of tissues.

Slug and Snail were the first transcription factors to be identified in the NC, about a decade ago.[18] Snail, slug, sox-9, sox-10 and Foxd-3 genes form a transcriptional network associated with down regulation of the cell adhesion molecules such as N cam, N-cadherin and cadherin 6B and also bring about break down of basement membrane through increase in integrins.[13-15,19-21]

BMP signaling, which is critical for NC induction, also plays a role in NC delamination. Furthermore, Delta-Notch signaling promotes Bmp4 expression and inhibits Slug expression and this could provide a mechanism for effectively controlling the formation and delamination of NC at the neural-epidermal junction.[22] RhoB is necessary for appropriate delamination of NCCs.[23] Cadherins control the timing of emigration, delamination and migration.[24]

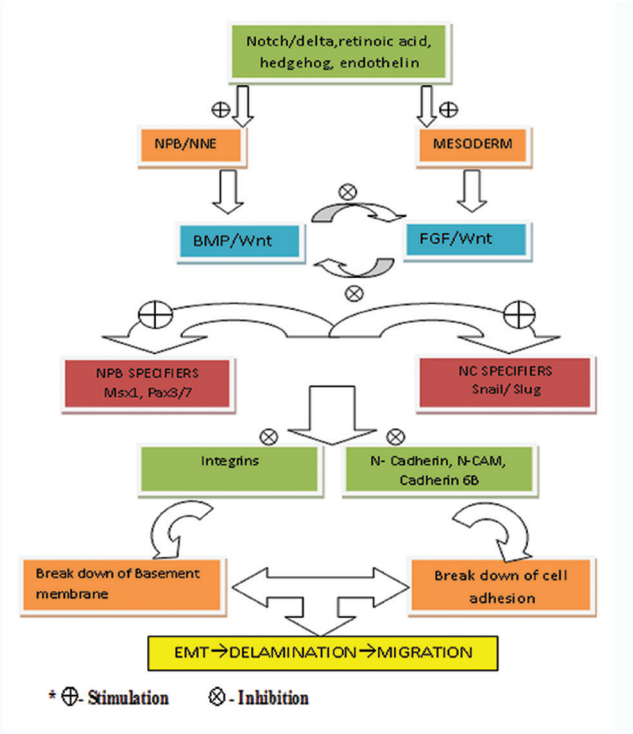

Figure 2: Molecular events in neural crest induction and emigration

Beside changes in cell-cell adhesion, the NCCs undergo a number of modifications in their interactions with the extracellular matrix that are believed to favor their release from the neural tube, which is evidenced by the expression of MMP-2 fostering NC delamination.[25,26]

MIGRATION OF NEURAL CREST CELLS

The capacity for long-range migration through the embryo is the defining feature of NCCs. The journey these cells take across the dynamic landscape of the developing embryo, exposes them to myriad signals from surrounding tissue microenvironments, which vary by developmental site and stage.[2]

During migration, NC cells are exposed to large number of positive and negative regulators that control where they go by modulating their motility and directionality [Table 1].[16] In addition, as most NC cells migrate collectively, cell–cell interactions play a crucial role in polarizing the cells and interpreting external cues. Cell cooperation eventually generates an overall polarity to the population, leading to directional collective cell migration.[12]

The pre-NCCs express range of phenotypical adoptions that help them during migration such as filopodia, blebs and the occasional "lobopodium."[27]

There is evidence of formation of a lamellipodium helping in cellular motility *in vitro*, along with attachment to a substratum, directional contractile forces and release of the trailing end.[28,29]

These evidences prove that the morphological alterations of NCCs provide them the locomotive properties.

REGIONS OF THE NEURAL CREST AND THEIR DERIVATIVES

The NC can be divided into four main functional (but overlapping) domains:

- The cranial (cephalic) NCCs produce the craniofacial mesenchyme that differentiates into the cartilage, bone, cranial neurons, glia and connective tissues of the face. These cells enter the pharyngeal arches and pouches to give rise to thymic cells, odontoblasts of the tooth primordia and the bones of middle ear and jaw[3]
- The trunk NCCs take one of two major pathways. NCCs of one path become the pigment-synthesizing

Table 1: Migration markers and their role

Phase	Genes	Proposed role	Reference
Migration	Hox genes	Maintain segmental identity of cranial NCCs[30]	Reviewed by Trainor and Krumlauf, 2000
Migration	Integrins	Mediate NCC motility on fibronectin in avian, Xenopus and mouse[31-33]	Alfandari *et al.*, 2003; Strachan and Condic, 2003; Strachan and Condic, 2008
Migration	Chemokines	Regulate cell migration and patterning in zebrafish[34]	Olesnicky Killian *et al.*, 2009
Migration	EphA4, EphB1 and ephrin-B2	Prevent intermingling of third and second arch Xenopus NCCs[35]	Smith *et al.*, 1997
Migration	Multiple Ephs and ephrins	Restricts avian and murine NCCs into streams by Inhibiting migration into NCC-free zones[36-38]	Adams *et al.*, 2001; Davy *et al.*, 2004; Mellott and Burke, 2008
Migration	Neuropilin-1 and semaphorin-3A,-3F	Avian and murine cranial NCCs express neuropilin-1 and are repelled by semaphorin-3A[39-41]	Eickholt *et al.*, 1999; Gammill *et al.*, 2007; Schwarz *et al.*, 2008
Migration	Neuropilin-1a,-1b,-2a,-2b and semaphorin-3Fa,-3Ga	Restricts zebrafish NCCs into streams by inhibiting migration into NCC-free zones[42]	Yu and Moens, 2005
Migration	Wnt11r	Promotes Xenopus cranial NCC migration[43]	Matthews *et al.*, 2008
Migration	Myosin-X	Promotes migration and segregation of Xenopus cranial NCCs[44]	Hwang *et al.*, 2009
Induction, Migration and Differentiation	BMPs	Multiple roles[45]	Reviewed by Nie *et al.*, 2006
Migration	Retinoic acid	Mediates the segmental migration of cranial NCCs[46,47]	Lee *et al.*, 1995; Pratt *et al.*, 1987
Migration	RhoA	Influences migration rate and filopodia dynamics[48]	Rupp and Kulesa, 2007
Migration and differentiation	Laminin alpha5	Required for proper migration and timely differentiation of a subset of murine cranial NCCs[49]	Coles *et al.*, 2006
Migration and differentiation	Disc1	Represses transcription of foxd3 and sox10[50]	Drerup *et al.*, 2009
Migration	ErbB4	Maintains the r3-adjacent NCC-free zone[51,52]	Golding *et al.*, 2004; Golding *et al.*, 2000
Migration	Chokh/rx3	Mutant chokh/rx3 zebrafish lack eyes and have disorganized NCC dorsal anterior migration[53]	Langenberg *et al.*, 2008
Target invasion	Neuropilin-1 and VEGF	VEGF attracts neuropilin-1 expressing NCCs into branchial arch 2[54,55]	McLennan and Kulesa, 2007; McLennan *et al.*, 2010
Target invasion	FGFR1	Provides a permissive environment for NCC migration into branchial arch 2[56]	Trokovic *et al.*, 2005
Target invasion	Endothelin-1 and endothelin A receptor	Required for proper migration into or within the arches[57-59]	Clouthier *et al.*, 2003; Pla and Larue, 2003; Clouthier *et al.*, 2000

*Modified from: Paul M. Kulesa, Caleb M. Bailey, Jennifer C. Kasemeier-Kulesa, and Rebecca McLennan. Cranial Neural Crest Migration: New Rules for an Old Road. Dev Biol. 2010 August 15; 344(2): 543-554. NCC: Neural crest cell, VEGF: Vascular endothelial growth factor, FGFR1: Fibroblast growth factor receptor 1, BMPs: Bone morphogenetic proteins

melanocytes; second migratory pathway takes the trunk NCCs ventrolaterally to each sclerotome and forms the dorsal root ganglia containing the sensory neurons. Those cells that continue more ventrally form the sympathetic ganglia, the adrenal medulla and the nerve clusters surrounding the aorta[3]

- The vagal and sacral NCCs generate the parasympathetic (enteric) ganglia of the gut[3,60]
- The cardiac NCCs can develop into melanocytes, neurons, cartilage and connective tissue (of the third, fourth and sixth pharyngeal arches). In addition, this region of the NC produces the entire musculoconnective tissue wall of the large arteries as they arise from the heart, as well as contributing to the septum that separates the pulmonary circulation from the aorta.[61]

The number of cell types that arise from the NC is truly astonishing as is the number of tissues and organs [Table 2][54] to which the NC contributes.[1,4]

CONTRIBUTION OF NEURAL CREST IN CRANIOFACIAL REGION

The majority of craniofacial connective tissues including those of the dental pulp and periodontal ligament, are formed by a special type of mesenchymal tissue, derived from the NC during embryonic development, thus termed ectomesenchyme.[63] Ectomesenchyme contributes to the generation of craniofacial structures, such as oral muscles, bones, tongue, craniofacial nerves; and teeth and dental ectomesenchymal stem cells (EMSCs). The

Table 2: A list of the cell types, tissues and organs derived from the neural crest[4]

Cell types	Tissues or organs
Cholinergic neurons	Spinal ganglia
Adrenergic neurons	Parasympathetic nervous system
Rohon-Beard cells	Sympathetic nervous system
Satellite cells	Peripheral nervous system
Schwann cells	Thyroid and parathyroid glands
Glial cells	Ultimobranchial body
Chromaffin cells	Adrenal gland
Parafollicular cells	Craniofacial skeleton
Calcitonin-producing (C) cells	Teeth
Melanocytes	Dentine
Chondroblasts	Connective tissue
Chondrocytes	Adipose tissue*
Osteoblasts	Smooth muscles
Osteocytes	Striated muscles
Odontoblasts	Cardiac septa, valves and aortic arches dermis
Fibroblasts (mesenchyme)	Eye cornea
Cardiac mesenchyme	Endothelia
Striated myoblasts	Blood vessels
Smooth myoblasts	Heart
Mesenchymal cells	Dorsal fin
Angioblasts	Brain
Merkel cells	Connective tissue of thyroid, parathyroid, thymus, pituitary, and lacrimal glands

*A recent investigation using both murine and Japanese quail embryos has demonstrated the origin of a population of adipocytes (fat cells) from cranial neural crest (Billon *et al.* 2007).[62] Adipocytes have long been thought to arise from cells within mesodermal lineages

ectomesenchyme, therefore, shares a common origin with NCCs.[64]

The cranial NCCs are central to the process of mammalian tooth development. They are the only source of mesenchyme able to sustain tooth development. Odontogenesis is regulated by a series of interactions between cranial NCCs and the oral epithelium. The oral epithelium provides the first instructive signals by secreting signaling molecules. These signals along proximodistal axis establish large cellular fields competent to form tooth of specific shape, along a rostrocaudal axis define an oral (capable of forming teeth) and nonoral mesenchyme and also helps in positioning the sites of future tooth development.[65]

MULTIPOTENCY AND STEMNESS OF NEURAL CREST CELLS

As NC can generate a great variety of cell and tissue types it represents a multipotent cell population. Several studies have been performed by Bronner-Fraser and Fraser (1989) and Frank and Sanes (1991) to address the developmental potential and the "stemness" of individual NCCs *in vivo*.[66,67] With the establishment of culture systems allowing the analysis of large cell numbers, it became apparent that multipotent cells are relatively frequent among the NCC population.[68] When grown in a rich medium containing serum, these cells differentiate into a number of NC derivatives.[5] Later, Stemple and Anderson coined the term neural crest stem cell (NCSC) and showed that NCCs *in vitro* not only have the ability to give rise to many tissue types but also to self-renew, a unique characteristic of stem cells.[69] Finally, Calloni *et al*. have demonstrated the existence of a highly multipotent cell predominantly found in cephalic NC and able to produce clones, comprising cell types as diverse as neurons, glia, melanocytes, chondrocytes, osteoblasts and smooth muscle.[70]

Researchers have attempted to identify signals supporting the self-renewal of NCSCs. Though still puzzling, the Wnt/BMP signaling with Sox 10 as the downstream target could be maintaining the undifferentiated state of early NCSCs. This theory is supported by the fact that mutation of these genes lead to multiple NC defects.[71-74]

TOOTH AS A SOURCE OF NC STEMCELLS

Umbilical cord, the bone marrow and adipose tissue, among others are the best known sources of multipotent mesenchymal stem cells (MSCs) in humans to date.[75] Whereas Dental and periodontal tissues constitute a relatively recently discovered source of NCSCs.[76]

Important features and facts of dental EMSC are:

- Substantial amount of EMSC are preserved in the dental pulp and periodontium of both deciduous and permanent teeth.[77] Amount of cells that can be obtained from a healthy human molar tooth pulp ranges between 500,000 and 2 million[76]
- As they present a neural crest phenotype they show promising use in nerve tissue restoration. They express neural-progenitor protein markers[64,78-80]
- Dental pulp stem cells (DPSCs) proliferate faster than bone marrow MSCs[76,77]
- DPSCs differentiate to multiple cell lineages including odontoblasts, chondroblasts, adipocytes, muscle cells and neurons *in vitro*[78,81]
- Dental pulp pluripotent stem cells have been recently isolated which express pluripotency markers such as Oct-4, Lin-28, Sox-2 and Nanog[82-84]
- Periodontal ligament stem cells (PDLSCs) are able to generate cementum and periodontal ligament-like structures including Sharpey's fibers[85]
- Apical papilla, dental sac or follicle, are the sources of EMSC from a developing toothgerm[86-88]
- EMSC population can also be isolated from exfoliated human teeth (milk teeth)[80]
- DPSCs and PDLSCs are good choice for their use in dental and periodontal tissue
- Engineering therapies.[89]

Table 3: Examples of neurocrestopathies according to compartment and type

Type of neurocrestopathy	Skin	Peripheral nervous system	Endocrine	Pharyngo cephalic	Dental anomalies
Cancer					
Isolated	Melanoma Merkel cell carcinoma	Neuroblastoma Schwannoma Neuroblastoma Paraganglioma	Pheochromocytoma Familialor sporadic Medullary thyroid carcinoma Chromaffin paraganglioma Carcinoid tumors	Hemangiocytoma Nonchromaffin paraganglioma (ear)	
Syndromic	Neurofibromatosis 1 Neurocutaneous melanosis	Hirshsprung	Multiple endocrine neoplasia $2A_1$ B_1	Congenital central hypoventilation	
Malformations					
Isolated	Congenital giant nevus Piebaldism	Hirshsprung		Cerebrodural arterio-venous malformation Cleft palate/lip Isolated contruncal cardiopathies Aplasia of lacrimal and salivary glands	Tooth aplasia[96] Generation of extra teeth[96] Cusp defects[96]
Syndromic	Sturge Weber	Waardenburg Familial dysautonomia type 2	Allgrove Barforth-Lazarus	Rieger Binder Moebius Johanson-Blizzard Treacher Collins-Franceschetti CHARGE Di George Pierre Robin Holoprocencephaly Kallmann Craniofrontonasal Goldenhar Orofacial digital Multiplesclerosis[96,97]	

*Modified from Etchevers, Heather C.; Amiel, Jeanne; Lyonnet, Stanislas (2006). "Molecular bases of human neurocristopathies". Neural Crest Induction and Differentiation, Volume 589. Advances in Experimental Medicine and Biology. pp. 213-34

The breakthrough achievement in regenerative dentistry would be to generate a whole functional replacement tooth, out of cultured and dissociated dental stem cells.[90,91] However, main hindrance in this research is a lack of consistent source of epithelial stem cells with odontogenic potential that can interact with the mesenchymal tissue.[92,93]

NEURAL CREST ABNORMALITIES: NEUROCRESTOPATHIES

Neurocrestopathy is a term coined by Bolande in 1974, referring to organ and tissue dysplasias with highly diverse clinical and pathological features caused due to abnormal migration, differentiation and division or survival of NCC [Table 3].[94,95]

CONCLUSION

The neural crest meets all the criteria used to define and identify a germ layer. Ectoderm and endoderm are primary germ layers: Mesoderm is a secondary germ layer formed after inductive interactions between ectoderm and endoderm. Like mesoderm, the neural crest arises early in development and gives rise to divergent cell and tissue types. Basically, neural crest arises by secondary induction from a primary germ layer, hence, meets the criteria of a secondary germ layer.[4] As the fourth germ layer, the neural crest is confined to vertebrates, which are therefore tetrablastic not triploblastic.[98] The mechanism of EMT and migration in NCC acts as a model to study malignant tumor cell metastasis as they share striking similarities at molecular level. The multipotency and stemness of NCC can help in regenerative tissue engineering. Hence, a thorough knowledge of NCC may help in understanding a disease process and address these pathological issues.

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Conflicts of interest

There are no conflicts of interest.

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