Mesenchymal potentials of the trunk neural crest cells

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Tese de doutorado apresentada por

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Tese em co-tutela entre a Universidade Federal do Rio de Janeiro e a Université Paris-Sud

O Potencial de Diferenciação Mesenquimal da Crista Neural Truncal

Defesa de tese de doutorado, dia 23 de abril de 2012 frente a uma banca composta por:

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Thèse de doctorat soutenue le 23 avril 2012 devant le jury composé de:

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ABBREVIATIONS

CN, crista neural / NC, neural crest

TN, tubo neural / NT, neural tube

PN, placa neural / NP, neural plate

AB, arco branquial / BA, branchial arch

TEM, transição epitélio-mesênquima / EMT, ephithelio-mesenchyme transition

ALP, alkaline phosphatase

βGP, β-glycerolphosphate

BMP, bone morphogenetic protein

C/EBP, CCAAT/enhancer-binding protein

CREB, cAMP-responsive element binding

Dlx, Distal-less homeobox

DMEM, Dulbecco's Modified Eagle Medium

FABP4, Fatty acid binding protein 4

FBS, fetal bovine serum

FGF, fibroblast growth factor

FGFR, fibroblast growth factor receptor

Glut4, insulin-responsive glucose transporter 4

HNK1, Human Natural Killer 1

Hox, homeobox

IBMX, 3-isobutyl-1-methylxanthine

IGF1, Insulin-like growth factor 1

Ihh, Indian hedgehog

KLF, Krüppel-like factor

MelEM, Melanocyte/melanoblast Early Marker

Msx, homeobox, msh-like

NICD, Notch intracellular domain

Pi, inorganic phosphate

Ppi, inorganic pyrophosphate

PPAR, peroxisome proliferator-activated receptor

Pref1, preadipocyte factor 1

QCPN, Quail cell PeriNuclear antigen

Runx, runt-related transcription factor

SATB2, special AT-rich sequence binding protein 2

Shh, Sonic Hedgehog

SMA, smooth muscle actin

SMP, Schwann cell myelin protein

Sox, SRY (sex determining region Y)-box

SREBP1, sterol regulatory element binding protein 1

T3, tri-iodo-thyronine

TGF, transforming growth factor

TH, tirosina hidroxilase

Wnt, wingless-related MMTV integration site

RESUMO

A crista neural (CN) é uma estrutura derivada das bordas dorsais do tubo neural (TN) dos vertebrados. Durante o desenvolvimento embrionário, essas células migram e contribuem para a formação de diferentes tecidos e órgãos do organismo. Ao longo do eixo ântero-posterior, a CN dá origem a neurônios e células gliais do sistema nervoso periférico e melanócitos. Além disso, a CN cefálica dá origem a tecidos mesenquimais, que formam a maior parte das cartilagens e ossos craniofaciais, derme facial, adipócitos e células musculares lisas na cabeça. No tronco dos vertebrados amniotas, os tecidos mesenquimais derivam do mesoderma, e não da CN. Nos vertebrados inferiores a CN truncal gera alguns tecidos mesenquimais, como os raios das nadadeiras do peixe-zebra. A questão que se apresenta, portanto, é se a capacidade das células da CN de produzir células mesênquimas foi totalmente perdida no tronco durante a evolução, ou se ainda pode ser alcançada em vertebrados amniotas em condições específicas. Algumas evidências apontam para um possível potencial de diferenciação mesenquimal das células da CN truncal. Desta forma, este trabalho se interessa em desvendar o potencial mesenquimal da CN truncal, com especial interesse na diferenciação de osteoblastos e adipócitos.

Nossa abordagem experimental consistiu em examinar os potenciais de diferenciação esqueletogênica e adipogênica das células da CN truncal de codorna em cultura de células *in vitro*. A diferenciação destas células foi mostrada por estudos de genes e marcadores específicos por hibridização *in situ*, imunocitoquimica e RT-PCR. As condições de cultura estabelecidas permitiram a observação do processo de esqueletogênese juntamente à adipogênese. A osteogênese foi caracterizada inicialmente através da expressão de *Runx2*, o primeiro fator de transcrição a ser expresso pelos osteoprogenitores, que tem seu RNAm detectado por hibridização *in situ* desde 5 dias de cultura. Quando estas células da CN truncal foram suplementadas com fatores osteogênicos e adipogênicos, observamos o processo de maturação dos osteoblastos com a expressão da proteína colágeno1, do RNAm de *osteopontina* e *fosfatase alcalina*, até a etapa de mineralização da matriz extracelular óssea como observado através da coloração com alizarin red. As células da CN truncal também realizam o processo de condrogênese, demonstrado pela expressão dos RNAm de *Sox9*, *agrecan* e *colágeno10*, e através do corante alciam blue. A observação das zonas de cultura mineralizadas e da condrogênese sugere que as células da CN truncal *in vitro* são capazes de realizar processos de ossificação endocondral e intramenbranosa. As células suplementadas com os fatores osteogênicos e adipogênicos também seguiram o processo de adipogênese. Adipócitos com gotículas lipídicas começam a se diferenciar a partir de 10 dias de cultura e podem ser identificados pelo corante oil red o. Entretanto, o RNAm de fatores de transcrição *PPAR*γ e *CEBP*α, essenciais para a adipogênese, e da proteína *FABP4* foram detectados por RT-PCR a partir de 3 dias de cultura. Para caracterização dos precursores de células ósseas e

adipociticas, avaliamos as potencialidades de diferenciação de células individuais da CN truncal. A análise dos tipos celulares das culturas clonais revelou que 76% dos clones geraram osteoblastos *Runx2*-positivos, e que as células da CN truncal compreendem progenitores multipotentes dotados tanto de potenciais neurais quanto osteogênico. Além disso, em outra condição de cultura clonal, adipócitos foram encontrados em 35,3% dos clones, e aproximadamente metade destes possui também potencial glial e/ou melanogênico.

Estes resultados mostram que *in vitro* as células da CN truncal são capazes de se diferenciar não só nos clássicos derivados encontrados *in vivo* (melanócitos, neurônios e células gliais), mas também em fenótipos mesenquimais, incluindo adipócitos e osteoblastos. Além disso, como nas células da CN cefálica, fenótipos mesenquimais podem se diferenciar de células progenitoras multipotentes, o que sugere que durante a evolução, as células-tronco da CN com potencial mesenquimal e neural tiveram a expressão do potencial mesenquimal inibido no tronco. Desta forma, embora não se manifeste nos vertebrados amniotas *in vivo*, o potencial mesenquimal esta presente de forma dormente nas células da CN truncal e pode se expressar *in vitro*.

RESUMÉ

La crête neurale (CN) est une structure dérivée de la partie dorsale du tube neural des Vertébrés. Pendant le développement embryonnaire, ces cellules migrent et contribuent à la formation de différents tissus et organes du corps. Le long de l'axe antéro-postérieur, la CN donne naissance aux neurones et cellules gliales du système nerveux périphérique, et aux mélanocytes. Par ailleurs, la CN céphalique est aussi à l'origine de tissus mésenchymateux qui constituent la plus grande partie des cartilages et os craniofaciaux, le derme de la face, et des adipocytes et cellules de muscles lisses dans la tête. Dans le tronc des Vertébrés amniotes, ces tissus mésenchymateux dérivent du mésoderme, et non de la CN. Chez les Vertébrés inférieurs, la CN troncale génère cependant des tissus mésenchymateux, comme les rayons des nageoires du poisson-zèbre. La question qui se pose est donc de savoir si la capacité des cellules de CN à produire des cellules mésenchymateuses a été totalement perdue dans le tronc au cours de l'evolution, ou bien si elle peut encore se manifester chez les Amniotes dans des conditions spécifiques. Certaines données ont suggéré que les cellules de la CN troncale pouvaient avoir un potentiel de différenciation mésenchymateuse. Ainsi, ce travail s'est intéressé à dévoiler le potentiel mésenchymateux de la CN troncale, avec un intérêt particulier pour la différenciation des ostéoblastes et adipocytes.

Notre approche expérimentale a été d'examiner le potentiel de différenciation squelettogénique et adipogénique des cellules de la CN troncale de caille en culture cellulaire in vitro. La différenciation de ces cellules a été démontrée par des études de gènes et marqueurs spécifiques par hybridation in situ, immunocytochimie et RT-PCR. Les conditions de culture mises en place ont permis l'observation des processus de squelettogenèse ainsi que d'adipogenèse. L'ostéogenèse a été initialement caractérisée par l'expression de *Runx2*, le premier facteur de transcription à être exprimé par les ostéoprogéniteurs, qui a été détectée par hybridation in situ à partir 5 jours de culture. Lorsque ces cellules de CN troncale ont été supplémentées avec des facteurs ostéogéniques et adipogéniques, nous avons observé la maturation des ostéoblastes, avec l'expression de la protéine collagen1, des ARNm de l'*ostéopontine* et de la *phosphatase alcaline*, jusqu'à l'étape de minéralisation de la matrice extracellulaire osseuse, identifiée par coloration avec l'Alizarin Red. Les cellules de CN troncale ont effectué également un processus de chondrogenèse, mis en évidence par l'expression des ARNm de *Sox9*, *aggrecan* et *collagène10*, et par la coloration au bleu Alcian. L'observation des zones minéralisées et des régions chondrogéniques suggère que les cellules de la CN troncale in vitro sont en mesure d'effectuer une ossification de types endochondral et intramembranaire. Les cellules supplémentées par des facteurs ostéogéniques et adipogéniques ont aussi engagé un processus d'adipogenèse. Les adipocytes avec des gouttelettes lipidiques ont commencé à se différencier à partir de 10 jours de culture et ont été identifiés par le colorant Oil Red O. Cependant, les ARNm des facteurs de transcription *CEBP*α et *PPAR*γ, essentiels pour l'adipogenèse, et de la

protéine *FABP4*, ont été détectés par RT-PCR dès 3 jours de culture. Afin de caractériser les précurseurs des cellules osseuses et adipocytiques, nous avons examiné le potentiel de différenciation des cellules individuelles de la CN troncale. L'analyse des types cellulaires dans les cultures clonales a montré que 76% des clones contiennent des ostéoblastes *Runx2*-positifs. Il est apparu que les cellules de CN troncale comprennent des progéniteurs multipotents doté à la fois de potentiels neuraux et ostéogénique. De plus, dans une autre condition de culture clonale, les adipocytes ont été trouvés dans la descendance de 35,3% des cellules, et environ la moitié de ces cellules de CN possédaient aussi un potentiel glial et/ou mélanogénique.

Ces résultats montrent que, in vitro, les cellules de la CN troncale sont capables de se différencier non seulement dans ses dérivés traditionnels trouvés in vivo (mélanocytes, neurones et cellules gliales), mais aussi dans des phénotypes mésenchymateux, y compris adipocytes et ostéoblastes. Comme dans les cellules de la CN céphalique, ces phénotypes mésenchymateux se différencient à partir de progéniteurs multipotents. Ceci suggère que, au cours de l'évolution, les cellules souches de la CN, dotées de potentiels à la fois mésenchymateux et neuraux, ont eu l'expression de leur potentiel mésenchymateux inhibée dans le tronc. Ainsi, chez les Vertébrés amniotes, même s'il ne se manifeste pas et reste dormant in vivo, un potentiel de différenciation mésenchymateuse est bien présent dans les cellules de la CN troncale et peut être révélé in vitro.

ABSTRACT

The neural crest (NC) is a structure derived from the dorsal borders of the vertebrate neural tube. During embryonic development, the NC cells migrate and contribute to the formation of different tissues and organs. Along the anteroposterior axis, the NC gives rise to neurons and glia of the peripheral nervous system and to melanocytes. Furthermore, the cephalic NC yields mesenchymal tissues, which form the large part of craniofacial cartilages and bones, facial dermis, fat cells and smooth muscle cells in the head. In the trunk of amniote Vertebrates, mesenchymal tissues are derived from the mesoderm, not from the NC. In lower Vertebrates, however, the trunk NC generates some mesenchymal tissues, such as in the dorsal fins of zebrafish. The question therefore is raised whether the ability of NC cells to produce mesenchymal cells was totally lost in the trunk of amniote Vertebrates during evolution, or if it can still be achieved under specific conditions. Some evidence pointed to a possible mesenchymal differentiation potential of the trunk NC cells. Thus, this work is interested in uncovering the mesenchymal potential of the avian trunk NC, with special interest in the differentiation into osteoblasts and adipocytes.

Our experimental approach was to examine the skeletogenic and adipogenic differentiation potentials of quail trunk NC cells after in vitro culture. Cell differentiation was evidenced by the analysis of lineage-specific genes and markers using in situ hybridization, immunocytochemistry and RT-PCR. The established culture conditions allowed observation of both skeletogenesis and adipogenesis. Osteogenesis was initially characterized by expression of *Runx2*, the first transcription factor specific of the osteoprogenitors, which was detected by in situ hybridization from 5 days of culture. When these NC cultures were supplemented with osteogenic and adipogenic factors, we observed osteoblast maturation, with the expression of collagen1 protein, *osteopontin* mRNA and *alkaline phosphatase* mRNA, until the bone matrix mineralization stage, as identified by Alizarin Red staining. The trunk NC cells also underwent chondrogenesis, as demonstrated by *Sox9*, *aggrecan* and *collagen10* mRNA expression, and Alcian blue staining. The observation of the mineralized areas and chondrogenesis suggested that the trunk NC cells in vitro are able to perform endochondral and membranous ossifications. Cells supplemented with osteogenic and adipogenic factors also showed adipogenesis. Adipocytes with lipid droplets began to differentiate from 10 days of culture and were identified by Oil Red O staining. The mRNAs of the *CEBP*α, *PPAR*γ and *FABP4* adipogenic markers were detected by RT-PCR from 3 days of culture. For the characterization of bone and adipocyte progenitors, we evaluated the differentiation potential of individual trunk NC cells. The phenotypic analysis of these clonal cultures showed that 76% of the cells generated *Runx2*-positive osteoblasts. Moreover, most of the clone-forming trunk NC cells were multipotent progenitors endowed with both neural and osteogenic potentials. Furthermore, in another clonal culture condition, adipocytes were found in 35.3% of the clones, and approximately half of them also contained glial and/or melanogenic cells.

These results show that the trunk NC cells in vitro are able to differentiate not only in their classical derivatives found in vivo (melanocytes, neurons and glial cells), but also in mesenchymal phenotypes, including adipocytes and osteoblasts. Importantly, as in cephalic NC cells, mesenchymal phenotypes differentiated from multipotent progenitor cells, suggesting that, during evolution, the NC stem cells intended for both mesenchymal and neural fates, had the expression of their mesenchymal potential inhibited in the trunk. Thus, although at the dormant state and not expressed in vivo, a significant mesenchymal potential is present in the trunk NC cells of amniotes Vertebrates and can be disclosed in vitro.

1. INTRODUÇÃO

A crista neural (CN) é uma estrutura que surge das bordas da placa neural (PN), no limite entre a região que formará o tubo neural (TN) e a região da ectoderme epidermal nos embriões dos vertebrados. Essa estrutura contribui para a formação de diferentes órgãos, o que mostra sua multipotencialidade. Ao longo de todo o eixo rostro-caudal, a CN dá origem a neurônios e células gliais do sistema nervoso periférico e melanócitos. Além destes, outros fenótipos são gerados pela CN que migra de regiões bem delimitadas. A CN cefálica, por exemplo, gera tecidos mesenquimais, que formam as cartilagens e ossos crâniofaciais e também derme, adipócitos e células de músculo liso (Le Douarin and Kalcheim, 1999).

A caracterização da multipotencialidade das células da CN é o interesse de muitos estudos. No laboratório da Prof. Dra. Nicole Le Douarin, trabalhos revelaram um progenitor altamente multipotente presente entre as células da CN cefálica de aves, que apresenta capacidades de diferenciação neural, melanocítica e mesenquimal.

Os tecidos mesenquimais do tronco dos vertebrados amniotas derivam da mesoderme, não da CN. Em contrapartida, a CN de vertebrados inferiores gera alguns tecidos mesenquimais no tronco, como o esqueleto dérmico das nadadeiras dorsal e caudal dos vertebrados teleósteos como o peixe-zebra. Logo, uma questão que se apresenta é se a capacidade das células da CN de produzir mesênquima no tronco foi perdida durante a evolução, ou se ainda existe nos vertebrados amniotas e pode ser revelada em condições específicas.

Para estudar o potencial das células da CN truncal é importante entendermos o processo de formação, especificação e determinação destas células. Estes aspectos, bem como o processo de evolução da CN e os mecanismos de diferenciação mesenquimal óssea e adipocítica serão abordados no capítulo de introdução desta tese.

1.1. A formação da crista neural e seus derivados

A CN é uma estrutura transitória multipotente dos embriões dos vertebrados, com células que se diferenciam em diferentes fenótipos e contribuem para a formação de diversas estruturas do organismo. Esta população celular foi descoberta por Wilhem His em 1868 em estudos com embriões de galinha. Ele descreveu a CN como um conjunto de células que saem do TN dorsal para formar os gânglios sensoriais da raiz dorsal, e por isso nomeou esta estrutura de crista gangliônica (His, 1868).

As células da CN são derivadas das bordas dorsais do TN no estágio de nêurula. Estas células passam por uma transição epitélio-mesênquima, que envolve mudanças nas propriedades de adesão intercelular e com os tecidos adjacentes, se dissociam do tubo neural, e seguem diferentes caminhos de migração para chegar aos locais específicos onde darão origem aos tipos celulares apropriados (**Figura1**) (Le Douarin and Kalcheim, 1999). O restante do TN guarda a estrutura neuroepitelial e forma o sistema nervoso central.

1.1.1. Indução e especificação da crista neural

O desenvolvimento da CN envolve uma complexa sucessão de eventos, desde a indução e formação das células da CN até a especificação e diferenciação

dos diferentes fenótipos. Estes eventos são guiados por uma rede de regulação gênica e de interações celulares, e podem ser divididos em 3 etapas: a indução da borda da PN, a especificação da CN, e mais tarde a geração da CN (Sauka-Spengler and Bronner-Fraser, 2008) (**Figura1**).

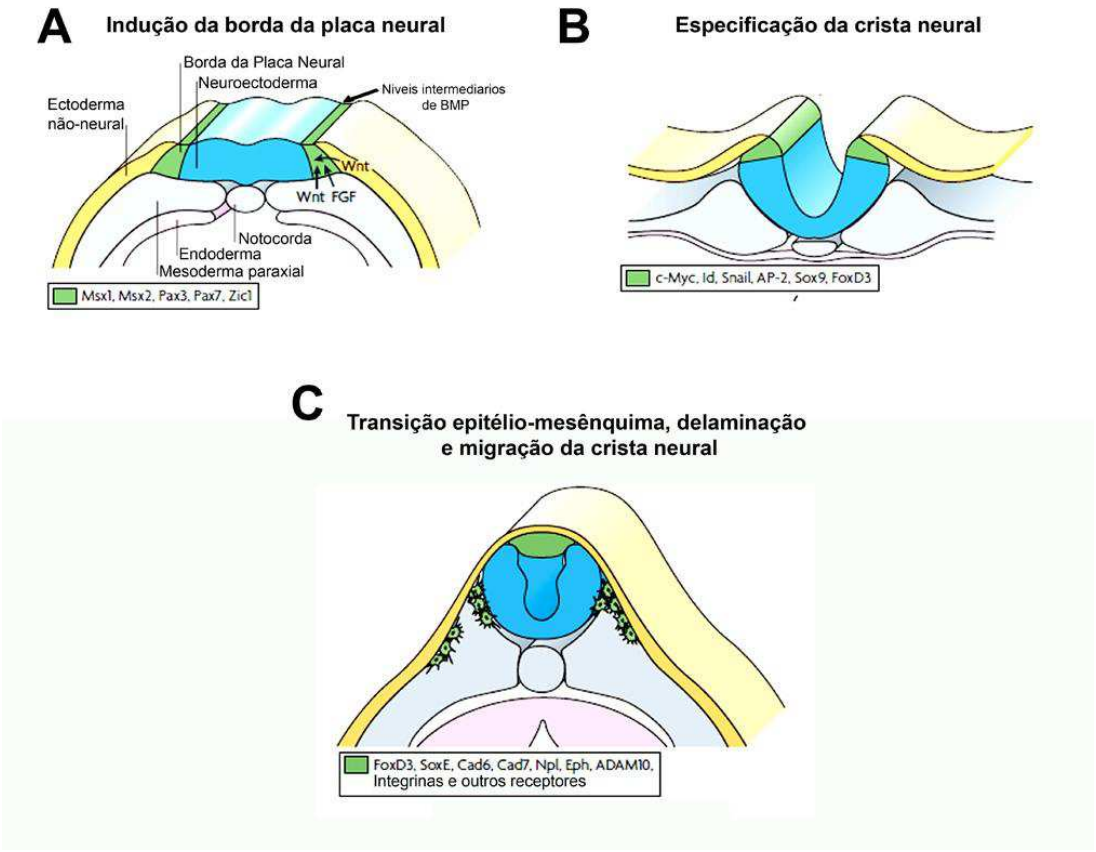

Figura 1 - **Etapas de formação da crista neural. (A)** A indução da borda da PN é mediada pela atuação de sinais derivados dos tecidos adjacentes, como FGF, Wnt e BMP. A ativação destas vias de sinalização induz a expressão dos genes especificadores da borda da PN. **(B)** Na etapa de indução da CN, os genes especificadores da borda da PN controlam a expressão dos genes especificadores da CN. **(C)** Os genes especificadores da CN promovem a segregação das células da CN do neuroepitélio dorsal, pois controlam eventos de proliferação celular, delaminação e o início da transição epitélio-mesênquima. (adaptado de Sauka-Spengler and Bronner-Fraser, 2008)

A formação da CN tem início na gastrulação, com a atuação de diferentes sinais provenientes dos tecidos adjacentes que promovem a indução da borda da PN. Um dos fatores de crescimento implicados é o BMP4 (proteína morfogenética do osso 4) expresso pelo ectoderma. Antagonistas de BMP (Chordin, Noggin e Follistatin), derivados do mesoderma, são importantes para gerar um nível intermediário de BMP essencial para a indução da CN na borda da PN (Marchant et al., 1998). Outras vias de sinalização atuam em conjunto com a sinalização BMP para induzir a formação da CN. Fatores FGF (fator de crescimento fibroblástico) também estão implicados na indução da CN. Foi mostrado em embriões de *Xenopus laevis* que FGF2 em combinação com antagonistas de BMP, aumenta a expressão do marcador de CN *Snail2* e induz a formação da CN (Mayor et al., 1997). Além disso, FGF8, expresso no mesoderma, induz transitóriamente células da CN (Monsoro-Burq et al., 2003). A via do receptor Notch atua na indução da CN antes da via de BMP para restringir a formação da CN à região da borda da PN (Endo et al., 2002). Além destes sinais, a via de sinalização Wnt também exerce um papel chave na formação da CN. Em embriões de *Gallus gallus* (galinha), Wnt6 presente na ectoderma não-neural adjacente à borda da PN, atua na indução da CN através da via canônica dependente de β-catenina (Garcia-Castro et al., 2002). No período da gastrulação, os sinais Wnt e BMP, expressos em domínios diferentes do ectoderma atuam em conjunto numa região específica da borda da PN para delimitar e induzir o território da CN. A região mais anterior da borda da PN, que recebe apenas os sinais BMP, dá origem aos placóides ectodermais, enquanto regiões mais centrais, sob influência somente da via de Wnt, formarão o sistema nervoso central (Patthey et al., 2008). Esta primeira etapa de indução da PN leva à ativação de fatores de transcrição específicos na região da borda da PN, chamados de

“especificadores da borda da PN”, que incluem *Dlx3, Dlx5, Msx1, Msx2, Pax3, Pax7* e *Zic1* (Sauka-Spengler and Bronner-Fraser, 2008) (**Figura1A**).

A combinação destes genes leva à especificação das células da borda da PN em células da CN através da ativação dos genes “especificadores da CN” (ou marcadores da CN), que incluem *Snail1, Snail2, Sox8, Sox9, Sox10, FoxD3, AP2, Twist, c-Myc* e membros da família *Id* (**Figura 1B**). Estes genes definem um novo estado regulatório destas células e ativam os genes efetores da CN, que guiam uma sequência complexa de eventos para especificar os diferentes destinos da CN. Os mecanismos de indução e controle dos genes especificadores da CN são pouco conhecidos. Entretanto, estudos em *Xenopus leavis* mostraram que a indução da expressão de *Snail2 e FoxD3* por *FGF8* é dependente da sinalização Wnt e mediada por *Msx1*. Além disso, *Snail2 e FoxD3* são regulados por Pax3 e Zic1, que por sua vez são dependentes de Wnt (Monsoro-Burq et al., 2005; Sato et al., 2005). Em embriões de camundongos, foi observado que *Sox10* é induzido e mantido por Pax3 e AP2, e também por Sox9 e por ele mesmo, através da ativação da via de Wnt (Werner et al., 2007).

Assim, a atividade dos genes especificadores da CN segrega estas células do neuroepitélio dorsal, pois controla eventos que conferem características específicas das células da CN, como a transição epitélio-mesênquima, o controle do tamanho da população celular, o controle do ciclo celular, e a diferenciação numa grande diversidade de derivados (**Figura 1C**).

A delaminação das células da CN do neuroepitelio envolve o processo de transição epitélio-mesênquima, que envolve mudanças nas propriedades adesivas, na forma e na mobilidade celular. O tempo de delaminação varia entre as subpopulações das células da CN localizadas em diferentes regiões do eixo ântero-

posterior. A CN cefálica delamina de uma só vez. Em embriões de camundongos e *Xenopus* este processo ocorre quando o TN ainda esta aberto, enquanto em embriões de aves coincide com a fusão das bordas neurais (Theveneau et al., 2007). A delaminação das células da CN truncal ocorre após o fechamento do TN e de forma progressiva, com as células deixando o neuroepitélio "uma de cada vez". Foi mostrado em embriões de galinha que as células da CN truncal mais anterior delaminam poucas horas após o fechamento do TN (Sela-Donenfeld and Kalcheim, 1999), enquanto as células mais caudais emigram um dia após o fim da neurulação (Osorio et al., 2009a).

Estudos realizados basicamente em embriões de galinha mostraram que na região do tronco, a delaminação das células da CN é desencadeada por uma cascata de sinalização ativada pelas vias de BMP e a via canônica de Wnt (Burstyn-Cohen et al., 2004; Cheung et al., 2005). Esta cascata promove a transição epitélio-mesênquima através da ativação de Snail2, FoxD3, Sox9 e Sox10. Estes fatores de transcrição atuam na troca da expressão das moléculas de caderina (N-caderina é substituida por caderinaB6, que é trocada porsteriormente por caderina 7 e caderina11), ativam integrinaβ1, modulam a expressão de RhoB, estimulam a síntese de laminina e a degradação da lâmina basal (Cheung et al., 2005;(Chalpe et al.)). A cascata de sinalização de BMP4 e Wnt1 promove a transição da fase G1 da mitose para a fase S do ciclo celular, que tem um papel permissivo na transição epitélio-mensêquima (Burstyn-Cohen et al., 2004). ADAM10, ativado pela via de BMP4, provoca a clivagem de N-caderina. A clivagem de N-caderina na porção extracelular diminui a adesão entre as células. A clivagem de um domínio citoplasmático leva a ativação de ciclina D1 que leva à progressão do ciclo celular e

contribui na transição G1/S do ciclo celular (Shoval et al., 2007). A ativação da cascada de BMP/Wnt está ligada à maturação dos somitos, que conduz ao bloqueio da expressão de Noggin (inibidor de BMP) no TN dorsal (Sela-Donenfeld and Kalcheim, 2000). Na CN cefálica e na CN truncal mais caudal, a delaminação das células é regulada de forma diferente. As células pré-migratórias da CN cefálica expressam vários inibidores de BMP e Wnt, e a maior parte delas não tem contato com os somitos (responsáveis pela inibição de Noggin no TN na região do tronco) (Tzahor et al., 2003). De fato, a superexpressão de Noggin e o receptores dominante negativos de BMP não são capazes de bloquear a delaminação da CN cefálica (Kalcheim and Burstyn-Cohen, 2005). Além disso, a emigração da CN cefálica não está relacionada à transição da fase G1 para S do ciclo celular (Osorio et al., 2009b). Na região mais caudal do tronco, as células da CN também não estão sincronizadas com a somitogênese. A diminuição de Noggin no TN dorsal não acontece em resposta à diferenciação dos somitos e a emigração ocorre com um atraso de 24h (Osorio et al., 2009b). Nesta região, a delaminação das células da CN esta sob controle de Wnt3a e é independente da ação combinada de BMP4/Wnt1.

Além dos genes especificadores da CN que mantém sua expressão em estágios mais tardios, as células da CN migratórias, podem ser identificadas através do anticorpo monoclonal HNK1 (human natural killer 1), que reconhece carboidratos que contém ácido glucurônico sulfatado em sua composição (Abo and Balch, 1981). Epítopos com estes carboidratos foram originalmente identificado em células NK (natural killer) humanas, e são expressos pelas células migratórias da CN (Tucker and Erickson, 1984) (**Figura 2B**). Mais tarde, a expressão de HNK1 foi observada em outros tipos celulares, como as células gliais, parte das células do TN, algumas

subpopulações neuronais e cardiomiócitos (Nakajima et al., 2001). Desta forma, a marcação com o anticorpo HNK1 permite a identificação das células da CN *in vitro* e *in vivo*.

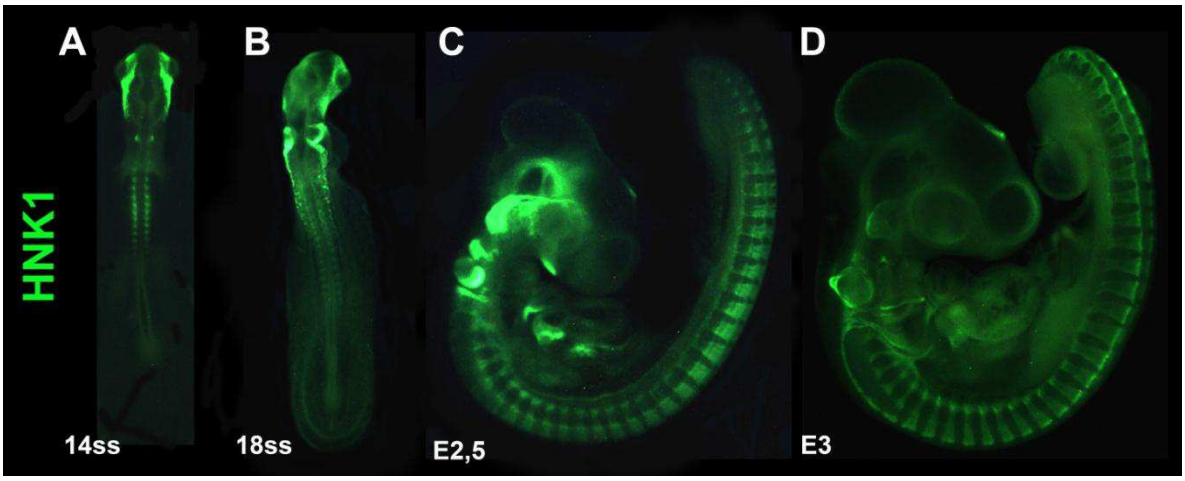

Figure 2 – Células migratórias da crista neural de codorna evidenciadas pelo anticorpo HNK1 *in toto*. (A) Embrião de 14 somitos onde podemos observar a CN cefálica já longe do TN e o começo de migração das células da CN truncal. **(B)** Embrião de 18 somitos onde observamos as células da CN cefálica cobrindo toda a regiao da cabeça e colonizando a região dos futuros arcos branquiais e a CN truncal anterior já em estado avançado de migração. **(C e D)** Embriões de 2,5dias e 3 dias, respectivammente, onde observamos células da CN presentes em seus locais de diferenciaçao, como, por exemplo, o gânglio sensorial trigêmeo, os gânglios da raiz dorsal e os gânglios simpáticos.

1.1.2. As quimeras codorna-galinha e os derivados da crista neural

De acordo com o nível ântero-posterior do TN de onde as células da CN se originam, elas serão fonte para a formação de tecidos e órgãos específicos. Estes derivados, bem como o nível ântero-posterior do qual cada um se origina, foram identificados em embriões de aves devido aos experimentos de quimeras galinha-codorna (Le Douarin, 1969; Le Douarin and Teillet, 1974; Le Lievre and Le Douarin, 1975); Le Douarin and Kalcheim, 1999), desenvolvidos no laboratório de Nicole Le Douarin à partir da década de 70.

A realização destes experimentos se tornou possível após a observação realizada por Nicole Le Douarin de que os núcleos das células de *Coturnix coturnix japonica* (codorna) tem a particularidade de possuir a heterocromatina condensada associada ao nucléolo (Le Douarin, 1969). Este DNA compacto pode ser bem visualizado com a coloração de Feulgen, que colore ácidos nucleicos. Nas outras espécies, incluindo *Gallus gallus* (galinha) a heterocromatina se encontra dispersa no núcleo no momento da intérfase. Mais tarde o anticorpo QCPN (*quail non-chicken perinuclear antigen*) também pode ser utilizado para distinguir as células de codorna nas quimeras heteroespecíficas (Lance-Jones and Lagenaur, 1987). Para identificação dos derivados da CN, estas quimeras são produzidas através do transplante de uma região particular do TN (ou das bordas dorsais do TN) do embrião de galinha, antes da migração da CN, no embrião de codorna (**Figura 3**).

Diversos estudos mostraram o domínio de origem de cada tipo celular derivado da CN e os caminhos de migração destas células. Os melanócitos, por exemplo, se diferenciam a partir de toda a extensão ântero-posterior da CN, enquanto derivados mesenquimais são originados apenas da região da CN cefálica (Le Douarin and Kalcheim, 1999). As células que contribuem para o SN periférico sensorial e autônomo e o SN entérico estão presentes apenas em algumas áreas do eixo neural (**Figura 4**).

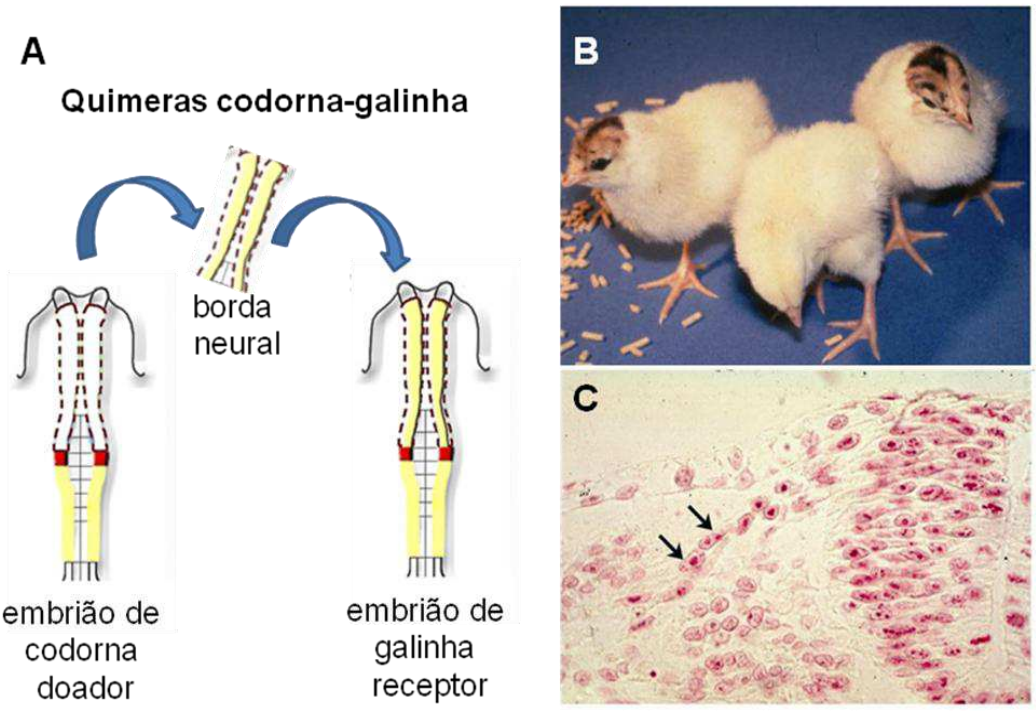

Figura 3 - Esquema da realização de quimeras de codorna-galinha. O tubo neural que é retirado de um embrião de codorna doador é implantado em um embrião de galinha no mesmo estágio embrionário. **(A)** Exemplo de transplante de parte do tubo neural cefálico de codorna para o embrião de galinha. **(B)** Galinhas quiméricas de raça branca resultantes de transplante realizado ao nível do cérebro anterior possuem penas pigmentadas somente na região da cabeça, devido à presença de melanócitos originados da região transplantada de codorna. Observamos dois pintos quiméricos e um pinto normal ao centro. **(C)** Coloração Feulgen de um corte histológico transversal do embrião de galinha receptor mostra a migração das células da crista neural (setas) à partir do tubo neural da codorna doadora, cujas células possuem uma cromatina mais condensada (adaptado de Le Douarin and Kalcheim, 1999).

Estudos de mapeamento dos derivados da CN também foram realizados em embriões de camundongos através de experimentos de recombinação genética que permitiram o rastreamento de longo prazo das células da CN. Inicialmente camundongos P0-Cre foram usados combinados com camundongos trangenicos que expressam genes repórteres (β-galactosidase ou GFP), o que confirmou a maior parte dos derivados da CN já observados em aves (Yamauchi et al., 1999). No entanto, a recombinação mediada por P0-cre ocorre apenas em subconjuntos de

células da CN e exibe expressão ectópica deixando muitas questões sobre a diferenciação da CN sem resposta. Pouco tempo depois, o promotor do gene Wnt1, mais específico para as células da CN, foi utilizado em combinação com camundongos com o repórter condicional ROSA26-lacZ ou o repórter para *EGFP* (Jiang et al., 2000); 2002).

Coletivamente as análises realizadas em aves, camundongos, peixes e anfibios demonstraram que as células da CN cefálica e truncal produzem diversos derivados, como neurônios, células gliais, células endócrinas, melanocitos, e derivados mesenquimais que se diferenciam apenas da CN cefálica, como células de músculo liso, ossos e cartilagem (Le Douarin and Kalcheim, 1999). Além da divisão em CN cefálica e truncal, ainda podemos destacar outras subregiões. As regiões vagal (7 primeiros somitos) e sacral (região posterior ao somito 28) são responsáveis pela formação do sistema nervoso entérico, que controla os movimentos peristálticos do sistema digestório (Le Douarin and Teillet, 1973). A CN cardíaca (romboncéfalo posterior, à nível dos 4 primeiros somitos) gera células de músculo liso que formam o septo que separa a aorta e a artéria pulmonar no desenvolvimento do coração (Le Lievre and Le Douarin, 1975; (Kirby and Waldo, 1995). Os resultados destes experimentos de mapeamento dos derivados em aves são resumidos na **Figura 4**.

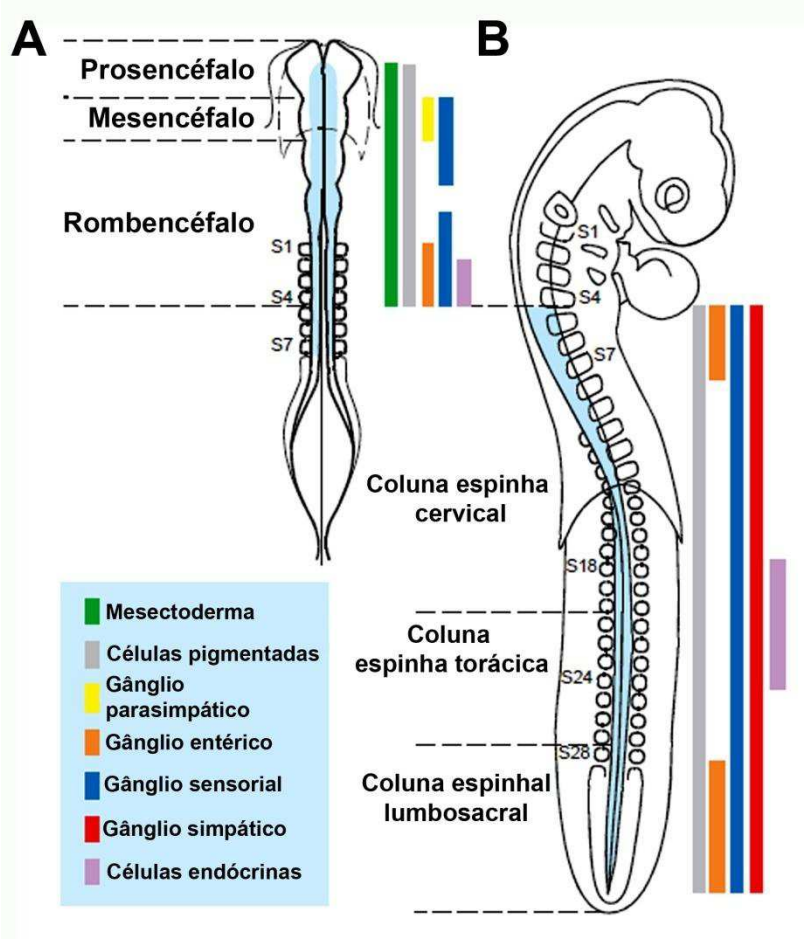

Figura 4 – Mapa dos derivados da crista neural. Os diversos fenótipos provenientes da CNC (A) e da CNT (B) ao longo do eixo anteroposterior. Podemos observar as células da CN em azul no embrião de 7 ss(A) e no embrião de 28 ss(B). A direita destes embriões observa-se os tecidos originários das células da CNC (A) e CNT (B) identificados por cores diferentes.

A CN cefálica compreende as células que migram do diencéfalo posterior, mesencéfalo e dos rombômeros até o quarto somito incluso (Couly et al., 1996). O padrão de migração da CN cefálica mostra poucas variações entre as diferentes espécies (**Figura 5A** – rotas de migração da CN aviária). As células da CN cefálica que contribuem com células mesenquimais para a região da cabeça e do pescoço foram designadas de mesectoderma (ou ectomesenquimas), a fim de distinguir estas células das células mesenquimais derivadas do mesoderma (Platt, 1983). As células que migram para o broto nasal e o primeiro arco branquial formam a cápsula nasal,

osso maxilar, mandíbula e o entoglosso (esqueleto da língua). A cartilagem hióide é formada pelas células que colonizam os arcos branquiais 2, 3, 4 (Couly et al, 1996). As células da CN cefálica que migram cobrindo o prosencéfalo atuam na formação da calota craniana, que é composta dos pares de ossos frontal, parietal, esquamosal e a parte anterior do osso occipital, ainda é discutida. Experimentos que utilizaram a técnica de transplantes para produção das quimeras galinha-codorna apontam que estes ossos são inteiramente derivados da CN (Couly et al., 1993). Entretanto, estudos em camundongos transgênicos que permitem o acompanhamento das células da CN devido à expressão do gene *βgalactosidade* associado ao promotor do gene *Wnt1* mostraram que os ossos parietais são de origem mesodérmica. Entretanto, as suturas cranianas sagital (entre os ossos parietais) e coronais (entre os ossos frontais e parietais) são formadas pela CN e se desenvolvem na interface entre células derivadas da CN e outras do mesoderma (Jiang et al., 2002). Além destes, existem outros derivados mesectodermais, como as células dermais da face, meninges, tecido adiposo, tendões, e tecido conjuntivo do timo, parte das glândulas tireóide e paratireóide, e de músculos da cabeça e do pescoço. A CN cefálica também dá origem a diferentes tecidos dos olhos e dos dentes, e participa de estruturas cardiovasculares formando as camadas de células de músculo liso adjacentes ao endotélio das grandes artérias dos arcos aórticos e dos vasos sanguíneos que irrigam a face e o prosencéfalo, e também participa da formação do septo cardíaco (Le Douarin and Kalcheim, 1999; (Chai et al., 2000; Etchevers et al., 2001); Jiang et al., 2000, 2002).

As células da CN cefálica também contribuem para a formação de neurônios e células gliais do sistema nervoso periférico, como neurônios do gânglio sensorial trigêmeo e os gânglio parassimpáticos, como o ciliar, por exemplo (Le Douarin and

Kalcheim, 1999). Também são derivadas da CN cefálica as células C, células endócrinas produtoras de calcitonina. Estas células se desenvolvem no corpo ultimobranquial em todos os vertebrados, exceto nos mamíferos, onde esta estrutura se junta à glândula tireóide.

No tronco, o tempo de emigração e as trajetórias seguidas pelas células da CN possuem algumas diferenças entre as diferentes espécies. A CN truncal pode seguir dois caminhos alternativos de migração: o caminho ventral e o caminho dorsolateral. Em embriões de galinha e camundongo, as células migram inicialmente pelo caminho ventral, entre o TN e a metade anterior do esclerótomo somítico, o que leva a um padrão de migração segmentado (Guillory and Bronner-Fraser, 1986; Loring and Erickson, 1987; Rickmann et al., 1985; Teillet et al., 1987) (**Figura 5B**). As células que migram por este caminho seguem uma ordem ventral para dorsal de colonização de seus derivados, começando por formar a cadeia de gânglios simpáticos, as células de Schwann associadas a todos os nervos periféricos do organismo, e finalmente os gânglios da raiz dorsal sensoriais (Serbedzija et al., 1994). A CN truncal que migra do nível ântero-posterior entre os somitos 18 a 24 gera também células da medula adrenal (células produtoras das catecolaminas - adrenalina e noradrenalina), e os grupos de nervos que cercam a aorta (Le Douarin and Teillet, 1974). As células que migram mais tarde seguem um caminho dorsolateral superficial, entre o ectoderma e o dermomiótomo, e se tornam melanócitos situados em toda a pele (Erickson et al., 1992). Em camundongos estas rotas são invadidas simultaneamente, enquanto em galinha as células começam a seguir o caminho dorsolateral aproximadamente 24h após o inicio da migração da CN truncal (Kuo and Erickson, 2010).

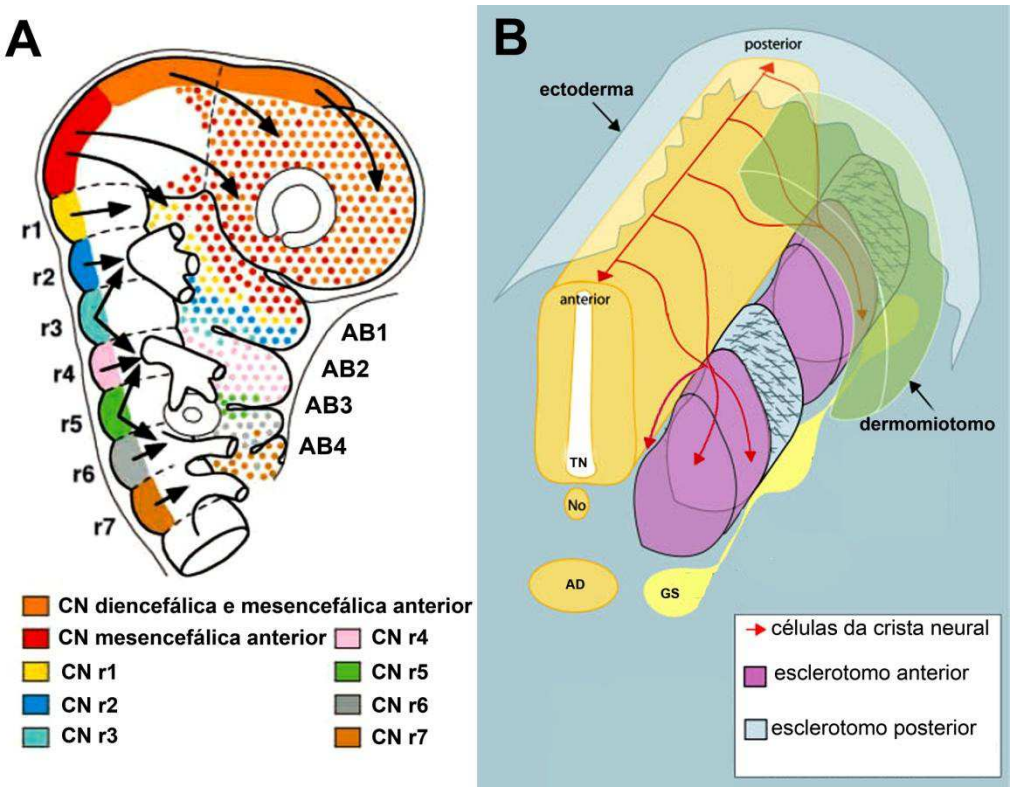

Figura 5 – Esquemas da migração das células da crista neural cefálica e truncal no embrião de ave. **(A)** Migração de células da CN cefálica. A origem das células encontradas nas regiões frontonasal e periocular e nos AB está diferenciada por cores. O mesencéfalo anterior contribui para as regiões frontonasal e periocular. O mesencéfalo posterior também participa nestas estruturas, e preenche a parte anterodistal do primeiro AB. A parte complementar do primeiro AB deriva dos rombômeros r1/r2, e de uma pequena contribuição do r3. A maior contribuição para o segundo AB vem do r4. As células dos r3 e r5 contribuem para dois AB adjacentes; células do r3 migram para o primeiro e o segundo AB; e as do r5 migram para o segundo e terceiro. Células dos r6 e r7 migram para o terceiro e quarto AB. **(B)** Migração ventral das células da CN truncal. Durante esta migração (setas vermelhas), moléculas no esclerótomo posterior bloqueiam a invasão das células nesta porção do somito, e estabelecem um padrão metamérico dos gânglios da raiz dorsal. As diferenças entre as metades anteriores e posteriores dos somitos também ditará a posição e a segmentação dos gânglios simpáticos (GS) primários. TN, tubo neural; No, notocorda; AD, aorta dorsal; GS, gânglios simpáticos. (Couly et al., 2002; Harris and Erickson, 2007).

Em embriões de peixe-zebra e *Xenopus* observamos algumas diferenças quanto à migração da CN truncal. No peixe-zebra, as células do caminho ventral migram pela parte medial dos somitos. A migração lateral começa quatro horas depois (Raible et al., 1992). No *Xenopus* a CN passa entre o TN e o somito

posterior, enquanto poucas células seguem o caminho dorsolateral (Collazo et al., 1993). Ao contrário do observado em galinhas e camundongos, precursores de melanoblastos podem seguir as rotas ventral e dorsal em *Xenopus* e zebrafish (Collazo et al., 1993; Kelsh et al., 2009). Além destas diferenças, algumas células da CN truncal de peixes e anfíbios migram em direção dorso-medial, onde contribuem com células pigmentadas e mesenquimais para as nadadeiras dorsais (Collazo et al., 1993; Raible and Eisen, 1994).

Ao contrário da CN cefálica, a CN truncal dos vertebrados amniotas não contribui para a formação dos derivados mesenquimais do tronco durante o desenvolvimento. Para estudarmos o potencial de diferenciação da CN truncal, é importante entendermos o processo de especificação e determinação da CN como ponto de partida.

1.2. O potencial de diferenciação da crista neural

A população de células da CN é capaz de se diferenciar em diferentes tipos celulares durante o desenvolvimento embrionário. Um ponto de discussão no processo de diferenciação destas células é o seu o potencial antes e durante o processo de migração a partir do TN dorsal. Dois modelos são debatidos quanto à origem das diversas linhagens celulares à partir da CN. O primeiro modelo sugere que as células da CN são originalmente células-tronco que se dividem e sofrem restrições progressivas no seu potencial até a diferenciação final, à semelhança da diferenciação dos diferentes tipos celulares do sistema hematopoiético (Bronner-Fraser and Fraser, 1989; Bronner-Fraser and Fraser, 1988; Trentin et al., 2004). A

opção apresentada pelo segundo modelo é a de que a CN pré-migratória seria composta por um mosaico de precursores já pré-determinados (Krispin et al., 2010).

Em experimentos de transplantes heterotópicos da CN truncal de codorna em embriões de galinha para substituição da CN cefálica, nenhuma célula mesectodermal se desenvolveu a partir da região transplantada. Quando a CN truncal é transplantada apenas de um lado da região cefálica, estas células migram junto com as células do hospedeiro e se diferenciam em miofibroblastos e células do tecido conjuntivo, mas nunca participam da esqueletogênese (Nakamura and Ayer-le Lievre, 1982). Estes dados apóiam o segundo modelo de potencial restrito de diferenciação das células da CN.

Entretanto, outros experimentos de transplantes heterotópicos demonstraram que algumas células pré-migratórias da CN tem uma grande plasticidade. Isto também é verdade para algumas populações celulares derivadas da CN já presentes em seus territórios definitivos. Quando a CN vagal (TN mais CN pré-migratória) de um embrião de galinha é trocada pela CN torácica de codorna, que normalmente não dá origem à células do sistema nervoso entérico, as células transplantadas colonizam o intestino e se diferenciam nos tipos neuronais apropriados colinérgicos, peptidérgicos e serotoninérgicos (Fontaine-Perus et al., 1982; Le Douarin et al., 1975). Outros experimentos de transplantes heterotópicos com embriões de aves demonstraram que as células gliais satélites do gânglio nodose derivadas da CN são capazes de migrar novamente quando colocadas na rota de migração das células da CN truncal em um embrião de galinha hospedeiro. As células transplantadas foram capazes de dar origem a neurônios autônomos e contribuiram para os gânglios simpáticos, as células da medula da glândula adrenal e gânglios entéricos (Ayer-Le Lievre e Le Douarin, 1982). Nestes casos, os alvos colonizados pelas células

transplantadas são determinados pelas rotas de migração características do nível axial onde foram colocadas. Desta forma, estes resultados reforçam a idéia de que as células da CN são originalmente multipotentes e possuem um amplo potencial de diferenciação diretamente influenciado pelo ambiente por onde as células passam e o encontrado nos sítios alvos.

Para compreendermos os mecanismos de segregação de diferentes linhagens a partir da CN, se faz necessários experimentos de análise dos fenótipos obtidos a partir de células individuais da CN. Experimentos *in vivo* foram realizados através da injeção de marcadores vitais nas células da CN pré-migratórias, e permitiram observar os fenótipos de sua progênie (Bronner-Fraser and Fraser, 1989; Bronner-Fraser and Fraser, 1988; Fraser and Bronner-Fraser, 1991; Krispin et al., 2010). *In vitro*, foram utilizados experimentos de clonagem celular das diferentes regiões da CN em diferentes contextos de cultivo, permitindo o estudo do potencial de diferenciação de cada uma das células clonadas (Baroffio et al., 1988; Baroffio et al., 1991; Calloni et al., 2007; Calloni et al., 2009; Sieber-Blum, 1989; Sieber-Blum and Cohen, 1980; Trentin et al., 2004).

1.2.1. Especificação e determinação das células da CN *in vivo*

Os primeiros trabalhos de rastreamento de linhagem derivada das células da CN *in vivo* foram realizados pelo grupo da pesquisadora Marianne Bronner-Fraser (Bronner-Fraser and Fraser, 1988, 1989; Fraser and Bronner-Fraser, 1991). Experimentos em embriões de galinha de microinjeção com marcador fluorescente vital em células individuais do TN dorsal mostraram que os descendentes clonais de células da CN truncal participavam da formação de diferentes tipos de derivados da

CN, produzindo após dois dias várias combinações de neurônios (nos DRGs e/ou gânglios simpáticos), células de schwann ao redor dos nervos, melanócitos e células adrenomedulares. Estes resultados argumentam fortemente em favor da multipotência das células pré-migratórias da CN.

Experimentos mais recentes de rastreamento de células do TN dorsal, realizados pelo grupo de Chaya Kalcheim, mostraram que a localização final das células da CN pode ser prevista por sua posição ventrodorsal no domínio pré-migratório do TN dorsal ou pelo seu tempo de delaminação. Além disso, as células da CN que emigram a partir da linha média dorsal do TN de embriões de galinha, geraram uma progênie que contém apenas um único tipo celular. O trabalho conclui que as células da CN possuem um potencial de diferenciação limitado já antes de sua emigração (Krispin et al., 2010).

A divergência entre esses resultados recentes e os precedentes é, provavelmente, devido a diferenças entre os estágios em que as células se encontravam no momento que foram marcadas. Nos trabalhos pioneiros, algumas das células pré-migratórias da CN devem ter sido acompanhadas desde momentos em que a segregação entre os tipos celulares do TN e da CN, ou entre os diferentes derivados da CN, ainda não tinha ocorrido (Bronner-Fraser and Fraser, 1988). Enquanto em Krispin et al., 2010, as células foram marcadas em estágios mais tardios e geraram uma progênie constituída de apenas um tipo celular.

O termo “especificação” muitas vezes é utilizado para designar células que além de orientadas para se tornar futuramente um fenótipo especifico também possuem um potencial de diferenciação restrito (Harris and Erickson, 2007). Este trabalho de tese considera denotações ligeiramente diferentes para os termos

“especificação” e “determinação”. Especificação se refere a “células que são competentes para se diferenciar num determinado fenótipo, mas pode ainda não estar comprometida com esta diferenciação” (Livesey and Cepko, 2001). A célula especificada não é definitivamente restrita o seu potencial de diferenciação e pode ser re-especificada para outro fenótipo. Determinação ou comprometimento, por sua vez, se refere a “uma decisão irreversível de produzir ou se tornar um tipo celular especifico” (Livesey and Cepko, 2001). Assim, uma célula já determinada para um tipo celular especifico, se desafiada com diferentes contextos ambientais não pode responder aos sinais que dirigem à diferenciação em outro fenótipo celular

Desta forma, a interpretação do trabalho do grupo de Kalcheim, que diz que as células da CN possuem um potencial de diferenciação limitado já antes de sua emigração, deve ser discutida. O fato de que as algumas células da CN serem especificadas e terem uma tendência para seguir um destino específico antes da emigração já foi mostrada por trabalhos anteriores *in vivo* (Dorsky et al., 2000; Le Lievre et al., 1980; Raible and Eisen, 1994) e *in vitro* (Ziller and Le Douarin, 1983). Todavia, a especificação celular, primeira etapa do comprometimento a um destino celular não esta diretamente relacionada a restrições no potencial de diferenciação. Além disso, durante sua migração, as células da CN são influenciadas pelos sinais do ambiente, que também atuam no processo de diferenciação. Para afirmarmos que uma célula é determinada, devemos desafiá-la com outro ambiente e observar que continuam o processo de diferenciação especificado inicialmente, o que significa que a célula não pode ser desviada para um novo destino. Para testar esta hipótese, Krispin e colaboradores testaram desviar as células da CN do caminho ventral para o caminho de migração dorsolateral melanocítico através da eletroporação do gene

de EDNRB2 (receptor B2 de endotelina3), que dirige a migração dorsolateral de melanoblastos (Krispin et al., 2010). Nestas condições, as células continuam seguindo o programa de diferenciação especificado inicialmente e adquirem características neurais e não melanocíticas. Entretanto, o receptor EDNRB2 é expresso em melanoblastos já especificados, e apenas estas células são capazes de entrar naturalmente na via de migração dorsolateral melanocítica (Erickson and Goins, 1995; Pla et al., 2005). Sendo assim, podemos concluir que a expressão forçada de EDNRB2 não foi suficiente para re-especificar estas células para o destino melanocítico e permitir que respondam aos sinais encontrados neste caminho. Novos experimentos seriam necessários para testar a irreversibilidade da decisão do fenótipo de células iniciais da CN.

O fato de uma célula estar especificada para um destino celular, não significa que não possa ser re-especificada por um contexto ambiental diferente e seguir outro destino celular. Esta possibilidade de especificação para destinos celulares diferentes é o que caracteriza o potencial de diferenciação de uma célula. Desta forma, os derivados obtidos a partir de uma célula *in vivo* não correspondem necessariamente ao seu completo potencial de diferenciação, que só pode ser de fato revelado através de testes *in vitro*, onde as células são expostas à ambientes permissivos.

Com o método de cultivo das células da CN *in vitro*, trabalhos anteriores de nosso grupo (Baroffio et al., 1988; 1991; (Dupin et al., 1990); (Dupin and Le Douarin, 1995; Lahav et al., 1998); Trentin et al., 2004; Calloni et al., 2007, 2009) confirmam o caráter multipotente da CN, mostrando que estas células da não tem seu destino determinado antes da emigração, e que um ambiente rico e permissivo possibilita a

especificação e diferenciação da progênie de uma única célula em múltiplos fenótipos.

Desta forma, a investigação do potencial de diferenciação mesenquimal em osteoblastos a adipócitos da CN truncal, objetivo desta tese, deve incluir experimentos de clonagem *in vitro*.

1.2.2. O potencial de diferenciação das células da crista neural *in vitro*

Devido aos diferentes tipos celulares que se originam da CN, essa população celular constitui um modelo apropriado para estudar a diversificação de linhagens a partir de células multipotentes. Os experimentos de transplante da CN e de rastreamento destas células durante sua migração no modelo de aves *in vivo* contribuíram muito para a compreensão de seus diferentes derivados. Contudo, para a compreensão de como elas interpretam os diferentes sinais recebidos do ambiente e, em particular, para estudar a descendência de células individuais, diferentes laboratórios realizaram análises de clonagem celular *in vitro* semelhantes aos ensaios de unidades formadoras de colônia (CFU) desenvolvidos à partir da década de 1950 para decifrar a origem da diversidade das células hematopoiéticas (Spangrude et al., 1988).

Em 1975, foi mostrado que as células da CN de codorna são capazes de migrar de explantes do TN *in vitro*. Estas células podem então ser ressuspendidas após a retirada do explante e clonadas (Cohen and Konigsberg, 1975). Ensaios clonais demonstraram, então, que as células da CN truncal são heterogêneas em seu potencial, e podem dar origem à progênie não-pigmentada, pigmentada ou mista (Cohen and Konigsberg, 1975; (Sieber-Blum and Cohen, 1980). Tanto os clones não

pigmentados quanto os mistos contém várias centenas de neuroblastos sensoriais e adrenérgicos, mostrando que a célula clonogênica era pelo menos tripotente (Sieber-Blum, 1989).

A utilização de novos anticorpos monoclonais contra proteínas especificas de neurônios e células gliais aviárias enriqueceu o entendimento das propriedades das células da CN, e as análises clonais *in vitro* destas células puderam progredir. Novos experimentos clonais com estas melhorias foram então realizados com células da CN de diferentes níveis do eixo ântero-posterior e de diferentes estados. Estes dados confirmaram a heterogeneidade das células da CN, mostrando que diferem nos potenciais de proliferação e diferenciação, e a maior parte delas geram diferentes combinações de dois ou mais tipos celulares (Sieber-Blum, 1989; (Sieber-Blum et al., 1993); Baroffio et al., 1988; Baroffio et al., 1991; Lahav et al., 1998; Dupin et al., 1990; Trentin et al., 2004; (Dupin et al., 2006).

Em nosso laboratório, os experimentos de culturas clonais de Elisabeth Dupin, Nicole Le Douarin e colaboradores, associados à utilização de dos marcadores celulares específicos, resultaram num modelo hierárquico da diversificação das linhagens da CN cefálica e da CN truncal de codorna. Estes experimentos são realizados por micromanipulação de precursores da CN isolados assim que surgem do TN cefálico ou truncal (**Figura 6**), realizado na maior parte das vezes com a utilização de um substrato de fibroblastos 3T3 de camundongos, que permite uma grande eficiência de clonagem e a diferenciação de um repertório com diversos fenótipos que podem ser obtidos a partir da CN. Com relação à CN cefálica e truncal, este conjunto de resultados é resumido na **Figura 7** (Baroffio et al., 1988,

1991; Dupin et al., 1990; Dupin and Le Douarin, 1995; Lahav et al., 1998; Trentin et al., 2004; Calloni et al., 2007; Calloni et al., 2009).

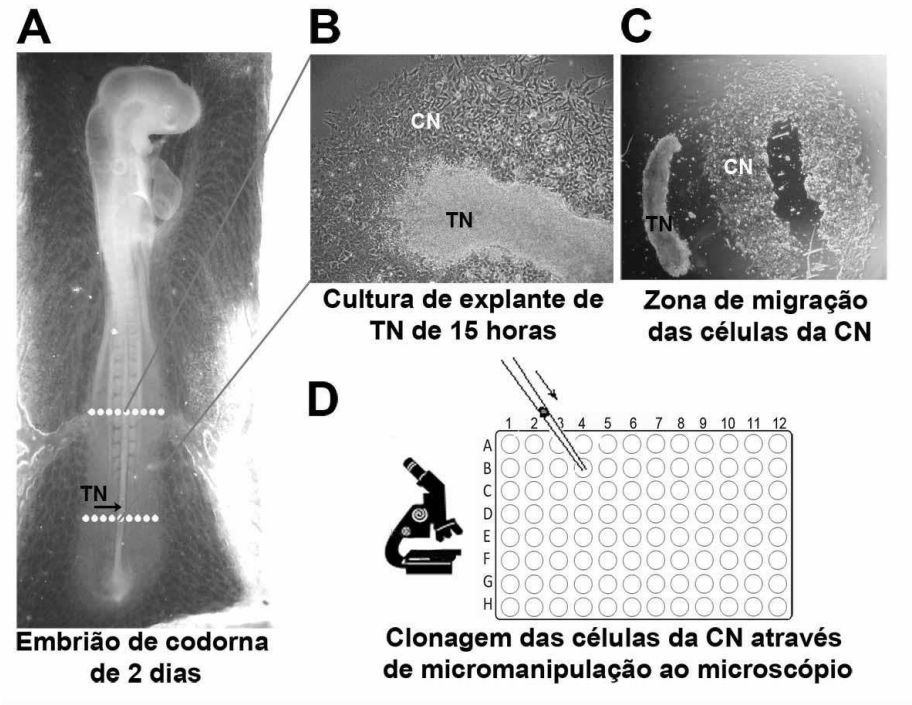

Figure 6 – **Esquema do método de cultura da crista neural truncal**. **(A)** Embrião de codorna HH14 (E2). A região do tronco a partir da qual o TN será isolado enzimaticamente das estruturas ao redor para a realização da cultura primária esta delimitada pela linha pontilhada. **(B)** Cultura primária de células da CN após 15h de migração a partir do TN *in vitro*. **(C)** Retirada do TN após o tempo estipulado para obtenção das células da CN que serão ressuspendidas com tripsina em seguida. **(D)** Clonagem das células da CN por micromanipulação sob controle microscópico.

Inicialmente Baroffio e colaboradores mostraram que alguns progenitores da CN cefálica do mesencéfalo geraram uma progênie diversa, que possuíam ao mesmo tempo capacidades neurais e condrogênicas (Baroffio et al., 1991). O modelo da CN cefálica atual compreende resultados mais recentes que mostraram que, ao isolar a CN cefálica mais precoce e adaptando as condições de cultura, uma grande parte destas células são multipotentes, capazes de produzir tipos celulares

pertencentes a todas as maiores linhagens derivadas da CN (neurais, melanocíticos e mesenquimais – incluindo esqueléticos) (**Figura 7A**) (Calloni et al., 2007; 2009).

Na CN truncal também foram descritos progenitores multipotentes para os 4 fenótipos encontrados a partir da CN truncal em cultura, melanócitos (M), miofibroblastos (F), e células gliais (G) e neurônios (N) do sistema nervoso periférico (Trentin et al., 2004; Calloni et al., 2007). Recentemente, trabalhos do nosso grupo mostraram que a CN de codorna, um vertebrado amniota, guarda algum potencial de diferenciação mesenquimal, mesmo se in vivo não contribuem com este tipo celular mesenquimal (Calloni et al., 2007). Estudos clonais mostraram que a CN truncal é capaz de se diferenciar em condrócitos, embora progenitores com esta capacidade sejam muito raros (em torno de 1 em 200) quando comparamos à CN cefálica (**Figura 7B**).

Os métodos de cultura *in vitro* da CN de aves foram adaptados para as células de mamíferos, e progenitores multipotentes também puderam ser identificados na CN truncal de embriões de ratos e camundongos (Ito and Sieber-Blum, 1993; Shah et al., 1996; Shah et al., 1994; Stemple and Anderson, 1992). As células da CN truncal de ratos isoladas com base na expressão do receptor de baixa afinidade de neurotrofinas p75, deram origem a progênie clonal com células gliais, neurônios autônomos e miofibroblastos. Estes precursores foram capazes de passar por sucessivos experimentos de clonagem, sempre repetindo o repertório de fenótipos encontrados em sua progênie, o que mostra a capacidade de auto-renovação destas células (Stemple and Anderson, 1992).

Em codorna, Trentin e colaboradores mostraram que alguns progenitores bipotentes da CN mesencefálica e truncal de aves também são dotados de propriedades de auto-renovação. Assim, progenitores glia-melanócitos (GM) e glia-

miofibroblastos (GF) se comportam como células-tronco que são capazes de se auto-renovar e também de gerar uma descendência restrita (Trentin et al., 2004) (**Figura 7B**).

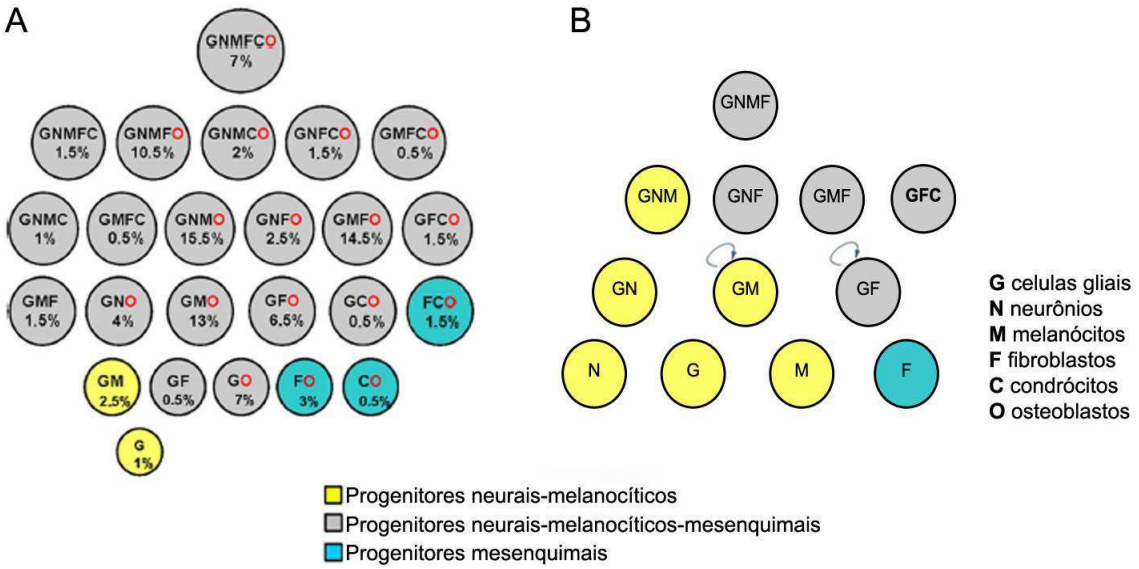

Figura 7 - **Hierarquia dos progenitores da CN identificados por análises clonais *in vitro* em embriões de codorna. Os progenitores são ordenados de acordo com o número de potenciais de desenvolvimento**. **(A)** Os diferentes tipos de células derivadas da CN cefálica surgem a partir de uma célula-tronco altamente multipotente (GNMFCO), que passa por progressivas restrições no seu potencial para gerar progenitores intermediários e células comprometidas (Calloni et al., 2009). **(B)** Na CN truncal a maior parte das células dá origem à glia, neurônios e melanocitos, e clones com potencial condrogênico são muitos raros. No entanto, vários progenitores miofibroblásticos estão presentes. Entretanto, muitos progenitores de miofibroblastos estão presentes, incluindo progenitores multipotentes GNMF. A auto-renovação (setas) foi demonstrada para alguns destes progenitores (Trentin et al., 2004; Calloni et al., 2007).

O potencial de diferenciação da CN cardíaca também foi investigado em codorna e camundongo. Esta região da CN cefálica é essencial para a formação do septo do coração. Nas colônias derivadas das células migratórias iniciais da CN cardíaca de codorna, foram identificados melanócitos, miofibroblastos, condrócitos e

neurônios sensoriais (Ito and Sieber-Blum, 1991). Resultados semelhantes foram obtidos com a CN cardíaca de camundongos (Youn et al., 2003).

Apesar de alguns fenótipos mesenquimais já terem sido reportados na CN truncal, como miofibroblastos e condrócitos, continuam desconhecidas as potencialidades dos progenitores da CN truncal capazes de originar outros tipos de fenótipos mesenquimais, como osteoblastos e adipócitos. Este trabalho tem especial interesse na investigação das capacidades osteogênica e adipogênica das células da CN truncal.

1.2.3. A manutenção da multipotência das células da CN

A CN é uma estrutura multipotente. Considerando progenitores individuais, alguns de seus progenitores já tiveram sua multipotencialidade e seu potencial de auto-renovação mostrado através de experimentos de clonagem *in vitro*. Células da CN de diferentes regiões do eixo ântero-posterior mostram plasticidade celular *in vivo*, se diferenciando em outros tipos celulares quando desafiadas com novas condições ambientais em experimentos de transplante. Além disso, células multipotentes derivadas da CN foram encontradas em diferentes regiões pós-migratórias da CN, inclusive em alguns tecidos adultos (Dupin and Sommer, 2012). Estes progenitores adultos são multipotentes *in vitro* e compartilham pelo menos alguns marcadores e propriedades de diferenciação com progenitores iniciais da CN.

Esta temática foi abordada em detalhes na revisão recente "Isolation and differentiation properties of neural crest stem cells" escrita por Dupin e Coelho-Aguiar, aceita pela revista *Cytometry A* e em fase de impressão (Anexo1 – pagina 213).

É de grande importância para as células-tronco que elas continuem indiferenciadas e mantenham o potencial de diferenciação. Alguns genes têm grande importância na manutenção e sobrevivência destas células. Nos precursores multipotentes da CN de *Xenopus*, observou-se que o gene *c-Myc* atua no controle do ciclo celular e controla a decisão entre proliferação e apoptose através de *Id3* (Light et al., 2005). *Sox9* também regula o ciclo celular e promove a sobrevivência das células pré-migratórias da CN truncal. Células da CN de camundongo nocaute para o gene *Sox9* sofrem apoptose devido à diminuição do fator anti-apoptótico *Snail1* (Cheung et al., 2005).

Os genes *FoxD3* e *Sox10* estão diretamente implicados na habilidade de auto-renovação e na manutenção do caráter multipotente da CN. A expressão de *Sox10* diminui após a diferenciação das células da CN, exceto na gliogênese, onde atua regulando diretamente a expressão de genes envolvidos neste processo. Além disso, foi mostrado que Sox10 tem um papel na manutenção do potencial neurogênico e na inibição da diferenciação neuronal, tanto *in vivo* quanto *in vitro* (Kim et al., 2003). *FoxD3* também é importante para a manutenção das células da CN. Na ausência deste fator de transcrição as células da CN não são mantidas, e o camundongo nocaute de *FoxD3* condicionado às células da CN morre por volta do nascimento com grande redução ou perda de estruturas derivadas da CN, como os derivados mesenquimais da CN cefálica, gânglios cranianos, os gânglios da raiz dorsal e a perda da CN no trato gastrointestinal (Teng et al., 2008). Assim, *Sox10* e *FoxD3* são extremamente importantes para a manutenção do estado indiferenciado e multipotente da CN.

As vias de sinalização de Wnt e BMP, além de atuarem no processo de indução da borda da PN, estão envolvidas também nesta fase de proliferação e auto-renovação das células migratórias da CN. Embora Wnt1 atue instruindo as células pré-migratórias da CN para o destino sensorial e BMP promova neurogênese autônoma, a combinação destas duas vias de sinalização mantém a expressão dos marcadores p75 e Sox10 implicados na multipotência, e impede a diferenciação celular (Kleber et al., 2005). As células multipotentes da CN mudam sua responsividade a estes fatores de crescimento após alcançarem seus alvos, e as células que se mantêm indiferenciadas e proliferativas adquirem a capacidade de responder ao fator mitogênico EGF (Fuchs et al., 2009). Assim, o desenvolvimento das células multipotentes da CN é regulado pela combinação de fatores de crescimento que refletem na atividade de sinais intracelulares específicos que estão em constante mudança.

1.3. A evolução da CN e de seus derivados esqueléticos

A CN é uma estrutura específica dos embriões dos vertebrados, e sua evolução está intimamente ligada à evolução deste grupo. Devido ao seu grande potencial de diferenciação e seus múltiplos derivados, foi proposto que a CN pode ser considerada uma quarta camada germinativa, já que a aparição desta estrutura foi a base para a evolução de tecidos e órgãos únicos dos vertebrados (Hall, 2000). A CN produz uma diversidade de células e tecidos ainda maior do que o mesoderme, incluindo células neurais, esqueléticas, tecido conjuntivo, células cardíacas, dentais, endócrinas e células pigmentadas da pele.

Em 1983, Northcutt e Gans elaboraram a hipótese da “nova cabeça”, que propõe que as novas estruturas derivadas do neuroectoderma, a CN e os placóides, permitiram o aparecimento da cabeça rostral dos vertebrados, que inclui órgãos sensoriais, o prosencéfalo, a calota craniana, o condrocrânio pré-cordal e a região mandibular e maxilar da face (Gans and Northcutt, 1983). Além disso, o aparecimento da CN e dos placóides foi determinante para a especialização do modo de vida predatório, quando os animais passaram de um modo de nutrição filtrante, presente nos cordados invertebrados, para uma alimentação por sucção devido ao aparecimento também de uma faringe muscularizada. Os novos derivados da CN e dos placóides ajudaram na adaptação destes animais a um estilo de vida predatório e móvel. De fato, foi mostrado mais tarde que o crânio pré-cordal e pelo menos uma grande parte do neurocrânio são de origem da CN (Couly et al., 1992; Couly et al., 1993). A evolução do crânio a partir da CN também foi importante para o desenvolvimento de órgãos sensoriais e o aumento do volume cerebral. Além disso, a CN tem grande influência na morfogênese do TN durante a formação do cérebro (Creuzet et al., 2006).

Devido à sua grande importância na evolução dos vertebrados, muitos estudos são realizados para compreender a origem das células da CN. Os principais modelos de estudo da evolução da CN são as lampréias (vertebrados ciclóstomos), e as ascídias (tunicados) e anfioxos (cefalocordados) [cordados invertebrados próximos filogeneticamente dos vertebrados] (**Figura 8**). Experimentos de análise do genoma de anfioxos mostraram que estes animais possuem genes homólogos da maioria dos genes envolvidos na indução da CN (Yu, 2010). Com base nestes dados, a explicação mais “parcimoniosa” para a evolução da CN diz que estas

células se desenvolveram a partir da modificação da rede gênica neuroepitelial pré-existente através da aquisição de genes já presentes no ancestral comum à todos os cordados (Baker, 2008). Entretanto, muitas moléculas associadas com a especificação e diferenciação dos derivados da CN só existem nos vertebrados. Desta forma, a hipótese atual divide a evolução da CN em 2 eventos: (1) a montagem da rede de regulação gênica inicial da CN, e (2) a diversificação dos derivados da CN durante a evolução dos vertebrados (Yu, 2010).

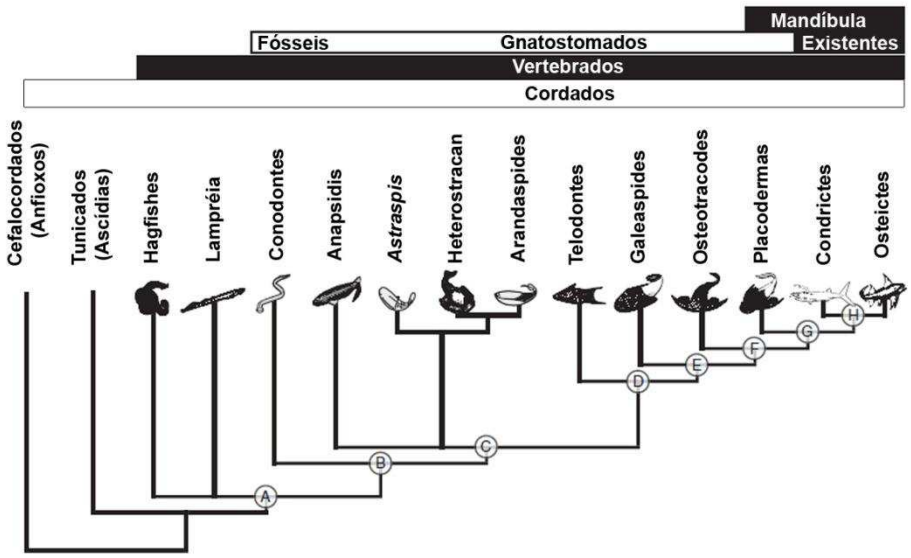

Figura 8 - **Arvore filogenética dos cordados.** Filogenia atual, mostrando que tunicados (que inclui ascídias) formam o grupo irmão dos vertebrados, enquanto cefalocordados (anfioxo) são os cordados mais basais. As letras indicam passos importantes na evolução do esqueleto dos vertebrados. (A) Origem de um esqueleto nos vertebrados, incluindo notocorda, raios nas nadadeiras e crânio, embora inteiramente composto por cartilagem não-mineralizada. (B) Origem do esqueleto mineralizado, dentina e esmalte que compreendem os odontódeos. (C) Origem de um de um esqueleto dermal mineralizado composto de odontódeos suportados por osso, impondo mineralização sobre as camadas colagenosas da derme. (D) Odontódeos associados com o viscerocrânio, incluindo que os arcos brânquia ou as aberturas nasais. (E) Origem de um neurocrânio mineralizado composto de cartilagem calcificada. (F) Neurocrânio mineralizado; osso intramembranoso celular também encontrado pela primeira vez no esqueleto dermal. (G) Viscerocrânio, o esqueleto axial, esqueleto apendicular e raios das nadadeiras mineralizados. (H) Osso endocondral. (modificado de Donoghue et al., 2006 e Baker, 2008).

A comparação da expressão de genes homólogos aos atuantes na indução da CN entre cordados invertebrados e vertebrados mostrou que as bordas das PNs de ascídias e anfioxos apresentam a expressão de genes especificadores da borda da PN, como *Dlx, Msx, Zic, Pax3* e *Pax7*. Entretanto, os genes especificadores da CN não são expressos em seguida aos especificadores da borda da PN. Estes genes são expressos no mesoderma e/ou endoderma, e o único que também é induzido na borda da PN, é o gene *Snail*, que provavelmente atua na sobrevivência e na mudança de forma das células (Langeland et al., 1998). O gene *FoxD* de anfioxo, por exemplo, esta expresso no mesoderma destes animais e sua região regulatória quando eletroporada em galinha induz a expressão do gene repórter associado à ele no mesoderma, mas não nas células da CN (Yu et al., 2008). Assim, o recrutamento de genes mesendodermais para a borda da PN foi um passo importante para a montagem completa da rede gênica de indução da CN. A aquisição dos genes especificadores da CN deve ter conferido novas propriedades às células neuroepiteliais dorsais, como multipotência, delaminação e migração.

Recentemente, células semelhantes à CN, capazes de migrar e se diferenciar em células pigmentadas, foram descritas em diferentes espécies de ascídias, sugerindo que o aparecimento da CN pode ter ocorrido antes da evolução dos vertebrados (Jeffery et al., 2004). Todavia, na espécie *Ciona intestinalis* estas células não são derivadas da borda da PN, e sim de uma região próxima, derivada dos blastômeros A7.6, que não expressa os genes especificadores da borda da PN (Jeffery et al., 2008). Por outro lado, estas células A7.6 e seus blastômeros precursores A6.3, que geram também células mesodérmicas e endodérmicas,

expressam os genes especificadores e efetores da CN. Embora a homologia entre estas células semelhantes à CN dos tunicados e as células da CN dos vertebrados ainda esteja sob discussão, estes dados mostram que células que reúnem os genes especificadores e efetores da CN surgiram evolutivamente antes das ascídias e após os anfioxos. Entretanto, a aquisição destes pelas células da borda da PN ocorreu somente nos vertebrados.

Após a evolução das células da CN à partir de células neuroepiteliais e sua aparição nos vertebrados, muitas mudanças ocorreram para que pudessem dar origem à tantos tipos celulares diferentes. De fato, a evolução dos derivados da CN ocorreu paralelamente à evolução dos vertebrados. Os vertebrados mais primitivos, as lampréias e “hagfishes”, não possuem alguns derivados da CN, como gânglios simpáticos, dentina e ossos, por exemplo. Entretanto, lampréias e “hagfishes”, já apresentam derivados mesenquimais (cartilagens cranial e faringeal) de origem endodérmica. De fato, *SoxE* e *colágeno-A* (semelhante ao colágeno2a1 dos vertebrados amniotas) estão expressos na faringe endodermal de anfioxos e hemicordados, sugerindo que um esqueleto cartilaginoso dependente de soxE, apareceu pelo menos junto com os deuterostomos.

Apesar de a hipótese atual para evolução da CN esquelética apontar para uma aquisição de rede gênica pré-existentes em células endodermais e/ou mesodermais, a expressão de genes específicos de cartilagem está presente também em estruturas ectodermais (Baker, 2008). As células pré-migratórias e migratórias da CN, bem como seus derivados gliais, expressam *Sox9*, *Sox5* e *Sox6*, que atuam em cooperação na ativação de *colágeno2a1* e *aggrecan*. Além disso, proteínas específicas de cartilagem, como colágeno2a1 e aggrecan são expressas

também no cérebro e na medula espinhal em desenvolvimento. Em anfioxos, *SoxE* e *colágenoA* também são expressos ao longo do TN em desenvolvimento. Desta forma, é possível que a evolução da cartilagem derivada da CN tenha ocorrido por modificações de uma rede gênica neuroepitelial pré-existente, com posterior aquisição desta rede gênica pelo mesoderma. Neste caso, a cartilagem acelular, derivada da endoderme faríngea de hemicordados e anfioxos, poderia representar uma co-opção independente da RRG ectodérmica primitiva (Baker, 2008).

Estudos dos fósseis evidenciaram uma rápida emergência de outros fenótipos ectomesenquimais após a divergência filogenética da lampréia e "hagfish". A primeira dentina, estrutura mineralizada secretada pelos odontoblastos, é encontrada nos vertebrados fósseis conodontes, nos odontodeos. E o primeiro osso apareceu em anaspides para conexão da dentina à armadura dermal (Baker, 2008). Assim estes estudos com fósseis mostraram que os primeiros componentes esqueléticos mineralizados dos vertebrados, os odontódeos dos conodontes, são de origem da CN. Dentina e esmalte são considerados marcadores da origem a partir da CN (Sansom et al., 1992). Vertebrados fósseis agnatos do período inicial do cambriano possuem uma armadura dermal ossificada com uma camada de material semelhante à dentina. Dentre os vertebrados fosseis conhecidos, arandaspids são os que primeiro apresentaram este tipo de esqueleto mineralizado, mas eles possuem apenas o exoesqueleto de osso intramenbranoso e seu endoesqueleto ainda é cartilaginoso (Sansom et al., 2005; Smith, 1991). Estes primeiros vertebrados com esqueleto mineralizado são chamados de ostracodermes. A maior parte dos ostracodermes, como arandaspids, não possuem endoesqueleto

calcificado. Entretanto, dois grupos de ostracodermes, os galeaspids e osteostracans possuem um endoesqueleto calcificado (Janvier, 2008).

Nos vertebrados amniotas, o esqueleto derivado da CN é reduzido ao crânio e aos ossos e cartilagens da face, enquanto nos peixes teleósteos, como o peixe-zebra, a capacidade do mesênquima derivado da CN de produzir tecido calcificado é conservada, e a CN truncal está na origem dos raios ósseos das nadadeiras dorsal e caudal (Smith et al., 1994; Mari-Beffa et al., 2010). A perda da armadura dermal mineralizada durante a evolução dos vertebrados coincide com a aparição do esclerotómo somítico que dá origem à coluna vertebral. Desta forma, pouco se sabe sobre o potencial mesenquimal da CN truncal dos vertebrados amniotas. Alguns trabalhos apontam para um potencial restrito, que possivelmente foi guardado na CN dos vertebrados superiores. Este trabalho de tese se interessa em estabelecer novos métodos de cultivo para revelar este potencial e permitir o avanço no entendimento da evolução dos tipos celulares derivados da CN.

1.4. Diferenciação do esqueleto e dos adipócitos

Nos vertebrados superiores, os derivados embrionários mesenquimais se diferenciam a partir da CNC, que dá origem à maior parte do mesênquima da cabeça, e do mesoderma, que forma todos os demais tecidos mesenquimais. Dentre os tecidos de origem mesenquimal são encontrados derme, tendões, tecido muscular, os elementos do esqueleto, com ossos e cartilagens, bem como o tecido adiposo (Le Douarin et al., 2004).

Neste capítulo serão apresentados os diferentes mecanismos de diferenciação e formação do esqueleto, bem como a diferenciação dos adipócitos;

para que possamos compreender como uma célula mesenquimal indiferenciada se torna comprometida com estes destinos celulares. Em seguida, serão abordados os mecanismos que dirigem o processo de diferenciação mesenquimal a partir da CN.

1.4.1. Ossificação Endocondral e Intramembranosa

1.4.1.1. As etapas da diferenciação óssea

No organismo adulo, o funcionamento adequado dos ossos é garantido por uma renovação contínua do tecido, chamada de remodelamento. O osteoclasto,é um tipo celular de origem hematopoiética monocítica, responsável pelo processo de reabsorção óssea, enquanto os osteoblastos reconstroem o osso reabsorvido pela elaboração de matriz extracelular que posteriormente se torna mineralizada (Karsenty and Wagner, 2002).

Os esqueletos apendicular (esqueleto dos membros) e axial (vertebras e costelas) são originários da placa lateral da mesoderme e do mesoderma paraxial, respectivamente. O crânio dos vertebrados consiste do neurocrânio (a base e calota craniana) e do viscerocrânio (mandíbula, maxilar e os outros derivados dos arcos branquiais), e é formado pelo mesênquima da cabeça derivado de duas fontes embrionárias destintas: o mesoderma e a CN.

A formação dos ossos pode ocorrer por dois mecanismos, a **ossificação endocondral**, que ocorre nos ossos longos dos membros, parte basal do crânio, vértebras, costelas e parte medial das clavículas (**Figuras 9F-9J**); e a **ossificação intramembranosa**, que acontece em vários ossos crâniofaciais e na parte lateral da clavícula (**Figuras 9A-9E**).

O processo de ossificação endocondral ocorre pela formação de um esqueleto cartilaginoso que posteriormente é resposto por osso pelos osteoblastos. A condrogênese tem início quando parte das células mesenquimais condensadas (**Figura 9F**) começam a expressar o fator de transcrição *Sox9* [SRY (sex determining region Y)-box 9] (Akiyama *et al*., 2002), que induz a expressão de *colágeno2a1* e do proteoglicano *aggrecan* (Lefebvre et al., 1997), e consequentemente induz a diferenciação em condrócitos proliferativos (**Figura 9G**). Pouco depois os condrócitos mais centrais param de proliferar, se tornam pré-hipertróficos, param a produção de colágeno2a1 e começam a produzir colágeno10. Ao se tornarem hipertróficos (**Figura 9H**), passam à expressar *osteopontina*, *bonesialoproteinaII* e *MMP13* (matriz metalopeptidase13) (Inada et al., 1999). Subsequentemente entram no processo de morte celular e favorecem a invasão de vasos sanguíneos, que trazem osteoblastos para o centro do molde de cartilagem (**Figura 9I**). Estes osteoblastos produzem uma matriz extracelular rica em colágeno1a1 sobre a matriz rica em colágeno10. Este processo de ossificação ocorre de forma centrífuga, e os condrócitos adjacentes à região de ossificação continuam proliferando e seguindo este processo de diferenciação. Dessa forma, condrócitos proliferativos e hipertróficos se organizam em colunas numa estrutura avascular chamada placa de crescimento, que é encontrada nas extremidades dos ossos em desenvolvimento e é responsável pela formação dos ossos longos (Karsenty and Wagner, 2002; (Kronenberg, 2003). Além disso, os condrócitos são necessários também nas articulações para permitir a mobilidade dos vários elementos do esqueleto.

Os osteoblastos, no caso da ossificação endocondral, se diferenciam a partir de osteoprogenitores presentes na região do pericôndrio, que se encontram ao redor da zona dos condrócitos proliferativos (**Figura 9I**). Estes osteoprogenitores

expressam *Runx2* (runt-related transcription factor 2), o fator de transcrição determinante da diferenciação dos osteoblastos, que induz a expressão de colágeno1a1 após a chegada destas células no centro do molde de cartilagem (Ducy et al., 1997). Na ossificação intramenbranosa (**Figuras 9A-9E**), os osteoblastos se diferenciam diretamente a partir do mesênquima condensado (Karsenty et al., 2009). Neste processo as células mesenquimais condensadas expressam *Runx2*, independentemente da presença do nódulo de cartilagem, e este induz colágeno1a1. Células mesenquimais imaturas e pré-osteoblastos tem uma fraca expressão de colageno1a1, que é aumentada em osteoblastos imaturos (Inada et al., 1999). Em estágios iniciais da diferenciação os osteoblastos apresentam também os marcadores osteopontina, osteonectina e bonesialoproteína (BSP). O estágio mais tardio é marcado pela expressão de fosfatase alcalina, osteocalcina e mineralização da matriz extracelular (Nakashima and de Crombrugghe, 2003).

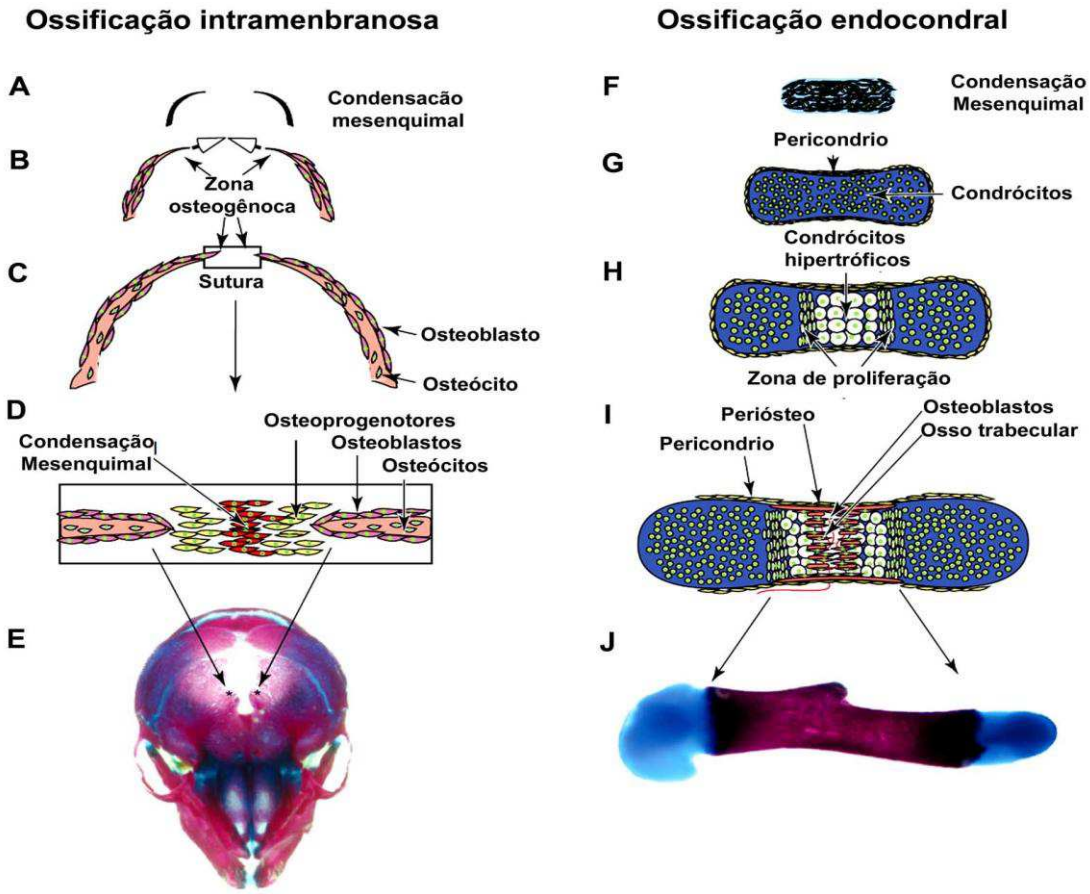

Figura 9 - **Ossificação intramembranosa e ossificação endocondral. (A)** A formação de ossos frontais começa em condensações mesenquimais na parte lateral da cabeça. **(B)** A partir desta condensação, a massa celular com atividade osteogênica se espalha para cima em direção ao topo do crânio (setas) e produz matriz óssea. **(C)** Posteriormente, as extremidades das massas de cada lado se encontram na linha média, onde a sutura é formada. **(D)** Na sutura, ocorre a diferenciação em células osteoprogenitoras, e depois em osteoblastos, que produzem moléculas de matriz óssea. Osteoblastos se diferenciam em osteócitos, que se enraízam na matriz óssea. **(E)** Visão frontal de um crânio de camundongo (E18.5) corado com alizarina red, e alcian blue. As setas apontam para frentes osteogênicas dos ossos frontais. **(F)** No inicio da formação dos ossos longos por ossificação endocondral ocorrem condensações mesenquimais. **(G)** Estas células dão origem a condrócitos, enquanto as células na periferia formam o pericôndrio. **(H)** Os condrócitos proliferativos saem do ciclo celular e se diferenciar em condrócitos hipertróficos. A diferenciação seqüencial dos condrócitos estabelece a placa de crescimento epifisária. **(I)** Na extremidade distal da placa de crescimento epifisária, condrócitos hipertróficos mineralizam sua própria matriz cartilaginosa. Em paralelo, as células do periósteo, que entornam a matriz da cartilagem, invadem a zona de condrócitos hipertróficos, juntamente com vasos sanguíneos e osteoclastos. A matriz cartilaginosa serve de modelo para a deposição da matriz óssea pelos osteoblastos. O osso endocondral contém um envelope exterior, o osso cortical, que circunda a cavidade medular e a placa de crescimento. O osso cortical é contínuo com o pericôndrio. **(J)** Úmero de camundongo corado com alizarin red e alcian blue (Nakashima and Crombrugghe, 2003).

1.4.1.2. Controle molecular da osteogênese

Dentre os principais fatores de transcrição que atuam no processo de ossificação encontramos *Sox9* e *Runx2*. *Sox9* é um fator de transcrição que possui um domínio HMG-box (grupo de alta mobilidade) de ligação ao DNA do grupo de alta-mobilidade, e atua na ativação de genes específicos de condrócitos, como *colágeno2a1*, *colágeno11a2* e *aggrecan* (Bridgewater et al., 1998; Lefebvre et al., 1997). *Sox9* esta expresso em todos os condroprogenitores e condrócitos, exceto nos condrócitos hipertróficos. A mutação de *Sox9* em humanos heterozigotos causa a displasia campomélica, caracterizada pela hipoplasia geral dos ossos endocondrais (Wagner et al., 1994). A inativação de *Sox9* nos brotos dos membros resulta na ausência de condensação mesenquimal e consequentemente da formação de cartilagem e osso. Sua atuação leva à formação do molde cartilaginoso, onde atua juntamente com *Sox5* e *Sox6*, na produção da matriz cartilaginosa (Akiyama et al., 2002). Assim, *Sox9* é necessário antes e após a condensação mesenquimal (Akiyama et al., 2005). *Runx2* é o primeiro fator específico dos osteoblastos a ser identificado (Ducy et al., 1997). Runx2 pertence à família Runt de fatores de transcrição, e é através do domínio Runt que se liga ao DNA e à muitas proteínas nucleares.

Runx2 é um gene determinante na diferenciação dos osteoblastos, como se nota com o camundongo nocaute para *Runx2*, que não apresenta osteoblastos em todos os elementos do esqueleto (Komori et al., 1997). Este fator de transcrição dirige as células mesenquimais multipotentes para a linhagem osteoblástica ativando a expressão de muitos promotores, incluindo os dos genes *colágeno1a1, osteopontina, osteocalcina* e *Mmp13*. *Runx2* mantém os osteoblastos num estágio

imaturo, já que sua superexpressão inibe a maturação dos osteoblastos e reduz a expressão dos genes de *colágeno1a1* e *osteocalcina*. Além disso, este fator de transcrição, juntamente com *Runx3*, é necessário para a maturação dos condrócitos e regula a expressão dos genes de *colágeno10, ostepontina, bone sialoproteina2* e *Mmp13* nos condrócitos hipertróficos (Komori, 2010). Estudos com o camundongo Sox9-Cre;ROSA26, que expressa o gene da *βgalactosidade* a partir do promotor de *Sox9*, revelaram que a expressão de *Sox9* antecede a do fator de transcrição *Runx2* durante a esqueletogênese. As células mesenquimais *Sox9*-positivas do broto do membro dão origem aos condrócitos e aos osteoblastos. Além disso, a ausência da marcação com alizarin red de elementos esqueléticos membranosos em camundongos transgênicos que tem o gene *osterix* (um fator de transcrição necessário para a diferenciação dos osteoblastos) inativado em células que expressam *Sox9* indica que células *Sox9*-positivas são as progenitoras dos osteoblastos também em ossos intramembranosos (Akiyama et al., 2005). *Runx2* é regulado por alguns genes, mas também por proteínas que interagem com ele. Dentre os genes reguladores de *Runx2* encontramos *Sox8, Dlx5,* o gene homeobox *Msx2 (*msh-like 2*)* e o gene homeobox *Bapx1. Sox8* regula *Runx2* negativamente e atenua a osteoblastogênese (Schmidt et al., 2005), enquanto *Dlx5* e *Bapx1* são reguladores positivos (Tribioli and Lufkin, 1999)Lee et al., 2005; Holleville et al., 2007). *Msx2* promove a proliferação e a diferenciação dos osteoblastos. Contudo, em osteoblastos mais maduros *in vitro*, ele inibe a atividade de *Runx2* (Newberry et al., 1998; Shirakabe et al., 2001).

As proteínas reguladoras de Runx2 compreendem Twist1/2, que se ligam à Runx2 e impedem seu acesso ao DNA, inibindo assim a diferenciação precoce dos osteoblastos (Twist1 no crânio e Twist2 no esqueleto apendicular) (Bialek et al.,

2004); e SATB2 (proteína ligadora da sequencia especial rica em AT 2), que interage com ele aumentando sua atividade (Dobreva et al., 2006).

Apesar da expressão de *Runx2* ser essencial e indispensável para a osteogênese, osteoprogenitores positivos para este fator de transcrição ainda não são totalmente comprometidos a se tornarem osteoblastos, para isso é necessário o fator de transcrição Osterix. Ele é considerado o segundo fator de transcrição específico dos osteoblastos, e pertence à família Sp das proteínas dedo-de-zinco tipo kruppel. Está presente em todos os progenitores de osteoblastos e é necessário para que progenitores se tornem comprometidos com a linhagem osteoblástica, e assim assegura a diferenciação em osteoblastos ao invés de condrócitos (Nakashima et al., 2002). *Osterix* é conhecidamente ativado por Runx2, porém um trabalho recente demonstrou que também pode ser ativado à jusante de *Msx2* em células mesenquimais deficientes para *Runx2* (Matsubara et al., 2008). Desta forma, Osterix pode ser regulado por mecanismos dependentes e independentes de Runx2.

Essa ativação sequencial de fatores de transcrição durante a formação dos ossos é guiada e induzida por diferentes vias de sinalização que atuam nas diversas etapas do processo de ossificação. O processo de diferenciação dos osteoblastos envolve as vias de Ihh, FGF, Notch, Wnt e BMP. Na ossificação endocondral, a maturação dos condrócitos e a diferenciação dos osteoblastos são interligados pela sinalização Ihh, que induz a expressão de *Runx2* e a diferenciação dos osteoblastos nas células pericondriais adjacentes (Yoshida et al., 2004). O fator de transcrição *Runx2* induz *FGF18*, que, além de atuar no desenvolvimento dos condrócitos, age na diferenciação dos osteoprogenitores do pericôndrio (Liu et al., 2002). Wnt previne a diferenciação de osteoprogenitores em condrócitos, e assim permite que sigam o processo de diferenciação óssea (Day et al., 2005; Hill et al., 2005). No

desenvolvimento do membro, Wnt também atua inibindo a condrogênese, e a via de FGF é essencial para o crescimento continuo do broto do membro já que promove a sobrevivência das células mesenquimais. Além disso, a combinação de Wnt e FGF tem um efeito diferente. Juntos, eles dirigem a proliferação das células mesenquimais do broto do membro e as mantém indiferenciadas com habilidade de diferenciação condrogênica (ten Berge et al., 2008). A via de Notch também tem uma atuação na osteogênese. O efetor da via, o domínio intracelular de Notch (NICD), vai ao núcleo e se liga à Runx2 inibindo sua atuação e a diferenciação dos osteoblastos (Engin et al., 2008; Hilton et al., 2008). As proteínas BMP são conhecidas por induzir a diferenciação osteoblástica de células pluripotentes mesenquimais, como células do estroma da medula óssea, culturas primárias de células da sutura craniana e linhagens celulares mesenquimais (Franceschi, 1999). Além disso, Hollevile e colaboradores mostraram que o gene *Dlx5 (*Distal-less homeobox 5) induzido por BMP2 nas células das bordas osteogênicas da sutura craniana derivadas da CN, leva a expressão de *Runx2*, e assim dá início a osteogênese (Holleville et al., 2007; Holleville et al., 2003). Também já foi mostrado que a expressão de *Dlx5* induzida por BMP2 induz diretamente *osterix*. Na morfogênese da mandíbula BMP4 do mesênquima derivado da CN dita o início da osteogênese (Merrill et al., 2008). O processo de ossificação de ossificação das células da CN será abordado em mais detalhes na seção 1.4.3.

1.4.1.3. Mineralização da matriz óssea

Durante o processo de formação óssea, condrócitos e osteoblastos mineralizam suas matrizes extracelulares. Este processo tem inicio com a formação

inicial de hidroxiapatita cristalina no interior das vesículas de matriz (Anderson, 2003). As vesículas de matriz germinam a partir de regiões específicas da membrana plasmática. A formação dos primeiros cristais minerais é aumentada pela atividade das fosfatases das vesículas de matriz encontradas dentro ou perto da membrana das vesículas, como fosfatase alcalina (ALP), trifosfatase adenosina e pirofosfatase, e de moléculas quelantes de cálcio. A próxima etapa é a secreção dos cristais de hidroxiapatita pré-formados para fora das vesículas. O fluido extracelular normalmente contém íons cálcio e fosfato, suficientes para suportar a continua produção de cristal, com os cristais pré-formados servindo de moldes para a formação de novos cristais por um processo de nucleação homóloga (Anderson, 2003). Este processo de mineralização depende do equilíbrio de uma série de fatores, como as concentrações de cálcio e fosfato inorgânico (Pi), a presença de proteínas da matriz, e a presença de inibidores de mineralização, como pirofosfato inorgânico (PPi), osteopontina e osteocalcina. No osso, ALP está confinada à superfície celular de osteoblastos e condrócitos, incluindo nas membranas das vesiculas de matriz. ALP gera Pi para a cristalização de hidroxiapatita e também hidrolisa PPi para facilitar a precipitação e crescimento do mineral (Fallon et al., 1980; Rezende et al., 1994).

Para observarmos o processo de mineralização de condrócitos e osteoblastos, precisamos fornecer as condições necessárias. Acido ascórbico (AA), β-glicerolfosfato (βGP) e dexametasona (Dex), são componentes conhecidos por favorecer a expressão do fenótipo osteoblástico em vários sistemas de cultura de células ósseas (Coelho and Fernandes, 2000). βGP induz a mineralização pois é hidrolisado por ALP para produzir níveis altos de íons fosfato fornecendo as condições químicas necessárias para a deposição de mineral (Bellows et al., 1991).

A presença de dexametasona resulta em aumento da proliferação e aumento da expressão de ALP (Coelho and Fernandes, 1999). Em células da linhagem osteoblástica ROS17/2.8, dexametasona induz a mineralização. Nestes experimentos, dexametasona aumentou a atividade transcricional de *Runx2*, o que induz um aumento da expressão dos marcadores osteoblásticos tardios, osteopontina e osteocalcina (Mikami et al., 2007). A presença de AA induz a síntese de colágeno. Dados sugerem que este aumento da acumulação da matriz extracelular leva a uma maior atividade de ALP e à habilidade de formar matriz mineralizada (Franceschi, 1992).

O processo de diferenciação óssea até sua etapa de mineralização será investigado nesta tese em cultura de células da CN truncal.

1.4.2. Adipogênese

Os adipócitos armazenam e mobilizam lipídios em resposta a vários hormônios. O tecido adiposo pode ser classificado em dois tipos, o branco, cuja principal função é o estoque energético de gordura, e o marrom, que queima a gordura para produzir calor. Além disso, os adipócitos tem também um papel muito importante devido à suas propriedades autócrina, parácrina e endócrina, tais como a secreção de leptina e adiponectina (Guerra et al., 1998; (Arner et al., 2011).

Os adipócitos são originados a partir de progenitores mesenquimais, que por mecanismos ainda pouco conhecidos se diferenciam em pré-adipócitos, e posteriormente em adipócitos maduros capazes de armazenar lipídeos. Este processo é denominado adipogênese e ocorre durante o desenvolvimento embrionário tardio e também no período pós-natal. Na idade adulta

aproximadamente 10% dos adipócitos são renovados à cada ano (Rosen and MacDougald, 2006; Spaldin *et al*., 2008).

No tronco e nos membros o mesênquima é de origem mesodérmica, logo, supõe-se que os tecidos adiposos que se desenvolvem nesta região são derivados do mesoderma, ainda que poucas regiões de tecido adiposo tenham tido seus progenitores identificados durante o desenvolvimento (Billon *et al*., 2007). Experimentos de transplantes de TN realizados em embriões de aves indicaram que as células da CN podem ser a origem dos adipócitos em algumas áreas da face e do pescoço (Le Lièvre e Le Douarin, 1975), o que foi confirmado em camundongos para os depósitos de adipócitos da glândula salivar e do ouvido (Billon et al., 2007).

O comprometimento das células mesenquimais à linhagem adipocítica é muito pouco estudada, já que a maior parte dos estudos *in vitro* foi feita com o uso de linhagens celulares de pré-adipócitos. A fator Pref-1 (pré-adipocítico 1) (ou DLK1, delta-like 1) é tido como o primeiro marcador de pré-adipócitos. A expressão desta proteína transmembrana deve diminuir durante o processo de diferenciação adipocítica e está ausente em adipócitos maduros. De fato, a expressão constitutiva de Pref-1 inibe a diferenciação dos adipócitos, e a ausência deste aumenta esta diferenciação *in vitro* (Smas e Sul, 1993; Smas et al, 1999).

A diferenciação dos pré-adipócitos em adipócitos tem sido extensivamente estudada *in vitro* (Otto and Lane, 2005; Rosen and MacDougald, 2006), devido principalmente ao estabelecimento de linhagens celulares de pré-adipócitos obtidas a partir da dissociação de embriões de camundongos ou do tecido adiposo adulto (Green and Kehinde, 1975, 1976; Green and Meuth, 1974; Negrel et al., 1978). Esta diferenciação é dirigida pela expressão seqüencial de um conjunto de fatores de

transcrição (**Figura 10**). O acúmulo de cAMP (adenosina monofosfato cíclica) nos pré-adipócitos leva ao aumento de CREB (elemento ligador responsivo à cAMP) fosforilada. Esta, por sua vez, ativa a transcrição de *C/EBPβ* (elemento ligador ao *enhancer* CCAAT). C/EBPβ fosforilada induz a expressão de *KLF5* (fator tipo Kruppel 5), e ambos promovem a expressão de dois fatores de transcrição adipogênicos chave, *C/EBPα e PPARγ2* (receptor ativado proliferador do peroxisoma). Além disso, estes dois últimos estimulam reciprocamente um ao outro. C/EBPα e PPARγ2, junto com SREBP1c (proteína ligadora do elemento regulador de esterol 1c), coordenam a ativação dos genes responsáveis pela maturação dos adipócitos, envolvidos no metabolismo de lipídeos e carbohidratos, como FABP4 (proteina ligadora de acido graxo 4), glut4 (transportador de glicose responsivo à insulina 4) e glicerolfosfato desidrogenase (Rosen et al., 2000; Farmer, 2006). A diferenciação terminal é acompanhada por mudanças na expressão de proteínas do citoesqueleto e da matriz extracelular (Spiegelman and Farmer, 1982). É importante notar que PPARγ é o único fator de transcrição necessário e suficiente para induzir a maturação dos adipócitos (MacDougald and Lane, 1995).

A diferenciação dos pré-adipócitos pode ser comumente estimulada *in vitro* por alguns fatores, que são normalmente mais eficientes quando combinados em coquetéis de diferenciação. Dentre os fatores usados nestes coquetéis encontra-se IBMX (3-isobutyl-1-methylxantine), um inibidor de cAMP fosfodiesterase que inibe a degradação de cAMP. Outro componente é a dexametasona, um glucocorticóide responsável pela expressão de *C/EBPδ,* que atua com C/EBPβ para induzir *PPARγ2* e *C/EBPα.* A insulina, também utilizada nos meios adipogênicos em concentração suprafisiológica atua através do IGFR1 (receptor do fator de crescimento tipo

insulina 1) na indução de *SREBP1c*, mimetizando o efeito biológico de seu ligante natural IGF1 (Smith et al., 1988). Por fim, T3 (tri-iodo-thyronine), outro componente do coquetel de diferenciação, atua na expressão de PPARγ através do receptor nuclear do hormônio da tireóide (Mishra et al., 2010). Recentemente, agonistas farmacológicos de PPARγ, como rosiglitazone, têm sido usados no coquetel de diferenciação (Albrektsen et al., 2002). Além de atuar na diferenciação dos pré-adipócitos, trabalhos indicam que alguns destes fatores também atuam no comprometimento de células mesenquimais à linhagem adipocítica. Existem evidências para a atuação de dexametazona, insulina e IBMX na indução de pré-adipócitos (Pantoja et al., 2008; Yang et al., 2008).

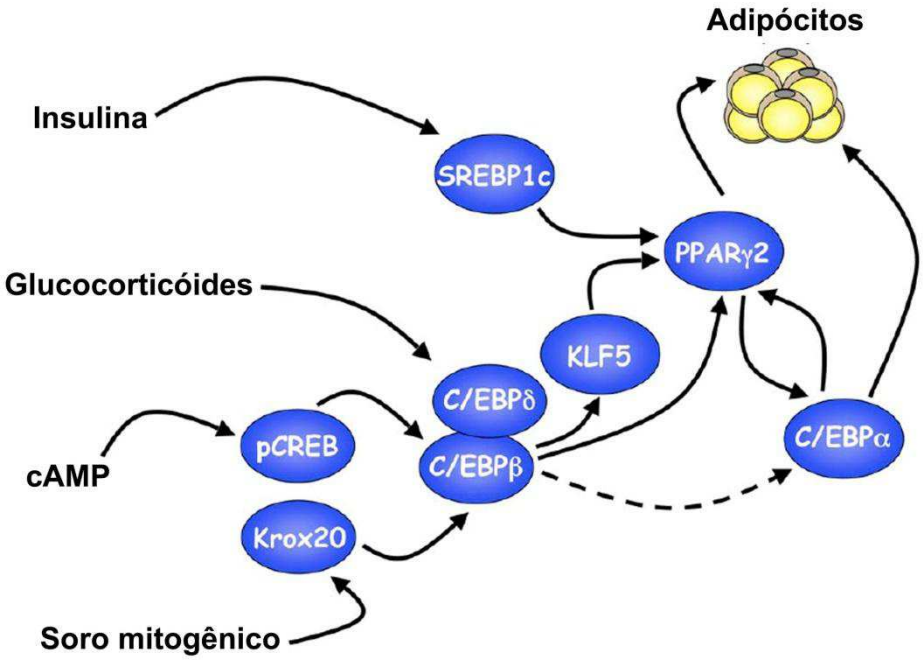

Figura 10 - **Indução de adipogênese por uma cascata de fatores de transcrição.** Exposição de pré-adipócitos à um coquetel de indutores adipogênicos composto por insulina, glucocorticóides, IBMX (agente que eleva o AMPc) e soro fetal bovino. O coquetel ativa a expressão de diversos fatores de transcrição que convergem para PPARγ. PPARγ induz então a expressão de C/EBPα. Juntos, estes fatores controlam a adipogênese terminal (Farmer, 2006).

O balanço entre diferenciação óssea e adipocítica não é conhecida durante o processo de diferenciação mesenquimal da CN. Na medula óssea, a diferenciação das células mesenquimais em adipócitos ou osteoblastos é competitivamente equilibrada. Mecanismos que promovem um destino celular ativamente suprimem os mecanismos que induzem a linhagem alternativa. Isso ocorre através da comunicação entre vias de sinalização, incluindo a via dos BMPs, das proteínas Wnts e Hedgehogs, a via de Notch, dos FGFs, e IGFs; e fatores de transcrição reguladores da diferenciação dos adipócitos e osteoblastos incluindo PPARγ e Runx2, respectivamente (Muruganandan et al., 2009). Assim, dependendo das condições encontradas, este progenitor mesenquimal pode seguir o caminho de diferenciação em osteoblasto ou adipócitos.

Neste trabalho de tese estudamos o potencial de diferenciação da CN truncal em diferentes fenótipos mesenquimais, incluindo a diferenciação em adipócitos.

1.4.3. A diferenciação esqueletogênica da CN

Durante a diferenciação das células da CN cefálica, as células que migram até regiões mais ventrais estão destinadas à formação de derivados ectomesenquimais, enquanto as células que se mantém mais dorsais próximas ao TN se diferenciam em neurônios e células gliais para formar os gânglios craniais. As células migratórias mais tardias param de migrar em posições mais dorsais e formam apenas os derivados não-mesenquimais (Baker et al., 1997).

Os mecanismos que segregam e induzem as células da CN começam a ser desvendados. Blentic e colaboradores mostraram que as células da CN cefálica assumem um fenótipo ectomesenquimal quando entram nos ABs e deixam de expressar os marcadores iniciais de CN Sox10 e FoxD3 e iniciam a expressão de Dlx2 indicativa do fenótipo mesenquimal. FGF é importante neste processo, já que células insensíveis ao FGF (eletroporadas com a construção dominante negativa do receptor FGFR1) não param de expressar Sox10 e FoxD3, mesmo depois de entrarem nos arcos branquiais, e não iniciam a expressão de Dlx2 (Blentic et al., 2008). Recentemente foi mostrado que FoxD3 atua na restrição do destino da CN, e que a perda da expressão deste fator de transcrição guia as células da CN para um destino mesenquimal (Mundell and Labosky, 2011). *In vitro*, FGF2 induz a diferenciação óssea endocondral e intramenbranosa da CN cefálica (Sarkar et al., 2001). Da mesma forma, mutações ativadoras dos receptores FGFR1 e FGFR2 induzem condrogênese nestas células (Petiot et al., 2002).

As células que migram para o primeiro AB dão origem à maior parte do maxilar e da mandíbula, à parte lateral do crânio, ao palato e aos ossos do ouvido médio. A formação da mandíbula envolve os fatores de crescimento BMP4, FGF8 e Shh. A presença destas moléculas no ectoderma do primeiro AB regula a diferenciação da cartilagem de Meckel. O nocaute condicional de BMP4 nesta região resulta em perda quase completa da mandíbula (Liu et al., 2005). Também foi mostrado que o controle da expressão de FGF8 por BMP4 ocorre de forma dependente da concentração, com níveis altos de BMP4 reprimindo a expressão de FGF8 e baixos níveis promovendo a transcrição deste gene (Liu et al., 2005). O equilíbrio entre os níveis de BMP4 e FGF8 é crucial para a formação e extensão da

cartilagem de Meckel (Liu et al., 2005; Stottmann et al., 2001). Shh tem dois papéis fundamentais para a formação da mandíbula. Este fator de crescimento, inicialmente expresso na placa pré-cordal e no endoderma faringeal, atua na sobrevivência das células da CN que migram para o primeiro AB (Brito et al., 2006). Além disso, Shh também tem a função de induzir FGF8, BMP4, além de sua própria expressão no ectoderma adjacente do primeiro AB. Quando uma segunda fonte de Shh é fornecida ao futuro território do primeiro AB, em embriões de galinha em estágios precoces do desenvolvimento (estágio 5-8), ocorre indução de uma mandíbula extra, posicionada como uma imagem de espelho em relação à outra (Brito et al., 2008). Além disso, foi mostrado que a molécula Shh promove a condrogênese em células da CN cefálica e truncal, favorece o desenvolvimento de progenitores da CN dotados de ambos os potenciais neural e esqueletogênico, e induz aumento da expressão de *Runx2* nas células que entornam os nódulos de cartilagem em cultura de CNC (Calloni et al., 2007; 2009).

Diferentes fatores de crescimento também participam da ossificação intramenbranosa do crânio. Moléculas TGFβ secretadas pela meninge dura-máter, por exemplo, estão implicadas nas interações teciduais entre a dura-máter e as suturas cranianas que são recobertas por ela. A sinalização TGFβ é aumentada durante o período de fusão da sutura frontal quando comparado com a sutura sagital ainda aberta (Most et al., 1998; Opperman et al., 2000). O fator de crescimento FGF2 também está implicado na osteogênese das suturas cranianas. A inibição da atividade de FGF2 levou à diminuição da osteogênese das suturas cranianas através da inibição da proliferação e diferenciação celular (Moore et al., 2002). BMP2 e BMP4 também regulam positivamente a osteogênese e estão expressos nas

frontes osteogênicas. BMP4 também esta presente no mesênquima da sutura sagital e na dura-máter (Kim et al., 1998). Noggin, antagonista de BMP, tem um papel na manutenção da sutura craniana. Os níveis de BMP sejam relativamente equivalentes entre suturas abertas e suturas em fase de fusão de camundongos, mas a expressão Noggin é principalmente limitada às suturas sagital e coronal, que ainda estão abertas. Além disso, o aumento da sinalização FGF suprime a expressão Noggin por osteoblastos de rato *in vitro* (Warren et al., 2003).

A via de sinalização Wnt também possui um papel crucial na formação dos ossos intramembranosos do crânio. A desregulação da via canônica de Wnt nas suturas cranianas frontal e sagital leva à craniossinostose, que é uma das deformidades congênitas craniofaciais humanas mais comuns (Yu et al., 2005). Os indivíduos com craniossinostose desenvolvem ossos anormais do crânio, devido a fusão prematura das suturas cranianas. Axina 2, por exemplo, é um regulador negativo da via de Wnt (promove a degradação do efetor da via βcatenina) que controla o tempo de fechamento da sutura. Em camundongos nocaute para *axina2* ocorre um aumento dos osteoprogenitores Runx2-positivos, estimulação da expressão de marcadores osteogênicos, aumento da mineralização da matriz extracelular e fechamento precoce das suturas cranianas (Yu et al., 2005). Outro trabalho confirmou que o ganho de função de *βcatenina* no mesênquima da sutura craniana promove o aumento dos osteoprogenitores Runx2-positivos, e aumento de mineralização. Estes processos envolvem aumento da fosforilação de smad1, 5, 8, e de FGF2 e seus receptores FGFR1 e FGFR2 (Mirando et al., 2010). Um trabalho recente mostrou que osteoblastos da frente osteogênica das suturas cranianas frontais tem um potencial osteogênico maior que os osteoblastos das suturas sagitais, que pode ser explicado pela sinalização canônica de Wnt ativada nas

células das suturas frontais. A ativação constitutiva da via de Wnt nos osteoblastos parietais confere um maior potencial osteogênico à estas células, como o encontrado nos osteoblastos frontais. Além disso, foi mostrado que o tratamento das células das suturas cranianas com FGF2 induz a fosforilação de GSK3, aumentando o nível de βcatenina no núcleo, sugerindo que a ativação da via de Wnt pode ser mediada por FGF (Quarto et al., 2011).

Diferentes fatores de transcrição atuantes no processo de diferenciação óssea a partir da CN são induzidos por estes fatores de crescimento produzidos pelos tecidos adjacentes. Os sinais derivados do ectoderma induzem a expressão regionalizada de fatores de transcrição nas células ectomesenquimais da mandíbula. A sinalização BMP no ectoderma, além de induzir a expressão de *Runx2* indispensável para a diferenciação dos osteoblastos leva à expressão dos fatores de transcrição *dHand, Msx1, Msx2* e diferentes genes *Dlx* (Brito et al., 2008; Liu et al., 2005). A expressão local destes fatores de transcrição resulta na correta padronização da mandíbula. Além disso, parte destes genes também está envolvida na morfogênese do crânio. No primeiro AB cada estrutura deve adquirir sua identidade no eixo próximo-distal. O código de genes *Dlx* atua na especificação regional destas estruturas. *Dlx1/2* são inicialmente expressos nos primórdios maxilar e mandibular, enquanto *Dlx5/6* estão expressos apenas no primórdio mandibular. *Dlx3/4* estão restritos à um pequeno domínio do primórdio mandibular. Assim *Dlx1/2* regulam o desenvolvimento do maxilar, enquanto *Dlx5/6* conferem o fenótipo mandibular (Depew et al., 2005; Jeong et al., 2008). Na sutura craniana, foi mostrado que *Dlx5* é ativado por BMPs nos precursores de osteoblastos. *Dlx5* induz, então, a expressão de *Runx2*, e desencadeia a sequência de genes envolvidos na

osteogênese do crânio, como *osteopontina* e *fosfatase alcalina* (Holleville et al., 2003 ; 2007). Os genes *Msx* também estão envolvidos no desenvolvimento dos ossos craniofaciais, onde atuam normalmente como repressores transcricionais (Bendall and Abate-Shen, 2000). Na região do primeiro AB, *dHand* induz a expressão de *Msx1*, importante também para a sobrevivência das células nesta região (Thomas et al., 1998). Em camundongos com dupla mutação para *Msx1* e *Msx2* não ocorre formação o osso craniano frontal. Nestes embriões a expressão de *Dlx5* é bloqueada, e *Runx2* não é ativado (Han et al., 2007). O gene *twist* é induzido por FGF2 nas suturas cranianas e inibe a atuação do fator de transcrição Runx2 (Bialek et al., 2004; Rice et al., 2000). Durante a aproximação e diferenciação das suturas cranianas *twist* tem sua expressão diminuída. Camundongos com mutação deletéria no gene *twist* tem fechamento precoce das suturas cranianas (Paznekas et al., 1998).

Os genes *Hox*, uma família de fatores de transcrição com homeodominios, são conhecidos por exercer papéis importantes na especificação da identidade rostrocaudal de muitos tecidos (Iimura and Pourquie, 2007). Dois domínios podem ser distinguidos nas células da CN que estão na origem do esqueleto crânio-facial. Ossos intramembranosos surgem das células Hox-negativas, enquanto condrócitos são originados tanto pela CN Hox-positiva quanto Hox-negativa. Foi demonstrado (Couly et al., 1998) que as células Hox-positivas da CN quando transplantadas para o domínio Hox-negativo anterior não são capazes de se diferenciar em cartilagem e osso. Ao contrário, as células da CN do domínio Hox-negativo transplantadas posteriormente respondem normalmente aos sinais locais, participando da formação de cartilagem hióide, enquanto mantém seu estado Hox-negativo. Um dos motivos

para esta maior capacidade osteogênica da CN Hox-negativa é sua capacidade de responder aos sinais produzidos pela endoderme anterior durante a produção dos diferentes componentes esqueléticos da face superior, do maxilar e da mandíbula. Células da CN que expressam Hox não respondem a esses sinais. No domínio da CN Hox-negativa rostral, um fragmento equivalente à apenas um terço da borda neural dorsal é capaz de criar um esqueleto facial completo (Couly et al., 2002). O domínio Hox-negativo rostral da CN (ou FSNC, para CN facial esqueletogênica), portanto, se comporta como um “grupo de equivalência". Desta forma, os potenciais da CN cefálica são limitados pela expressão do gene Hox. De fato, em camundongos nocaute para *Hoxa2*, as células da CN do segundo AB se comportam como as células Hox-negativas que preenchem o primeiro AB, e formam pedaços ectópicos de esqueleto da mandíbula (Gendron-Maguire et al., 1993; Rijli et al., 1993).

Além disso, os genes *hox* têm sido relacionados com a ausência de diferenciação esqueletogênica pelas células da CN truncal. Foi demonstrada condrogênese em culturas de longa duração da CN truncal. Além de *Hoxa2* diminuir e *Hoxd10* inibir completamente a condrogênese em culturas de CN mesencefálica, também se observou que o gene *Hoxb4*, expresso pelas células da CN truncal pré-migratórias tem sua expressão diminuída apos 14 dias de culturas, e poucos condrócitos col2-positivos apresentaram este gene. Além disso, a superexpressão de *Hoxd10* também impede a condrogênese da CN truncal *in vitro* (Abzhanov et al., 2003). Outro trabalho observou que a condrogênese de células da CN truncal de camundongos é induzida *in vitro* por FGF2, e que este processo implica a diminuição de *Hoxd9* ao longo da cultura e aumento do gene de CN cefálica *Id2* (Ido and Ito, 2006). Foi demonstrado também que os sinais extracelulares acido retinóico (AR) e

Wnt induzem *Hoxa2 e Hoxd9,* respectivamente, na CN mesencefálica, que normalmente não expressa nenhum gene *Hox,* e que a diminuição de *Hoxd9* provoca diminuição da diferenciação de condrócitos colágeno2-positivos. AR e Wnt também induzem a manutenção destes genes *hox* nas células da CN truncal (Ishikawa and Ito, 2009).

Vários aspectos da diferenciação da CN cefálica em condrócitos e osteoblastos já são conhecidos. Entretanto, pouco se sabe sobre o potencial de diferenciação esqueletogênica, bem como da diferenciação em adipócitos, das células da CN truncal. A compreensão deste potencial e de como estes tipos celulares são obtidos a partir de células multipotentes da CN truncal, tema central desta tese, trará novas informações para entendermos o processo de especificação e determinação da CN, e permitira o avanço na compreensão do processo de evolução dos vertebrados amniotas.

I. INTRODUCTION

The neural crest (NC) arises in vertebrate embryos from the neural plate (NP) borders, at the boundary between the neuroepithelium that will form the central nervous system (CNS) and the ectoderm. This structure contributes to the formation of different organs and tissues throughout the body, which shows its multipotentiality. Along the whole rostrocaudal axis, the NC gives rise to neurons and glial cells of the peripheral nervous system (PNS) and to melanocytes. In addition, other phenotypes are generated by the NC cells that migrate along well delimited paythways. In addition to pigment cells and PNS cells, the cephalic NC generates mesenchymal cells that will form craniofacial cartilages and bones and also dermis, fat cells and smooth muscle cells in the head and neck (Le Douarin and Kalcheim, 1999).

The characterization of NC multipotentiality is an issue of longstanding interest. In the laboratory of Prof. Nicole Le Douarin, a number of studies have revealed the presence of a highly multipotent progenitor among the cephalic NC cells of birds, which has capabilities for neural, mesenchymal and melanocytic cell differentiation.

The mesenchymal tissues of the trunk in amniote Vertebrates derive from mesoderm, not from the NC. In contrast, the NC from lower Vertebrates generates some mesenchymal tissues in the trunk, such as the distal skeleton of the dorsal and caudal fins of teleost Vertebrates such as zebrafish. Therefore, a question arises as to whether the capacity of NC cells to produce mesenchyme in the trunk was lost during evolution, or if it still exists in amniotes Vertebrates and may be disclosed under specific conditions.

To study developmental potential of the trunk NC cells, it is important to understand the induction, specification and determination processes of these cells.

These aspects, as well as the evolution of the NC and the mechanisms of bone and adipocyte differentiation, will be discussed in the Introduction chapter of this Thesis.

1.1. The formation of the neural crest and its derivatives

The NC is a transient multipotent structure of vertebrate embryos, which comprises cells differentiating into different phenotypes and contributing to the formation of various structures of the body. This cell population was discovered by Wilhelm His in 1868 in studies with chicken embryos. He described the NC as a set of cells that leave the neural tube (NT) to form the dorsal root ganglia, so he named this structure “ganglionic crest” (His, 1868).

The NC cells are derived from the dorsal edges of the NT at the neurula stage. These cells undergo an epithelial-mesenchymal transition (EMT), which involves changes in intercellular adhesion and in interactions with the surrounding tissues. As a consequences, NC cells delaminate from the NT and follow different migration paths to reach specific locations, where they will generate the appropriate cell types (**Figure 1**) (Le Douarin and Kalcheim, 1999). The NT keeps the neuroepithelial structure and forms the CNS.

1.1.1. Induction and specification of the neural crest

NC development involves a complex sequence of events, from the induction and formation of the crest cells, until their specification and their differentiation into the various NC derivatives. These events are guided by a network of gene regulation and cellular interactions, and can be divided into three stages: induction of the NP border, NC specification, and later, generation of the NC (Sauka-Spengler and Bronner-Fraser , 2008) (**Figure 1**).

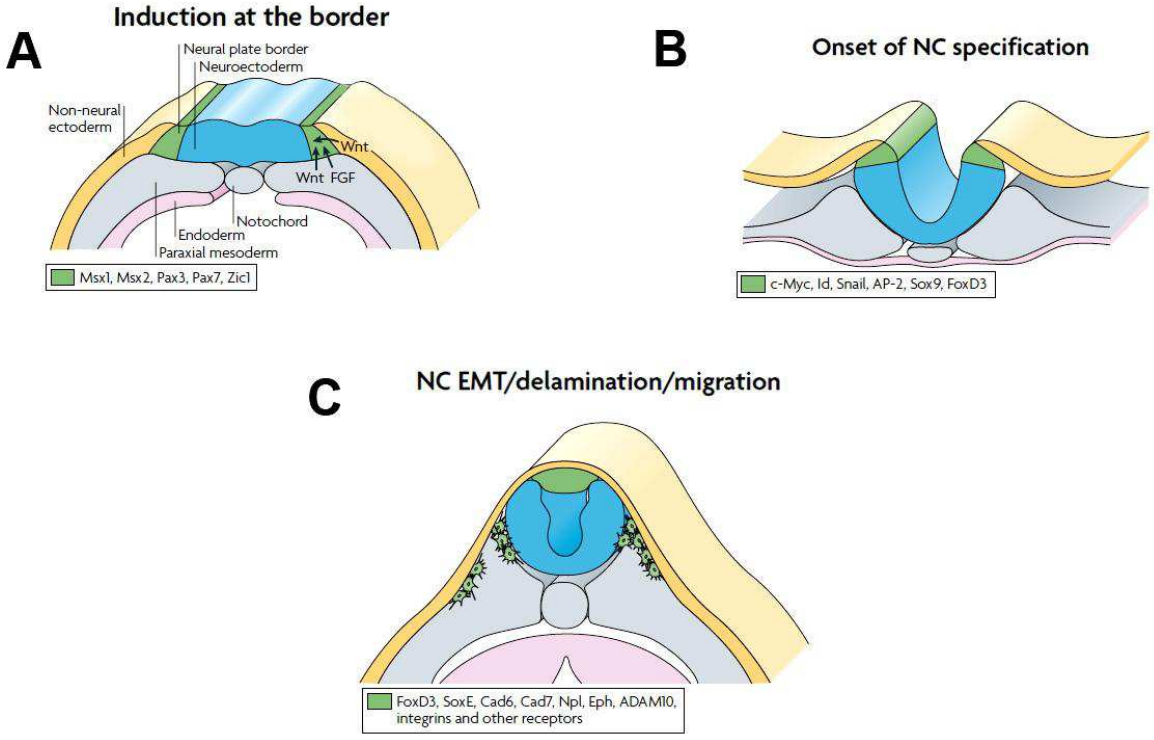

Figure 1 – Formation of the neural crest. (A) induction of the NP border is mediated by the action of signals derived from adjacent tissues such as FGF, BMP and Wnt. The activation of these signaling pathways induces the expression of NP border specifier genes. (B) In the NC induction step, the NP border specifiers genes control the expression of NC specifiers genes. (C) NC specifiers genes promote the segregation of the NC cells from the dorsal neuroepithelium by controlling cell proliferation, delamination and the onset of the epithelial-mesenchymal transition (adapted from Sauka-Spengler and Bronner-Fraser, 2008).

The NC formation begins at gastrulation, with different signals from adjacent tissues that promote the induction of the NP border. BMP4 (bone morphogenetic

protein 4), expressed by the ectoderm, is one of the implicated growth factors. BMP antagonists (Chordin, Follistatin and Noggin), derived from mesoderm, are important to generate an intermediate level of BMP, essential for induction of the NC in the NP border (Marchant et al., 1998). Other signaling pathways act together with BMP to induce NC formation. FGF (fibroblast growth factor) factors are also implicated in the NC induction. It was shown in *Xenopus laevis* embryos that FGF2, in combination with BMP antagonists, increases the expression of the NC marker Snail2 and induces the NC formation (Mayor et al., 1997). Furthermore, FGF8, expressed in the mesoderm, temporarily induces NC cells (Monsoro-Burq et al., 2003). The Notch receptor pathway acts on NC induction before BMP, to restrict NC formation to the NP border region (Endo et al., 2002). In addition to these signals, Wnt signaling pathway also plays a key role in NC formation. In *Gallus gallus* embryos (chicken), Wnt6 from the non-neural ectoderm adjacent to the NP border, plays a role in NC induction through the canonical βcatenin pathway (Garcia-Castro et al., 2002). During gastrulation, Wnt and BMP signals, expressed in different domains of the ectoderm, act together in a specific region of the NP edge, to delimit and induce the NC territory. The anterior region of the NP edge, that receives only BMP signals, gives rise to ectodermal placods, while central regions, under the influence only of the Wnt pathway, will form the CNS (Edlung et al., 2008). This first step of NP induction leads to the activation of specific transcription factors in the region of the NP edge, called "edge specifiers of NP", including *Dlx3, Dlx5, MSX1, MSX2, Pax3, Pax7* and *Zic1* genes (Sauka-Spengler and Bronner-Fraser, 2008) (**Figure 1A**).

The combined action of these genes leads to the specification of cells from the NP border into NC cells through the activation of "NC specifier genes" (or NC markers), which include *Snail1, Snail2*, *Sox8, Sox9, Sox10, FoxD3, AP2, Twist, c-*

Myc and *Id* family members (**Figure 1B**). These genes define a new regulatory state of these cells and activate the NC "effector genes", which guide a complex sequence of events to specify different NC fates (Sauka-Spengler and Bronner-Fraser, 2008).. The mechanisms of induction and control of NC specifier genes are poorly understood. However, studies in *Xenopus Leavis* showed that the induction of *FGF8, FoxD3* and *Snail2* expression is dependent on Wnt signaling and mediated by Msx1. Furthermore, *Snail2* and *FoxD3* are regulated by *Zic1* and *Pax3*, which in turn are dependent on Wnt (Monsoro-Burq et al., 2005, Sato et al., 2005). In mouse embryos, it was observed that *Sox10* is induced and maintained by Pax3 and AP2, and also by Sox9 and itself, through the activation of the Wnt pathway (Werner et al., 2007).

Thus, the activity of the NC specifier genes segregates these NC cells from the dorsal neuroepithelium, as these genes control events that confer NC specific characteristics such as EMT, regulate cell population size, cell cycle control, and differentiation into a wide variety of phenotypes (**Figure 1C**).

The NC delamination from the neuroepithelium involves the EMT process that implies changes in cell adhesive properties, shape and motility. The delamination time varies between NC cell subpopulations from different regions of the anteroposterior axis. The cephalic NC delaminates at once. In mice and *Xenopus* embryos, this process occurs when the NT is still open, while in avian embryos it coincides with the fusion of the neural folds (Theveneau et al., 2007).

The trunk NC cell delamination occurs after NT closure and in a gradually manner, with the cells leaving the neuroepithelium "one by one". It was shown that, in chicken embryos, the most anterior trunk NC cells delaminate few hours after the NT closure (Sela-Donenfeld and Kalcheim, 1999), while later, more caudal cells migrate one day after the end of neurulation (Osorio et al., 2009b). Studies carried out in

chicken embryos showed that in the trunk region, NC cell delamination is triggered by a signaling cascade activated by BMP and canonical Wnt pathways (Burstyn-Cohen et al., 2004; Cheung et al ., 2005). This cascade promotes EMT through *Snail2, FoxD3, Sox9* and *Sox10* activation. These transcription factors act in switching expression of cadherin molecules (N-cadherin is replaced by cadherinB6, which is replaced posteriorly by cadherins 7 and 11), they also activate integrinβ1, modulate RhoB expression and stimulate laminin synthesis and basal lamina degradation (Cheung et al., 2005; Chalpe et al., 2010). The BMP4 and Wnt1 signaling cascades promote the transition from G1 to S cell cycle phase, which has a permissive role in the EMT (Burstyn-Cohen et al., 2004). ADAM10 metalloproteinase, activated by BMP4 pathway, promote N-cadherin cleavage in its extracellular portion, resulting in a decreased adhesion between the cells. Cleavage of a cytoplasmic domain leads to cyclin D1 activation that allows cell cycle progression and contributes to the G1/S transition (Shoval et al., 2007). The activation of BMP/Wnt cascade is linked to somites maturation, which leads to Noggin (BMP inhibitor) expression blockage in the dorsal NT (Sela-Donenfeld and Kalcheim, 2000). In the cephalic NC and more caudal NC, the cell delamination is differently regulated. The premigratory cephalic NC cells express many BMP and Wnt inhibitors, and most of these cells have no contact with the somites (responsible for the inhibition of Noggin in the trunk NT) (Tzahor et al., 2003). Indeed, Noggin overexpression and dominant negative form of BMP receptors are not able to block the delamination of the cephalic NC cells (Kalcheim and Burstyn-Cohen, 2005). Moreover, the cephalic NC cell emigration is not related to G1 to S cell cycle transition (Osorio et al., 2009b). In the most caudal portions of the trunk, the NC cells are also not synchronized with somitogenesis. The decrease of Noggin in the dorsal NT does not occur in response to somitic differentiation and NC

cells emigration occurs with a delay of 24 hours (Osorio et al., 2009a). In this region, NC delamination is under control by Wnt3a and is independent of the BMP4/Wnt1 combined action.

In addition to the NC specifier genes that maintain their expression at later stages, the migratory NC cells can be identified using the monoclonal antibody HNK1 (human natural killer 1) in several species, including birds and rat (**Figure 2**). HNK1 recognizes carbohydrates containing sulfated glucuronic acid, originally identified in human NK (natural killer) cells (Abo and Balch, 1981). After NC migration, HNK1 expression is observed in other cell types such as glial cells, some NT cells, some neuronal subpopulations (Tucker et al., 1984) and cardiomyocytes (Nakajima et al., 2001). Thus, the HNK1 labeling thus allows the identification of NC cells in vitro and in vivo (**Figure 2**).

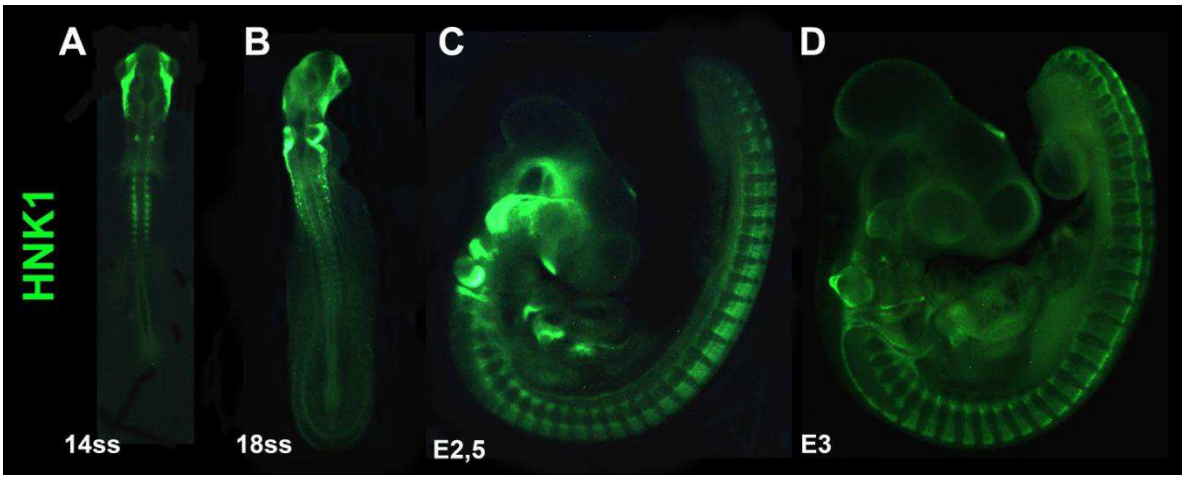

Figure 2 - Quail migratory neural crest cells evidenced in toto by HNK1 antibody. (A) Embryo of 14 somites, where we can observe the cephalic NC cells already away from the NT and the onset of trunk NC cell migration. (B) Embryo of 18 somites showing the cephalic NC cells that cover the entire cephalic region and colonize the prospective branchial arches. The anterior trunk NC cells are already in an advanced stage of migration. (C and D) 2.5 days- and 3 days-embryos respectively, where we notice NC cells present in regions of differentiation, as in trigeminal sensory ganglia, dorsal root ganglia and sympathetic ganglia.

1.1.2. The quail-chick chimeras and the neural crest derivatives

According to the antero-posterior level of NT from which they originate, the NC cells are the source for the formation of distinct specific organs and tissues. These derivatives, as well as their origin in the NT, were identified in avian embryos thanks to the chick-quail chimeras experiments (Le Douarin, 1969; Le Douarin and Teillet, 1974; Le Lievre and Le Douarin, 1975, Le Douarin and Kalcheim, 1999), which were developed by Nicole Le Douarin and collaborators from the 70s.

These experiments became possible after the observation made by Nicole Le Douarin that the nuclei of the *Coturnix coturnix japonica* (quail) cells exhibit a particular condensed heterochromatin associated with the nucleolus (Le Douarin, 1969). This compact DNA can be recognized after Feulgen staining of nucleic acids. In other species, including *Gallus gallus* (chicken), the heterochromatin is dispersed in the nucleus during interphase. Later on, the QCPN antibody (non-chicken quail perinuclear antigen) could also be used to distinguish quail cells in the chimeras (Lance-Jones and Langenaur, 1987). To identify the NC derivatives, chimeras were produced by isotopically grafting a particular region of the NT (or the dorsal edges of the NT) taken from the quail embryo before NC migration, into a host chick embryo of same stage (**Figure 3**).

Several of these studies have thus shown the domain of origin for each NC-derived cell type and the NC cell migration paths in the developing avian embryo. Melanocytes, for example, differentiate from NC cells from all levels of the antero-posterior axis, while mesenchymal derivatives are derived only from the cephalic NC (Le Douarin and Kalchein, 1999). Cells contributing to the sensory and autonomic

nervous system, and to the enteric nervous system, are derived from the NC in restricted areas of the neural axis (**Figure 4**).

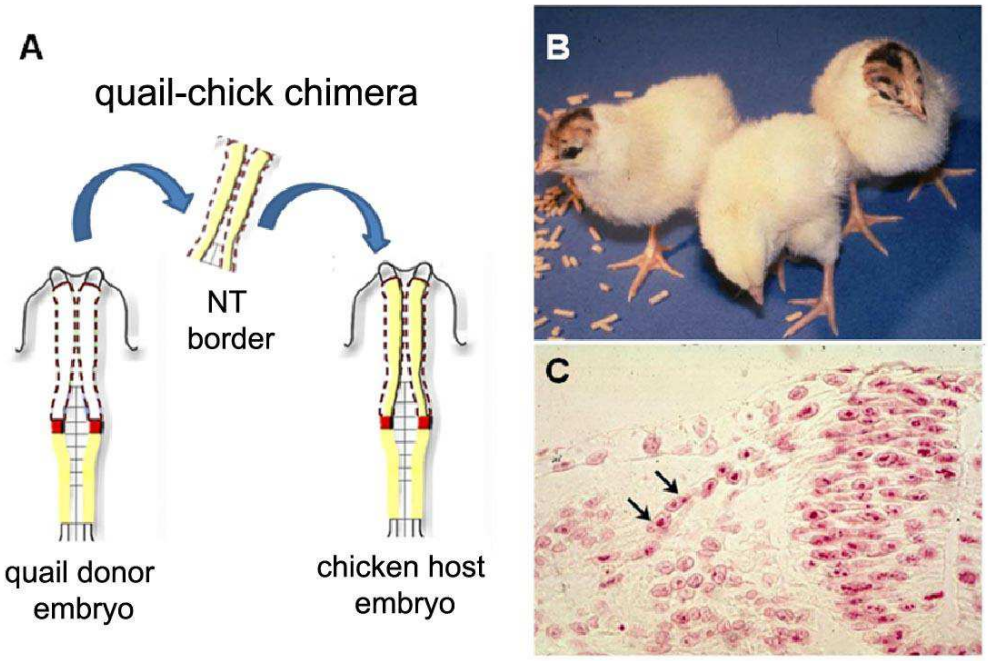

Figure 3 - Scheme of quail-chick chimeras experiments. The neural tube (NT) is removed from a donor quail embryo and implanted into a chick embryo of the same embryonic stage. (A) Example of cephalic NT/NC graft. (B) White Leghorn chimeric chickens achieved after grafting of the cephalic level of the NT; they have pigment cells only in the head due to the presence of melanocytes originated from the quail transplanted NC area. We observed two chimeric chickens and a non-grafted control one in the center. (C) Histological sections with Feulgen staining of the host chick embryo show NC cells (arrows) migrating from the donor quail NT, which have a more condensed chromatin (adapted from Le Douarin and Kalcheim, 1999).

More recently, fate mapping studies of the NC derivatives were performed also in mouse embryos, through genetic recombination experiments that allowed long-term tracking of the NC cells. P0-Cre mice combined with conditional reporter lines, have confirmed the majority of the NC phenotypes already found in avians (Yamauchi et al., 1999). However, recombination mediated by P0-Cre occurs only in

a subset of NC cells and it displays ectopic expression, leaving many questions concerning the NC differentiation unanswered. The promoter of *Wnt1* gene, more specific for the early NC cells, was since widely used in combination with conditional ROSA26-lacZ reporter or EGFP reporter (Jiang et al., 2000, 2002).

Collectively, the analyses conducted in birds, mice, fish and amphibians have shown that the cephalic and trunk NC cells produce a large set of derivatives including neurons, glial cells, endocrine cells, melanocytes, and mesenchymal derivatives, which differentiate only from the cephalic NC, forming smooth muscle, bone and cartilage (Le Douarin and Kalcheim, 1999). Besides division into cephalic and trunk NC, we can highlight other subregions. The vagal (the first 7 somites) and sacral regions (posterior to somite 28) are responsible for the formation of the enteric nervous system, which controls the digestive peristalsis (Le Douarin and Teillet, 1973). The cardiac NC (at posterior rhomboncephalon level, corresponding to the first 4 somites) yields the smooth muscle cells of the cardiac septum that separate the aorta and pulmonary artery in heart development (Le Lievre and Le Douarin, 1975; Kirby et al. 1995). The results of fate mapping experiments in avian embryos are summarized in **Figure 4.**

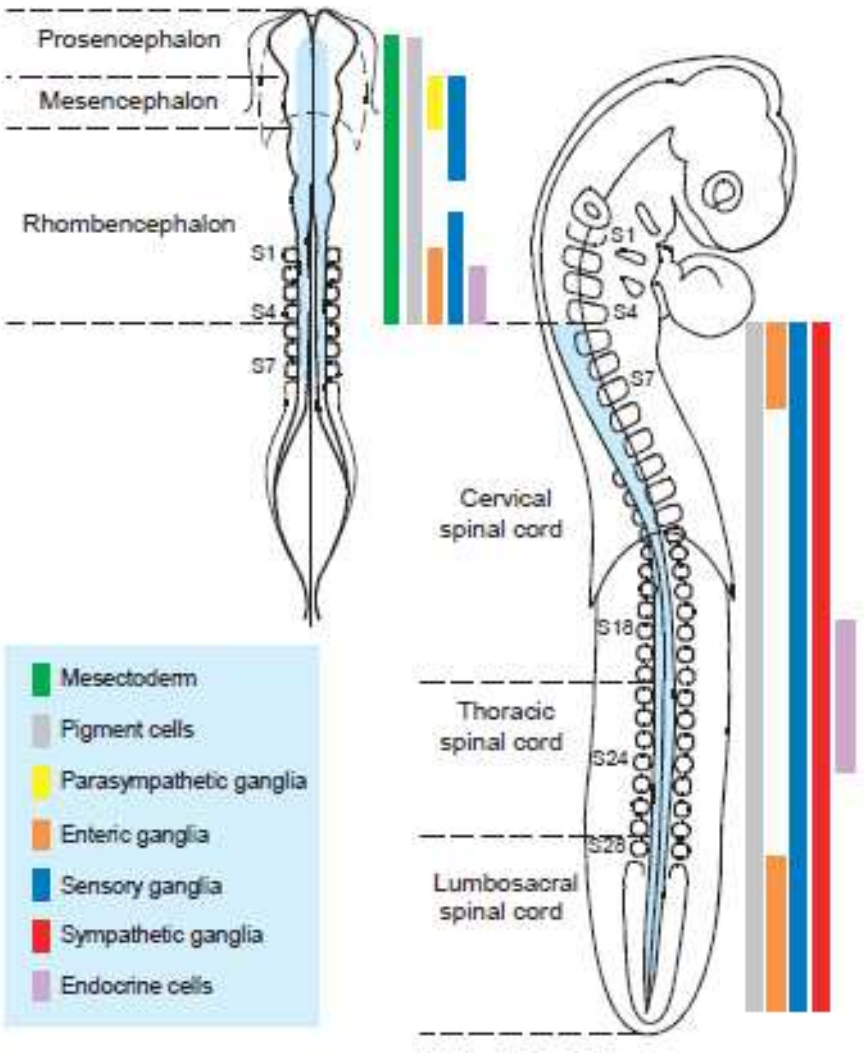

Figure 4 – Fate map of NC derivatives. The different phenotypes derived from cephalic (A) and trunk (B) NC along the anteroposterior axis are shown. The NC is indicated in blue in 7 somites-stage (ss) (A) and 28ss (B) embryos. To the right, the distinct tissues originated from cephalic (A) and trunk (B) NC are listed and identified by different colors.

The cephalic NC comprises cells that migrate from the posterior diencephalon, midbrain and hindbrain until the fourth somite included (Couly et al., 1996). The cephalic NC migration pattern shows little variation between different species (**Figure** 5A). The cephalic NC cells that contribute mesenchymal cells have been designated mesectoderm (or ectomesenchyme) in order to distinguish them from the mesoderm-derived mesenchymal cells (Platt, 1983). The cells that migrate to the nasal bud and

the first branchial arch (BA) will form the nasal capsule, and maxillary, mandible and entoglossum (the tongue skeleton) bones. The hyoid cartilage is formed by NC cells that colonize BA 2, 3 and 4 (Couly et al, 1996). The cephalic NC cells that migrate covering the forebrain act in skull formation. The skull vault is composed of frontal, parietal and squamosal pairs of bones and of the anterior part of the occipital bone. Quail-chicken chimeras experiments have indicated that these bones are entirely derived from the NC (Couly et al., 1993). However, in transgenic mice where NC cell progeny is traced due to βgalactosidase gene expression associated with the promoter of Wnt1 gene, it has been shown that the parietal bones are of mesodermal origin. Sagittal (between the parietal bones) and coronal (between the frontal and parietal bones) skull sutures are formed by NC cells and develop on the interface between NC- and mesoderm-derived cells (Jiang et al., 2002). In addition to skeletal elements, there are other NC mesectodermal derivatives, such as facial dermal cells, forebrain meninges, adipose tissues, tendons, smooth muscle cells and connective tissues in the thymus, the thyroid and parathyroid glands. The cephalic NC gives rise also to different tissues of the eyes and teeth, and participates in cardiovascular structures by forming the layers of smooth muscle cells adjacent to the endothelium of large arteries from the aortic arch and of blood vessels irrigating the face and brain (Le Douarin and Kalcheim, 1999; Chai et al., 2000; Etchevers et al., 2001; Jiang et al., 2000, 2002).

The cephalic NC cells also yield neurons and glial cells in the PNS, such as in the trigeminal sensory ganglion and the parasympathetic ciliary ganglion (Le Douarin and Kalcheim, 1999). Furthermore they give rise to the parafollicular C cells, a type of endocrine cells producing the hormone calcitonin. These cells develop in the

ultimobranchial body in all Vertebrates except in Mammals, where this structure fuses with the thyroid gland.

- In the trunk, the emigration time and the trajectories of NC cells exhibit some differences between species. The trunk NC cells can follow two alternative paths of migration: the ventral one and the dorsolateral one. In chicken and mouse embryos, cells migrate initially along the ventral path between the NT and the anterior half of the somitic sclerotome, which leads to a segmented migration pattern (Rickmann et al., 1985; Guillory and Bronner-Fraser, 1986; Loring and Erickson, 1987; Teillet et al., 1987) (**Figure 5B**). These migrating cells follow a dorsal to ventral order for the colonization of their targets, starting by forming sympathetic ganglia ventrally, then Schwann cells associated with peripheral nerves, and finally, the sensory dorsal root ganglia in a dorsal position close to the NT (Serbedzija et al., 1994). The trunk NC from the anteroposterior level between somites 18 to 24 also generates the adrenal medulla cells (cells that produce catecholamines - adrenaline and noradrenaline) (Le Douarin and Teillet, 1974). The NC cells that migrate later, follow a superficial dorsolateral path, between the ectoderm and dermomyotome, and will become melanocytes located in the whole skin surface (Erickson et al., 1992).

In mice both ventral and dorsolateral routes are invaded simultaneously by the NC cells, while in chicken, cells begin to follow the dorsolateral path approximately 24 hours after the onset of the trunk NC migration (Kuo and Erickson, 2010).

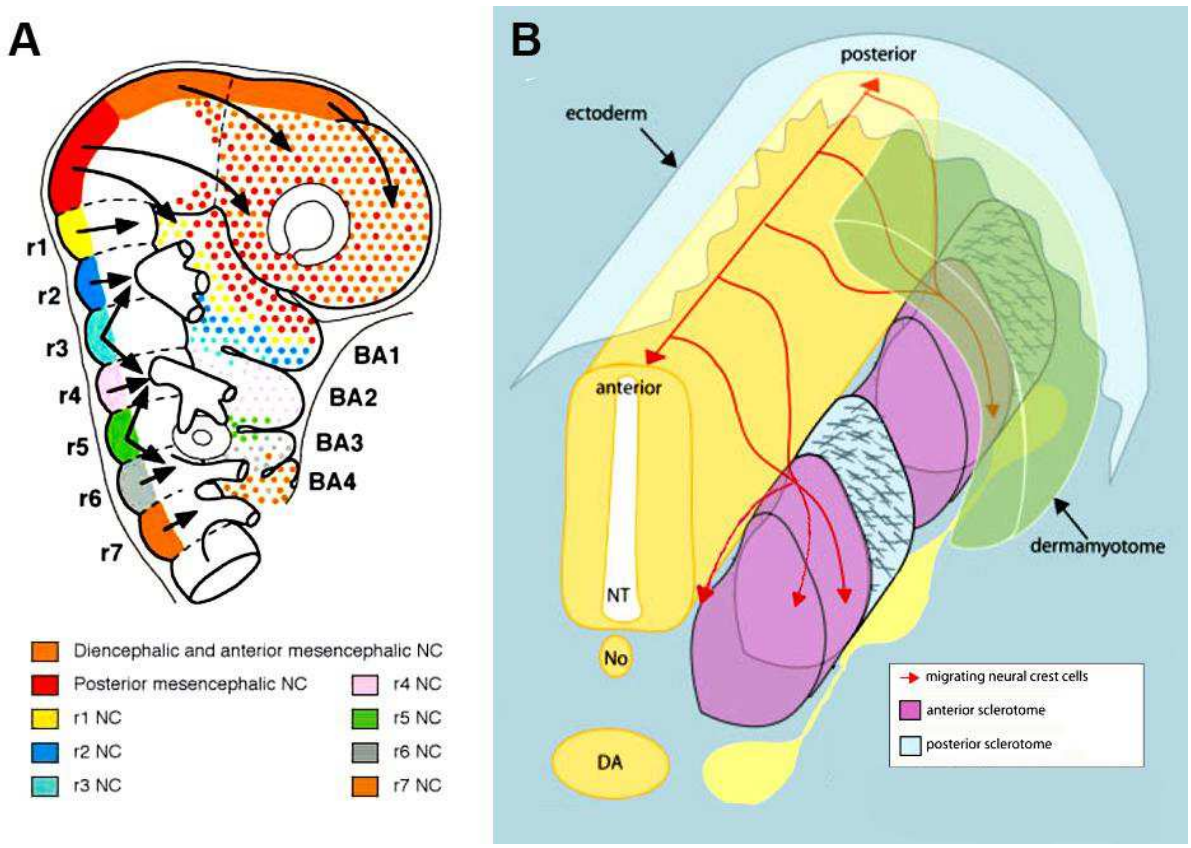

Figure 5 - **Migration pathways of the cephalic and trunk NC cells in the avian embryo.** (A) Cephalic NC cell migration. The origin of the cells found in the frontonasal and periocular regions, and in BAs are differentiated by colors. The anterior midbrain contributes to the frontonasal and periocular regions. The NC of the posterior midbrain also participates in these structures, and it fills the anterodistal part of the BAs. The complementary part of the BAs is derived from the first rhombomeres (R) R1/R2, with a small contribution of R3. The largest contribution to the second BA comes from R4. The cells of R3 and R5 contribute to the two adjacent BAs; R3 NC cells migrate to the first and second BA, and cells from R5 migrate to the second and third BAs. NC cells of R6 and R7 migrate into the third and fourth BAs. (B) Ventral migration of the trunk NC cells. During this migration (red arrows), molecules in the posterior sclerotome block invasion by NC cells of this portion of the somite, and establish a metameric pattern of the dorsal root ganglia. The differences between the anterior and posterior halves of the somites also dictate the position and segmentation of the primary sympathetic ganglia (SG). NT, neural tube; No, notochord; DA, dorsal aorta; SG, sympathetic ganglia. (Couly et al., 2002; Harris and Erickson, 2007).

In zebrafish and *Xenopus* embryos, there are some differences in the trunk NC migration. In zebrafish, the cells migrating in the ventral pathway pass through the medial part of somites. After 4 hours, the lateral migration starts (Raible et al.,

1992). In Xenopus, the NC cells migrate between the posterior somite and the NT, and few cells follow the dorsolateral path (Collazo et al., 1993). In opposite to what is observed in the chick and mouse, melanocyte precursors can follow both ventral and dorsal routes in Xenopus and zebrafish (Collazo et al., 1993; Kelsh et al., 2009). Moreover, some trunk NC cells in fish and amphibians migrate medio-dorsally to the dorsal fin, where they contribute to pigment cells and mesenchymal cells (Collazo et al., 1993, Raible and Eisen, 1994).

These findings indicate that, unlike the cephalic NC, the trunk NC of amniotes Vertebrates does not contribute to the formation of mesenchymal derivatives during development. To study the differentiation potential of the trunk NC, it is important to understand the process of specification and determination of the NC cells as a starting point.

1.2. The differentiation potentials of the NC cells

The population of NC cells is able to differentiate into different cell types during embryonic development. A discussion point in understanding the differentiation process of these cells is the extent of their potentials before and during their migration from the NT. Two models can be proposed for the cellular origin of the different derivatives from the NC. The first model suggests that the NC cells are originally stem cells that divide and undergo progressive restrictions of their lineage potential to the final differentiation, similar to the stem cell model of the development of hematopoietic cell types (Bronner-Fraser and Fraser, 1988, 1989; Trentin et al., 2004). The second model would be that the pre-migratory NC is composed of a mosaic of distinct and already determined precursors (Krispin et al., 2010).

In heterotopic transplantations experiments wherein quail trunk NC replaced the cephalic NC in chicken embryos, it appeared that no mesectodermal cell developed from the graft. However, if the trunk NC was grafted to the cephalic region only unilaterally, these cells migrated with the host cells and could differentiate into myofibroblasts and connective tissue cells, but they did not participate in skeletogenesis (Nakamura and Ayer-le Lievre, 1982). These data thus support the second model and the restricted differentiation potential of the trunk NC cells.

However, other heterotopic transplantation experiments have shown that some pre-migratory NC cells have a great plasticity. When the vagal NC (NT and pre-migration NC) in a chick embryo is replaced by quail thoracic NC (that do not give rise to enteric nervous system cells), the grafted cells colonize the intestine and differentiate into the appropriate cholinergic, peptidergic and serotonergic neuronal types (Fontaine-Perus et al., 1982, Le Douarin et al., 1975).

Other heterotopic grafts experiments with avian embryos demonstrated that This plasticity is also true for some NC-derived cell populations already located in their definitive territories such as the PNS ganglia. Indeed, satellite glial cells derived from the NC in the nodose cranial ganglion are able to re-migrate when implanted in the migration routes of the trunk NC cells in a younger host chicken embryo. These transplanted non-neuronal cells were then able to give rise to autonomic neurons in the sympathetic ganglia and adrenal medulla cells, and also colonize the enteric ganglia (Ayer-Le Lievre and Le Douarin, 1982). In these experiments, the target sites colonized by the grafted cells are determined by the NC migration routes specific for the axial level where they were placed. Thus, these results reinforce the idea that the NC cells are initially multipotent and possess a broad differentiation potential, which

is influenced by the environment from the migration paths and from their sites of homing.

To understand the mechanisms of segregation of the different NC lineages, it is necessary to analyze the phenotypes obtained from an individual NC cell. In vivo experiments were performed by the injection of vital markers in pre-migratory NC cells, which allowed to follow the progeny phenotypes (Bronner-Fraser and Fraser, 1988, 1989; Fraser and Bronner-Fraser, 1991; Krispin et al., 2010). In vitro, cell clonal cultures from different NC regions and in various culture conditions, allowed the differentiation potential of each cloned NC cells to be revealed (Baroffio et al., 1988; Baroffio et al., 1991; Calloni et al., 2007; Calloni et al., 2009; Sieber-Blum, 1989; Sieber-Blum and Cohen, 1980; Trentin et al., 2004)

1.2.1. Specification and determination of the NC cells *in vivo*

The first experiments of NC lineage tracing in vivo were performed by Bronner-Fraser's group (Bronner-Fraser and Fraser, 1988, 1989, Fraser and Bronner-Fraser, 1991). Experiments in chicken embryos, where individual dorsal NT cells were microinjected with a vital fluorescent marker, showed that the clonal descendants of trunk NC cells participated in the formation of different types of NC derivatives, producing after two days different combinations of neurons (in dorsal root and/or sympathetic, ganglia), glial cells along nerves, melanocytes, and adrenomedullary cells. These results strongly argued in favor the multipotency of pre-migratory NC cells.

Recent experiments of dorsal NT cell tracking, conducted by Kalcheim's laboratory, showed that the final location of trunk NC cells can be predicted by their

dorsoventral position within the pre-migratory domain of the dorsal NT or by their time of delamination. Moreover, the NC cells labeled in the dorsal midline of the NT generated a clonal progeny containing only a single cell type. This work thus concluded that the NC cells have a limited differentiation potential as early as before their emigration (Krispin et al., 2010).

The divergence between the results obtained by these two research groups is probably due to differences between the stages when dorsal NT cells were marked. In the pioneer work (Bronner-Fraser and Fraser, 1988), some of the pre-migratory NC cells must have been followed from an early time point, when the segregation between the NT and the NC cell types, or between the different NC derivatives, had not yet occurred. While in the more recent work (Krispin et al., 2010), NT/NC cells were marked at later stages and generated progeny consisting of only one cell type.

The term “specification” is often used to describe cells that are oriented to become a specific cell type and also have a limited potential to differentiate (Erickson and Harris, 2007). In this Thesis, we consider slightly different denotations for the terms “specification” and “determination”. Specification will refer to “a cell that is competent to make a particular cell type and might begin to differentiate along the pathway to become that cell type, but it might not be committed to that fate” (Livesey and Cepko, 2001). Although specified, a cell is not definitely restricted in its differentiation potential and can be re-specified to another fate. “Determination” or “commitment”, in turn, refers to “an irreversible decision to produce or become a particular cell type” (Livesey and Cepko, 2001) and thus, a cell already determined for a particular fate, if challenged by different environments, can no longer respond to signals driving cell differentiation into another cellular phenotype.

Thus, the interpretation of the work by Kalcheim's group, which conclude that the NC cells have a limited differentiation potential at the premigratory stage, must be discussed. The fact that, before emigration, some NC cells are already specified and have a tendency to follow a particular destination, has been shown previously in a number of studies in vivo (Dorsky et al., 2000; Le Lievre et al., 1980, Raible and Eisen, 1994) and in vitro (Ziller and Le Douarin, 1983). However, cell specification, the first step of commitment to a cellular fate, is not directly related to restrictions in the differentiation potential. Moreover, during their migration, the NC cells are influenced by environmental signals, which could drive them towards alternative fates. To prove that a cell is determined, we must challenge it with another environment and observe the continuation of the initial differentiation process, meaning that the cell can not be diverted to new fate. To test this hypothesis, Krispin and colleagues changed the direction of migration of the NC cells from the ventral path to the dorsolateral pathway, through the electroporation of the melanocytic gene EDNRB2 (endothelin3 receptor B2), that drives dorsolateral migration of melanoblasts (Krispin et al., 2010). Under these conditions, the NC cells initially destined to colonize the PNS, kept the differentiation program originally specified and acquire neural features. EDNRB2 receptor is known to be expressed in already specified melanoblasts, and only these cells are able to enter the dorsolateral melanocytic migration path (Erickson and Goins, 1995; Pla et al., 2005). We can conclude that forced expression of EDNRB2 was not enough to re-specify these NC cells to the melanocytic fate. Thus, the early NC cells were not able to respond to the signals found in this pathway, which does not mean that they would not be responsive to anothers challenging environments. Further experiments would be required to test the irreversibility of early NC cell fate decision.

If a cell is specified for a specific cell fate, does not mean that it can not be re-specified by a different environmental context and follow a different cell fate. This possibility of specification to different destinations is what characterizes the differentiation potential of a cell. Thus, the derivatives obtained from a cell in vivo do not necessarily correspond to their full potential of differentiation, which in fact can only be revealed by in vitro tests, where the cells are exposed to permissive environments.

With an in vitro culture approach, previous studies from our group (Baroffio et al., 1988, 1991; Dupin et al., 1990; Dupin and Le Douarin, 1995; Lahav et al., 1998; Trentin et al., 2004; Calloni et al., 2007, 2009) confirmed the NC multipotent characteristic, showing that these cells do not have their fate already determined before the emigration, since a rich and permissive environment allowed the progeny of a single NC cell to differentiate along multiple phenotypes.

Thus, research on the potential of mesenchymal differentiation in osteoblasts and adipocytes from the trunk NC, objectives of this Thesis work, should include cell cloning experiments in vitro.

1.2.2. The differentiation potential of the neural crest cells *in vitro*

Due to the different cell types that originate from the NC, this cell population is an appropriate model to study the diversification of lineages from multipotent cells. To understand how NC cells interpret the different environment signals and, in particular, to study the progeny of individual cells, several laboratories performed in vitro cell cloning similar to the colony forming unit (CFU) assays developed from the 1950s to decipher the origin of the hematopoietic cell diversity (Spangrude et al., 1988).

In 1975, it was shown that the quail NC cells are able to migrate from NT explants in vitro; these cells could then be resuspended following explant removal and cloned (Cohen and Konigsberg, 1975). These clonal experiments showed, then, that the trunk NC cells are heterogeneous in their potential, and can give rise to non-pigmented, pigmented or mixed progeny (Cohen and Konigsberg, 1975; Sieber-Blum and Cohen, 1980). Both non-pigmented and mixed clones contained several hundreds of sensory and adrenergic neuroblasts, showing that the clonogenic NC cell was at least tripotent (Sieber-Blum, 1989).

The use of novel monoclonal antibodies specific for avian proteins of neurons and glial cells enriched the understanding of the NC cell properties and in vitro clonal analysis of NC cell phenotypes could improve. Further clonal experiments with these improvements were later performed with NC cells of distinct levels of the anteroposterior axis and of different stages. Together, these data confirmed the NC cell heterogeneity, showing that these cells differ in proliferation and differentiation potentials, and most of them produce different combinations of two or more cell types (Sieber-Blum, 1989; Sieber-Blum et al., 1993; Baroffio et al., 1988, 1991; Lahav et al., 1998; Dupin et al., 1990; Trentin et al., 2004; Dupin et al., 2006).

In our laboratory, the clonal culture experiments by Elisabeth Dupin, Nicole Le Douarin and colleagues, in association with the use of specific cell lineage markers, resulted in a hierarchical model of cell diversification in the cephalic and trunk NC. These experiments were performed by micromanipulation of NC precursors, isolated when they arise from the cephalic and trunk quail NT (**Figure 6**), and the NC cells held in most cases were grown on a cellular substrate of 3T3 fibroblasts, which

allowed a high cloning efficiency and the differentiation into the diverse phenotypic repertoire that can be obtained from the NC.

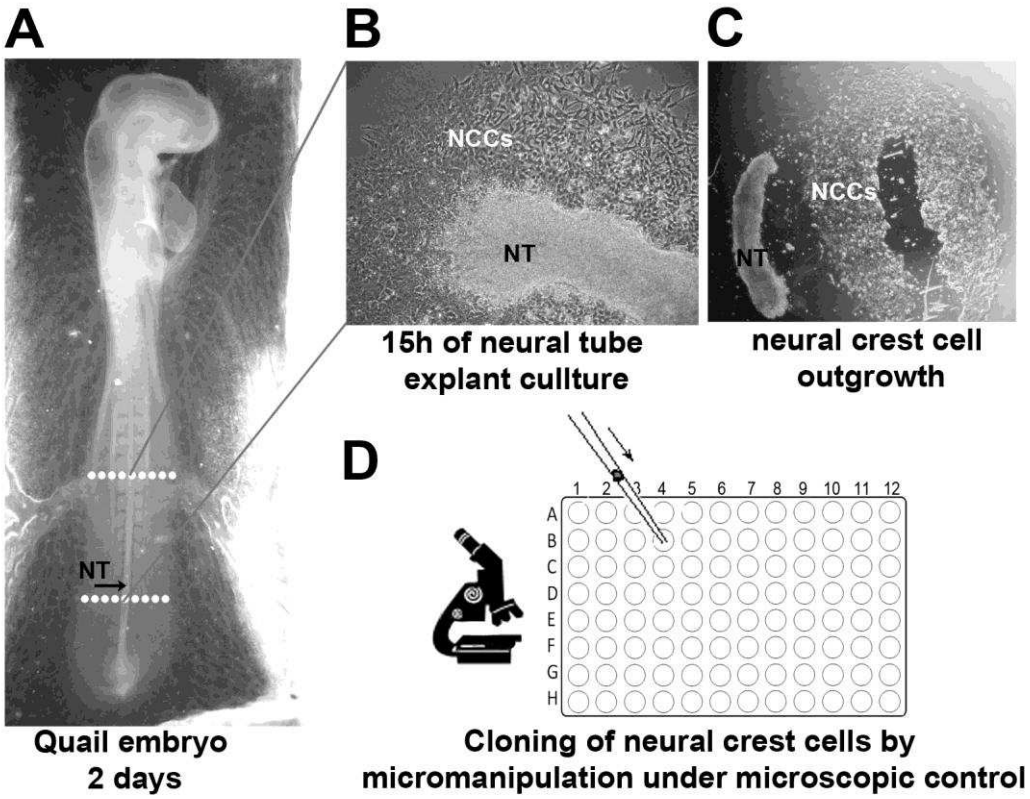

Figure 6 - Trunk neural crest culture scheme. (A) HH14 quail embryo (E2). The trunk region from which the NT is enzymatically isolated from surrounding structures is indicated by dotted lines. (B) Primary culture of NC cells after 15h of migration from NT in vitro. (C) Removal of NT after the time stipulated to obtain the migratory NC cells that are then resuspended with trypsin. (D) Cloning of NC cells by micromanipulation under microscopic control.

Figure 7 summarizes the results obtained with cephalic and trunk quail NC cells (Baroffio et al., 1988, 1991 ; Dupin et al., 1990; Dupin and Le Douarin, 1995; Lahav et al., 1998; Trentin et al., 2004; Calloni et al., 2007; Calloni et al., 2009).

Initially Baroffio and colleagues showed that some mesencephalic NC progenitors generate a diverse progeny, showing both neural and chondrogenic capacities (Baroffio et al., 1991). The current cephalic NC model comprises recent

results that showed, by isolating earlier cephalic NC cells and adjusting the culture conditions, that a large proportion of these cells are multipotent and capable to produce cell types belonging to all major lineages deriving from the NC (neural, melanocytic and mesenchymal, including skeletal) (**Figure 7A**) (Calloni et al., 2007, 2009).

In the trunk NC, it was described multipotent progenitors for 4 phenotypes found in the trunk NC in culture: melanocytes (M), myofibroblasts (F), glial cells (G) and neurons (N) of the PNS (Trentin et al., 2004; Calloni et al., 2007). Recently, our group has shown that trunk NC cells from the quail, an amniote Vertebrate, keep some mesenchymal differentiation potential even if in vivo, these cells do not contribute to mesenchyme (Calloni et al., 2007). Trunk NC cells were able to differentiate into chondrocytes in vitro, although the progenitors with this capability were very rare (about 1 in 200) in single cell cultures when comparing to the cephalic NC (Figure 7B).

The methods of avian NC in vitro cultures have been adapted for mammalian cells, and multipotent progenitors could also be identified in the trunk NC of mouse and rat embryos (Ito and Sieber-Blum, 1993; Shah et al., 1996; Shah et al., 1994; Stemple and Anderson, 1992). Rat trunk NC cells isolated based on expression of the neurotrophin low affinity receptor p75, gave rise to clonal progeny with glial cells, myofibroblasts and autonomic neurons. These NC precursors were able to undergo successive in vitro cloning experiments while maintening the repertoire found in their progeny, which showed the self-renewal capacity of these NC cells (Stemple and Anderson, 1992).

In the quail NC, Trentin and colleagues showed that some bipotent progenitors from mesencephalic and trunk NC are also endowed with self-renewal properties. Thus, glia-melanocyte progenitors (GM) and glia-myofibroblasts (GF) behave as stem cells (Trentin et al., 2004) (**Figure 7B**).

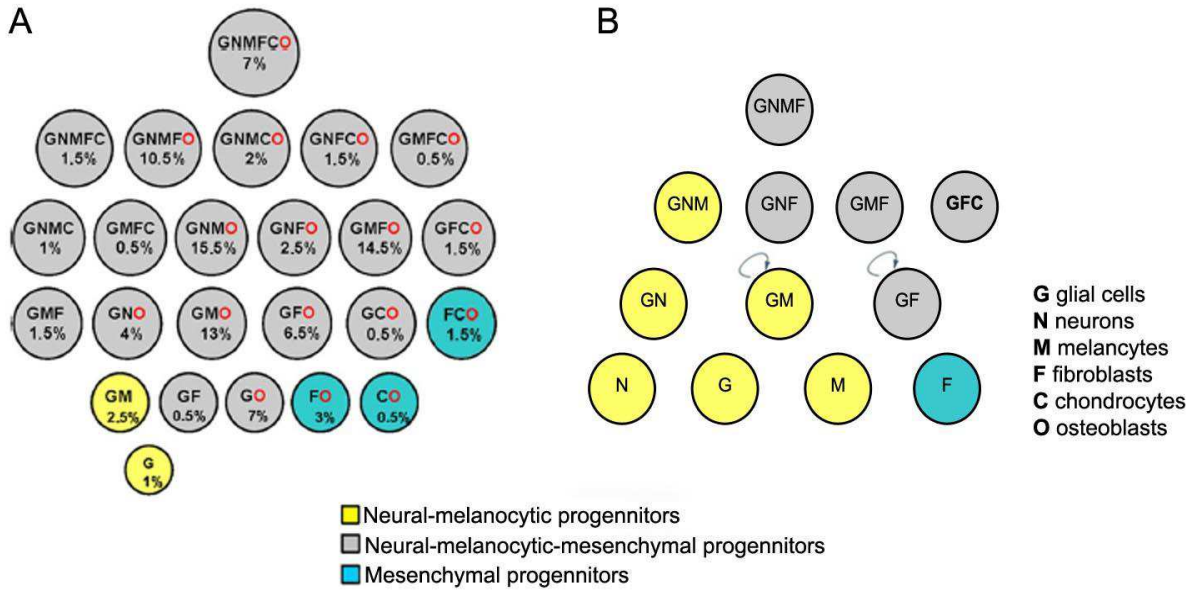

Figure 7 – NC progenitor hierarchy identified by in vitro clonal analysis of quail NC cells. Progenitors are organized according to their number of developmental potentials. (A) In cephalic NC cells, the different cell types arise from a highly multipotent stem cell (GNMFCO), which undergoes progressive restrictions of its potentials to generate intermediate progenitors and finally unipotent cells (Calloni et al., 2009). (B) In the trunk NC, most progenitors generate glia, neurons and melanocytes; chondrogenic clones were very rare. However, many myofibroblastic progenitors are present, including multipotent GNMF progenitors. The self-renewal (arrow) had been shown for some of these progenitors (Trentin et al., 2004; Calloni et al., 2007).

The differentiation potential of the cardiac NC cells was also investigated in quail and mouse. This posterior cephalic NC region is essential for heart septum formation. In colonies derived from the early migratory cardiac NC cells, melanocytes, myofibroblasts, chondrocytes and sensory neurons were identified (Ito

and Sieber-Blum, 1991). Similar results were obtained with the mouse cardiac NC (Youn et al., 2003).

Although some mesenchymal phenotypes had already been reported in the in vitro progeny of trunk NC cells, like myofibroblasts and chondrocytes, it remains unclear whether some trunk NC progenitors are able to give rise to other mesenchymal phenotypes such as osteoblasts and adipocytes. This Thesis work is specially devoted to the investigation of the osteogenic and adipogenic capacity of the trunk NC cells.

1.2.3. Maintenance of the multipotentiality in neural crest cells

The NC is a multipotent structure, and, at the single cell level, some of the NC progenitors have been identified as multipotent cells with self-renewal potential in vitro. These NC cells may therefore be regarded as stem cells. The early NC cells also showed lineage plasticity in vivo, differentiating into other cell types when challenged with new environmental conditions in graft experiments. Furthermore, multipotent NC-derived cells were recently found also in different sites of the post-migratory NC, including some adult tissues (Dupin and Sommer, 2012). These adult progenitors are multipotent in vitro and share at least some of the markers and differentiation properties with early NC progenitors.

These issues have been addressed in details in our recent review "Isolation and differentiation properties of neural crest stem cells" by Dupin and Coelho-Aguiar, accepted by Cytometry A and in press (Annex 1 - page 213).

It is of great importance for the stem cells that they remain undifferentiated and keep their differentiation potential. Some genes have important function in the maintenance and survival of these cells. In multipotent NC progenitors of Xenopus, it was observed that the gene *c-Myc* acts on the cell cycle control, and influences the decision between proliferation and apoptosis through *Id3* (Light et al., 2005). Sox9 also regulates the cell cycle and promotes cell survival of pre-migratory trunk NC, and NC cells of *Sox9* knockout mice undergo apoptosis due to decreased anti-apoptotic factor Snail1 (Cheung et al., 2005).

FoxD3 and *Sox10* genes are directly implicated in the self-renewal and maintenance of the NC multipotent character. *Sox10* expression decreases after NC cell differentiation, except in gliogenesis, where it acts directly to regulate the expression of genes involved in this process. Furthermore, it was shown that Sox10 has a role in maintaining the neurogenic potential and in inhibiting neuronal differentiation in vivo and in vitro (Kim et al., 2003). FoxD3 is also important for NC cell maintenance. In the absence of this transcription factor, the NC cells are not maintained, and the knockout of *FoxD3* in the mouse NC dies around birth with significant reduction or loss of numerous NC-derived populations, like the mesenchymal derivatives of the cephalic NC, cranial ganglia, dorsal root ganglia and NC cells in the gastrointestinal tract (Teng et al., 2008). Thus, Sox10 and FoxD3 are extremely important for maintaining the undifferentiated and multipotent state of the NC.

The Wnt and BMP signaling pathways, beside their early action in the NP border induction, are also involved in the proliferation and self-renewal of pre-migratory NC cells. Although Wnt1 acts by instructing pre-migratory NC cells to a

sensory fate, and BMP promotes autonomic neurogenesis, the combination of these two signaling pathways maintains the expression of p75 and Sox10 markers involved in multipotency, and prevents cell differentiation (Kleber et al., 2005). Multipotent NC cells change their responsivity to these growth factors after reaching their targets, and the cells that remain undifferentiated and proliferative acquire the ability to respond to the mitogenic factor EGF (Fuchs et al., 2009). Thus, the development of multipotent NC cells is regulated by combination of growth factors which reflect specific intracellular signal activity that are constantly changing.

1.3. The evolution of the NC and its skeletal derivatives

The NC is a structure specific of the Vertebrates, and its evolution is closely linked to the evolution of this group. Because of its great differentiation potential and multiple derivatives, it was proposed that the NC might be considered a “fourth” germ layer, since the appearance of this structure was the basis for the development of organs and tissues specific of Vertebrates (Hall, 2000). The NC produces a variety of cells and tissues even greater than the mesoderm, including neural cells, skeletal cells, conjunctive tissue, cardiac, dental and endocrine cells, and pigmented cells of the skin.

In 1983, Northcutt and Gans put forward the of hypothesis of the "new head”, which proposes that new structures derived from the neuroectoderm, i.e., NC and placodes, allowed the emergence of the rostral head of Vertebrates, including sensory organs, forebrain, part of skull, pre-chordal chondrocranium, and the maxillar and mandibular face regions (Gans and Northcutt, 1983). Furthermore, the appearance of NC and placodes was crucial to acquire a predatory lifestyle, when the filter nutrition way of invertebrate Chordates was changed for suction-feeding, due to

the development of a well muscled pharynx. Indeed, it was later shown that the pre-chordal skull and at least a large part of neurocranium are derived from the NC (Couly et al., 1992; Couly et al., 1993). The skull evolution from the NC was also important for the development of sensory organs and the increase in brain volume. In addition, the NC has crucial influence on NT morphogenesis and patterning during brain formation (Creuzet et al., 2006). Because of its key role in Vertebrate evolution, many studies are aimed at understanding the NC cell origin. The main models for the study of NC origin during evolution, are lampreys (cyclostomes Vertebrates), ascidians (Tunicates) and the amphioxus (belonging to Cephalochordates, that is invertebrate Chordates phylogenetically close to Vertebrates) (**Figure 8**). Amphioxus genome analysis revealed that these animals have genes homologous to most of those involved in NC induction (Yu, 2010).

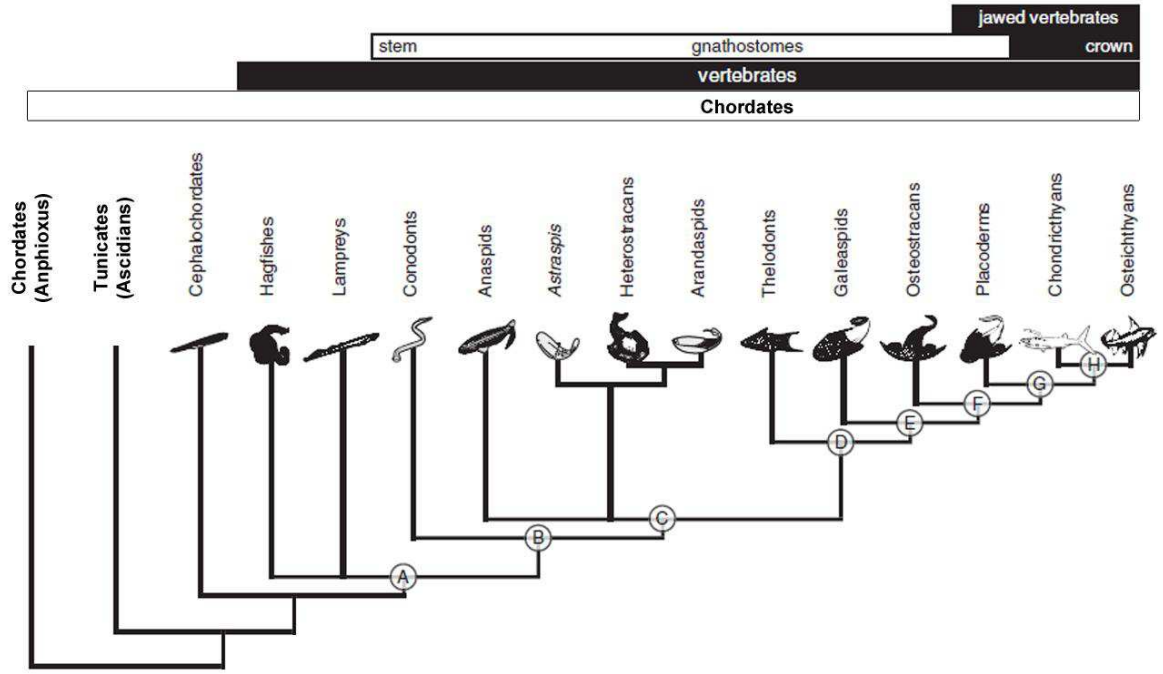

Figure 8 - Phylogenetic tree of Chordates. Current phylogeny, showing that Tunicates (ascidians) form the sister group of Vertebrates, while Cephalochordates (amphioxus) are the most basal chordates. The letters indicate important steps in the vertebrate skeleton evolution. (A) Origin of a skeleton in Vertebrates, including notochord, fin rays, and a skull, although entirely composed of non-mineralized cartilage. (B) Origin of the mineralized skeleton, dentine and enamel comprising the odontodes. (C) Origin of a dermal mineralized skeleton composed of odontodes supported by bone, placing mineralization on the collagenous layers of dermis. (D) Odontodes associated with viscerocranium, including branchial arches and nasal openings. (E) Origin of a mineralized neurocranium composed of calcified cartilage. (F) Mineralized neurocranium; celllular intramembranous bone first found in the dermal skeleton. (G) Visceroskull, axial and appendicular skeleton and fin rays mineralized. (H) Endochondral bone. (modified from Donoghue et al., 2006 and Baker, 2008).

Based on these data, the most parsimonious explanation for the NC evolution is that these cells developed from modification of the pre-existing neuroepithelial gene network, through the acquisition of genes already present in the common ancestor of all Chordates (Baker, 2008). However, many molecules associated with the specification and differentiation of the NC derivatives exist only in Vertebrates. Thus, the current hypothesis divides the evolution of the NC in two events: (1) The initial NC gene regulatory network assembly, and (2) the diversification of NC derivatives during Vertebrate evolution (Yu, 2010).

The comparison between invertebrate Chordates and Vertebrates of the expression of homologous genes that act in inducing the NC, showed that the NP borders of ascidians and amphioxus present expression of NP border specifier genes, such as *Dlx, Msx, Zic, Pax3* and *Pax7*. However, the NC specifier genes are not expressed after the NP border specifier genes. These genes are expressed in mesoderm and/or endoderm, and the only one that is also induced in the NP border is *Snail*, which probably acts on cell survival and cell shape changes (Langeland et al., 1998). The amphioxus *FoxD* gene, for example, is expressed in the mesoderm

and when electroporated in chicken, the *FoxD* regulatory region induces the expression of the associated reporter gene in the mesoderm, but not in the NC cells (Yu et al., 2008). Thus, the recruitment of mesendodermal genes to the NP border was an important step for the complete assembly of the gene network necessary for the NC induction. NC specifier gene acquisition must have conferred new properties to the dorsal neuroepithelial cells, like multipotency, delamination and migration.

Recently, NC-like cells, able to migrate and differentiate into pigmented cells, have been described in different species of ascidians, suggesting that the emergence of the NC may have occurred prior to the development of Vertebrates (Jeffery et al., 2004). However, in the ascidian *Ciona intestinalis*, these pigment cells are not derived from the NP border, but from a nearby region, originating from A7.6 blastomeres, which do not express NP border specifier genes (Jeffery et al., 2008). Moreover, these A7.6 cells and their A6.3 blastomeric precursors, which also generate mesodermal and endodermal cells, express NC specifier and NC effector genes. Although the homology between these NC-like cells of Tunicates and the vertebrate NC cells is still under discussion, these data show that the assembly of NC specifier and NC effector genes evolutionarily arose before ascidians and after amphioxus. However, the acquisition of these genes by the NP border occurred only in Vertebrates.

After the evolution of NC cells from the neuroepithelial cells and their appearance in Vertebrates, further changes led to the many different NC cell types. In fact, evolution of the NC derivatives occurred in parallel with vertebrate evolution. The most primitive Vertebrates, lampreys and hagfishes, do not present some NC derivatives such as sympathetic ganglia, dentin and bone. However, lampreys and

hagfishes already acquired mesenchymal derivatives (cranial and pharyngeal cartilages) of endodermal origin. In fact, *SoxE* and *collagen-A* (similar to collagen2a1 of amniote Vertebrates) are expressed in the endodermal pharynx of amphioxus and hemichordates, suggesting that a cartilaginous soxE-dependent skeleton appeared at least in Deuterostomes.

Although the current hypothesis for the skeletal NC evolution points to acquisition of a pre-existing network from endodermal and/or mesodermal cells, the expression of cartilage-specific genes is also present in ectodermal structures (Baker, 2008). Pre-migratory and migratory NC cells, as well as their glial derivatives, express *Sox9, Sox5* and *Sox6*, which cooperate to activate *collagen2a1* and *aggrecan*. Furthermore, cartilage-specific proteins, such as aggrecan and collagen2a1 are also expressed in the brain and spinal cord during development. In amphioxus, SoxE and collagenA are also expressed throughout the NT development. Thus, it is possible that evolution of the NC-derived cartilage resulted from the modification of a neuroepithelial pre-existing regulatory gene network, with subsequent acquisition of this gene network by the mesoderm. In this case, the pharyngeal acellular cartilage derived from the endoderm in amphioxus and hemichordates, would represent a co-independent choice of the regulatory gene network of primitive ectoderm (Baker, 2008).

Studies of fossils suggested a rapid emergence of other ectomesenchymal phenotypes after lamprey and hagfish phylogenetic divergence. The first dentin, the mineralized structure secreted by odontoblasts, is found in the odontodes of the vertebrate fossils, Conodonts. The first bone appeared in Anaspids (Ostracodermal fishes) with dentin connected to the superficial dermal armor (Baker, 2008). Thus

these studies indicated that the earliest mineralized components in Vertebrates, the odontodes of Conodonts, originated from the NC. Enamel and dentin are considered markers of a NC origin (Sansom et al., 1992). Vertebrate fossils of Agnates from the initial Cambrian period, thus possessed a dermal ossified armor with a layer of material similar to dentin. Among the known vertebrate fossils, Arandaspids are the first which presented this kind of mineralized skeleton, but they have only the membranous exoskeletal bone and their endoskeleton remains cartilaginous (Sansom et al., 2005; Smith, 1991). These early Vertebrates with mineralized skeleton are called Ostracoderms. Most Ostracoderms like Arandaspids do not have calcified endoskeleton; however, two ostracoderm groups, the Galeaspids and Osteostracans had a calcified endoskeleton (Janvier, 2008) (**Figure 8**).

In amniote Vertebrates, the NC-derived skeleton is reduced to the skull and facial bones and cartilages, while in teleost fishes, such as zebrafish, the trunk NC produces calcified tissue and is at the origin of the bony rays of dorsal and caudal fins (Smith et al., 1994, Mari-Beffa et al., 2010). The loss of dermal mineralized armor during vertebrate evolution coincided with the appearance of the somitic sclerotome that gives rise to the backbone.

Thus, little is known about the mesenchymal potential of the trunk NC in amniote Vertebrates. Some studies suggest a limited potential, which possibly has been retained in the trunk NC of higher Vertebrates. This Thesis work is interested to reveal the extent of this potential by establishing new culture methods to analyze the mesenchymal developmental potentials of NC cells and thereby provide further insights in understanding the evolution of NC-derived cell types.

1.4. Skeletal and adipogenic differentiation

In higher Vertebrates, the embryonic mesenchymal derivatives differentiate from the cephalic NC, which gives rise to most of the head mesenchyme, and from the mesoderm, which forms all other mesenchymal tissues. Among the mesenchymal tissues we found dermis, tendon, muscle, skeletal elements with bones and cartilages, and the adipose tissue (Le Douarin et al., 2004).

In this chapter, we will present the different mechanisms of skeletal celland adipocyte differentiation, so that we can understand how an undifferentiated mesenchymal cell becomes committed to these cell fates. Then, we discuss the mechanisms that drive the mesenchymal differentiation process from the NC cells.

1.4.1. Endochondral and membranous ossification

1.4.1.1. Bone differentiation steps

In the adult organism, the proper functioning of the bones relies on continuous renewal of this tissue, called remodeling. The osteoclast is an hematopoietic cell type of monocytic origin, responsible for bone resorption, while osteoblasts rebuild the resorbed bone by the production of extracellular matrix that subsequently becomes mineralized (Karsenty and Wagner, 2002).

The appendicular (limbs skeleton) and axial (vertebrae and ribs) skeletons originate from the lateral plate and paraxial mesoderm, respectively. The vertebrate skull consists of the neurocranium (the base of skull and the cranial vault) and the viscerocranium (upper jaw, lower jaw and other BA derivatives), and is formed by the head mesenchyme derived from two different embryonic sources: the mesoderm and the NC.

Bone formation can occur by two mechanisms, the endochondral ossification, which occurs in long bones of the limbs, the basal portion of the skull, vertebrae, ribs and

part of the medial clavicle (**Figure 9F-9J**), and the intramembranous ossification, which occurs in several craniofacial bones and on the side of the clavicle (**Figure 9A-9E**).

The endochondral ossification occurs by the formation of a cartilaginous skeleton, which is then replaced by bone built by the osteoblasts. Chondrogenesis begins when part of the condensed mesenchymal cells (**Figure 9F**) start to express the transcription factor *Sox9* [SRY (sex determining region Y)-box 9] (Akiyama et al., 2002), which induces the expression of collagen2a1 and that of the proteoglycan aggrecan (Lefebvre et al., 1997), leading to differentiation of proliferative chondrocytes (**Figure 9G**). Shortly after, the more central chondrocytes stop proliferating, become pre-hypertrophic, stop collagen2a1 synthesis and start producing collagen10. When they become hypertrophic (**Figure 9H**), the chondrocytes begin to express osteopontin, bonesialoproteinII, and MMP13 (matrix metallopeptidase13) (Inada et al., 1999). Subsequently the chondrocytes undergo cell death and trigge blood vessel invasion, which bring osteoblasts to the center of the cartilage template (**Figure 9I**). These osteoblasts produce a collagen1a1 extracellular matrix on the initial collagen10 matrix. This process occurs in a centrifuge ossification, and chondrocytes adjacent to the ossification region continue to proliferate following this differentiation process. Thus, proliferative and hypertrophic chondrocytes are organized into columns in an avascular structure called growth plate, which is found during development at the bone extremities and is important for the long bone formation and growth (Karsenty and Wagner, 2002; Kronenberg, 2003). Furthermore, the chondrocytes are also needed at the joints to allow mobility of the different skeletal elements.

Osteoblasts, in the case of endochondral ossification, differentiate from osteoprogenitors in the perichondrium region, around the proliferative chondrocytes area (**Figure 9I**). These osteoprogenitors express Runx2 (runt-related transcription factor 2), the determining transcription factor for osteoblast differentiation, which

induces the expression of collagen1a1 after the arrival of these cells in the center of the cartilage mold (Ducy et al., 1997). In membranous ossification (**Figure 9A-9E**), osteoblasts differentiate directly from the condensed mesenchyme (Karsenty et al., 2009). In this process, the condensed mesenchymal cells thus express *Runx2* gene irrespective of the presence of cartilage nodule, and thereafter Runx2 induces collagen1a1. Mesenchymal cells and immature pre-osteoblasts have a poor expression of collagen1a1, which is increased in immature osteoblasts (Inada et al., 1999). During differentiation, osteoblasts display osteopontin and osteonectin expression. The later stage is characterized by the expression of alkaline phosphatase and osteocalcin, and by extracellular matrix mineralization (Nakashima and de Crombrugghe, 2003).

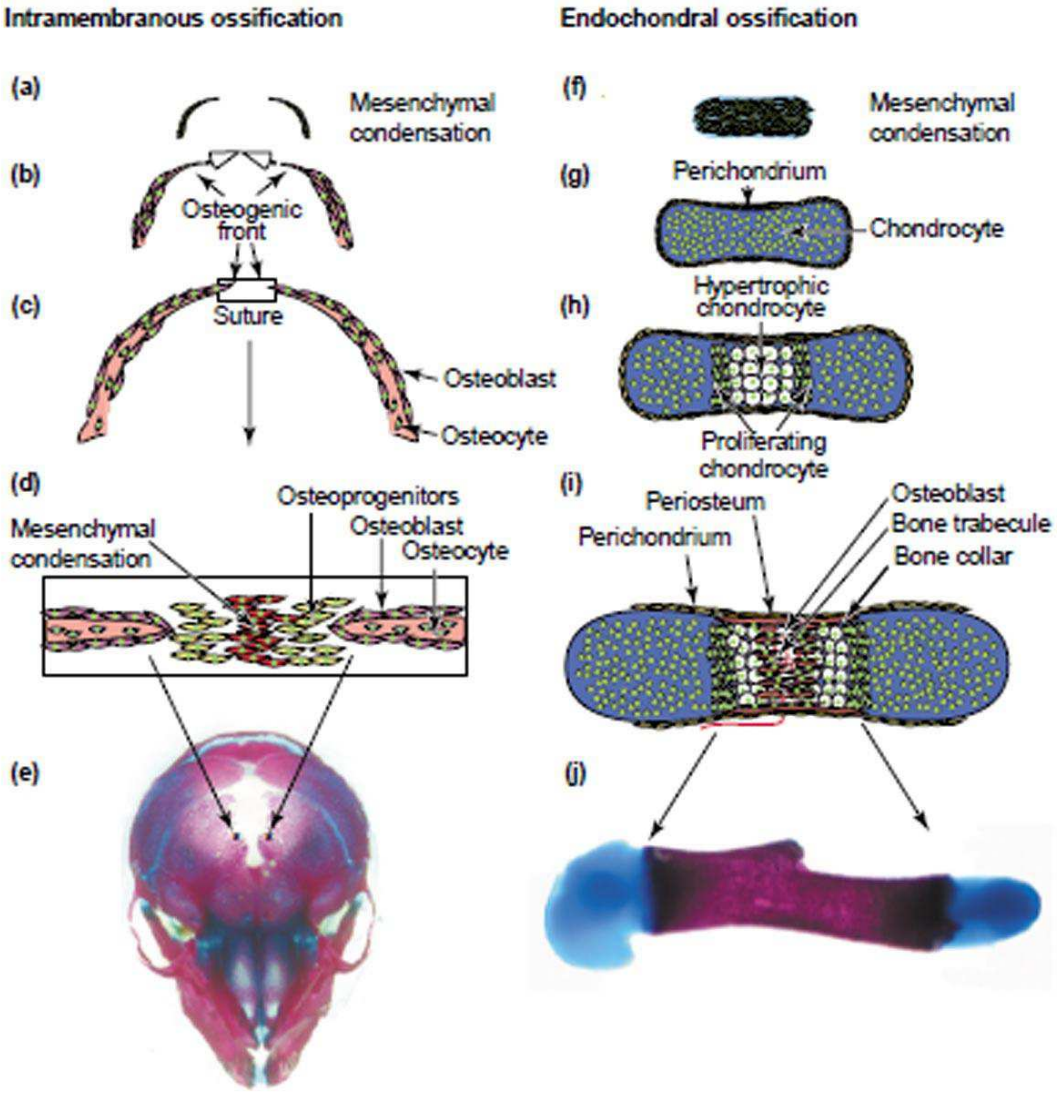

Figura 9 - **Intramembranous and endochondral ossification**. (a–d) Craniofacial bones are formed directly from condensations of mesenchymal cells. (a) Formation of frontal bones starts in mesenchymal condensations on the lateral side of the head. (b) From the mesenchymal condensation, the cellular mass with osteogenic activity spreads upward toward the top of the skull (arrows). Osteoblasts differentiate and produce bone matrix. (c) After, the ends of the cellular masses that originate on each side meet at the midline, where a suture is formed. (d) In the suture, cells differentiate into osteoprogenitor cells. Osteoprogenitors then differentiate into osteoblasts that produce bone-matrix molecules. Osteoblasts differentiate into osteocytes, which become embedded within the bone matrix. (e) A schematic frontal view of a mouse skull (E18.5) stained with alizarin red and alcian blue. Asterisks indicate osteogenic fronts of frontal bones. (f–j) By contrast, long bones are formed by endochondral ossification. (f) Development of endochondral bones starts with the formation of mesenchymal condensations. (g) These cells give rise to chondrocytes whereas cells

at the periphery form a perichondrium. (h) Proliferative chondrocytes exite the cell cycle and differentiate into hypertrophic chondrocytes. The sequential differentiation of chondrocytes establishes the epiphyseal growth plate. (i) At the distal end of the epiphyseal growth plate, hypertrophic chondrocytes further mineralize their own cartilaginous matrix. In parallel, cells from a thin layer of mesenchymal cells, called the periosteum, invades the zone of hypertrophic chondrocytes in addition to blood vessels and osteoclasts. The partially degraded, mineralized cartilage matrix forms a template for deposition of bone matrix by osteoblasts. Endochondral bone contains an outer envelop of cortical bone (the bone collar) that surrounds the marrow cavity and growth plate. The cortical bone is contiguous with the perichondrium. (j) A mouse humerus stained with alizarin red and alcian blue (Nakashima and Crombrugghe, 2003).

1.4.1.2. Molecular control of osteogenesis

Among the major transcription factors that act in the ossification process, we found *Sox9* and *Runx2.* Sox9 is a transcription factor that has an HMG (high mobility group)-box domain of DNA binding, and acts in the activation of specific genes in chondrocytes, *collagen2a1, collagen11a2* and *aggrecan* (Bridgewater et al., 1998; Lefebvre et al., 1997). Sox9 is expressed in all chondroprogenitors and chondrocytes except hypertrophic chondrocytes. Mutation of *Sox9* in heterozygous human causes campomelic dysplasia, characterized by general endochondral bone hypoplasia (Wagner et al., 1994). *Sox9* inactivation in the limb bud results in the absence of mesenchymal condensation and hence impairs the formation of cartilage and bone. Sox9 action leads to the formation of the cartilage mold, where it operates with Sox5 and Sox6 in cartilaginous matrix production (Akiyama et al., 2002). Thus, Sox9 is necessary before and after mesenchymal condensation.

Runx2 is the first specific transcription factor of osteoblasts to have been identified (Ducy et al., 1997; Komori et al., 1997). Runx2 belongs to the Runt transcription factor family and acts through its Runt domain that binds to DNA and nuclear proteins. *Runx2* is an essential gene in osteoblast differentiation, as shown in the knockout mice, in

which osteoblasts do not produce differentiated skeletal elements (Komori et al. 1997, Ducy et al., 1997). This transcription factor directs multipotent mesenchymal cells to the osteoblastic lineage by activating the expression of many gene promoters, including that of *collagen1a1, osteopontin, osteocalcin* and *Mmp13* genes. Runx2 maintains the osteoblasts in an immature stage, and its overexpression inhibits the maturation of osteoblasts and reduces the expression of *collagen1a1* and *osteocalcin* genes. Furthermore, this transcription factor, together with Runx3, is necessary for the maturation of chondrocytes and regulates expression of *collagen10, ostepontin, bonesialoprotein2* and *Mmp13* genes in hypertrophic chondrocytes (Komori, 2010). Studies with the Sox9-Cre/ROSA26 mice that express *βgalactosidade* driven by the *Sox9* promoter, revealed that *Sox9* expression precedes that of *Runx2* during skeletogenesis. Mesenchymal Sox9-positive cells in the limb bud give rise to chondrocytes and osteoblasts. Furthermore, alizarin red-stained membranous skeletal elements are missing in transgenic mice where the gene *osterix* (a transcription factor required for the differentiation of osteoblasts down stream of Runx2) is inactivated in cells expressing Sox9; this finding indicates that Sox9-positive cells are the progenitors of osteoblasts also in intramembranous bones (Akiyama et al., 2005). Runx2 is regulated by several genes, but also by proteins that interact with it. Among the genes regulating *Runx2*, are *Sox8, Dlx5*, the homeobox gene *Msx2* (msh-like 2) and the homeobox gene *Bapx1*. Sox8 negatively regulates *Runx2* and attenuates osteoblastogenesis (Schmidt et al., 2005), while Dlx5 and Bapx1 are positive regulators (Tribioli and Lufkin, 1999; Lee et al., 2005; Holleville et al., 2007). Msx2 promotes proliferation and differentiation of osteoblasts. However, in mature osteoblasts in vitro, it inhibits the activity of *Runx2* (Newberry et al., 1998; Shirakabe et al., 2001).

The regulatory proteins of Runx2 include Twist1/2, which bind to *Runx2* and prevent access to the DNA, thereby inhibiting the early differentiation of osteoblasts (Twist1 acts in the skull and Twist2 in the appendicular skeleton) (Bialek et al., 2004) and SATB2 (binding protein of special aT-rich sequence 2), which interacts with Runx2, increasing its activity (Dobreva et al., 2006).

Although Runx2 expression is essential and indispensable for osteogenesis, osteoprogenitors expressing this transcription factor are not yet fully committed to become osteoblasts, and they require the action of the transcription factor Osterix. It is considered the second specific transcription factor of osteoblasts, and belongs to the Sp family of zinc finger-type kruppel proteins. Osterix is present in all osteoblast progenitors and required for engagement towards the osteoblastic lineage, and thus Osterix drives progenitor differentiation into osteoblasts, instead of chondrocytes (Nakashima et al., 2002). Osterix is known to be activated by Runx2, but recent work has shown that it can also be activated downstream of Msx2 in mesenchymal cells that are deficient for *Runx2* (Matsubara et al., 2008). Thus, Osterix can be regulated by Runx2-dependent and Runx2-independent mechanisms.

This sequential activation of transcription factors during bone formation is regulated and guided by different signaling pathways that act at different stages of the ossification process. The osteoblastic differentiation involves the Ihh, Fgf, Notch, Wnt and BMP signaling pathways. In endochondral ossification, chondrocyte maturation and osteoblast differentiation are interconnected by Ihh signaling, which is expressed by chondrocytes and induces *Runx2* expression in adjacent perichondrial cells (Hartmann, 2009). The transcription factor Runx2 induces FGF18, which, besides acting in the development of chondrocytes, also plays a role in the

differentiation of perichondrial osteoprogenitors (Liu et al., 2002). Wnt prevents osteoprogenitor differentiation into chondrocytes and thus promotes the bone differentiation process (Day et al., 2005; Hill et al., 2005). In the developing limb, Wnt also inhibits chondrogenesis, and FGF pathway is essential for the continuous growth of the limb bud, as it promotes the survival of mesenchymal cells. Furthermore, the combination of Wnt and FGF has a different effect. Together, they drive cell proliferation and the maintenance of undifferentiated state in mesenchymal cells with chondrogenic differentiation ability (ten Berge et al., 2008).

The Notch pathway also influences osteogenesis. The intracellular domain of Notch (NICD) goes to the nucleus where its binds to Runx2 and inhibits its activity (Engin et al., 2008, Hilton et al., 2008). BMP proteins are known to induce osteoblastic differentiation of pluripotent mesenchymal cells such as bone marrow stromal cells, suture cells in primary cultures of skull and mesenchymal cell lineages (Franceschi, 1999). Furthermore, Hollevile and coworkers showed that the gene *Dlx5* (Distal less homeobox-5), induced by BMP2 in osteogenic calvarial suture cells, leads to expression of *Runx2*, and thus initiates osteogenesis (Holleville et al., 2003; Holleville et al., 2007). During morphogenesis of the jaw, mesenchyme-derived BMP4 dictates the onset of osteogenesis (Merril et al., 2008).

The ossification process of NC cells is discussed in more details in section 1.4.3.

1.4.1.3. Bone matrix mineralization

During bone formation, chondrocytes and osteoblasts mineralize their extracellular matrices. This process starts with the initial formation of hydroxyapatite crystals within the

matrix vesicles (Anderson, 2003). The matrix vesicles germinate from specific regions of the plasma membrane. The formation of the first mineral crystals is increased by matrix vesicle phosphatase activity, such as alkaline phosphatase (ALP), adenosine triphosphate, and pyrophosphatase, and calcium chelating-molecules, found within or near the membrane vesicles. The next step is the secretion of preformed hydroxyapatite crystals. The extracellular fluid typically contains calcium and phosphate ions, sufficient for the continuous production of crystals, with preformed crystals serving as templates for the formation of new ones through a process of homologous nucleation (Anderson, 2003). This mineralization process depends on the balance between a variety of factors, such as calcium and inorganic phosphate (Pi), matrix proteins and inhibitors of mineralization, such as inorganic pyrophosphate (PPi), osteopontin and osteocalcin (Meyer, 1984). In bone, ALP is confined to the cellular surface of osteoblasts and chondrocytes, including the membranes of matrix vesicles. ALP generates Pi for hydroxyapatite crystallization and also hydrolyses PPi (Fallon et al., 1980; Rezende et al., 1994).

To observe the mineralization process of chondrocytes and osteoblasts, we need to provide the necessary conditions. Ascorbic acid (AA), β-glycerolphosphate (βGP) and dexamethasone (Dex), are components known to favor the osteoblastic phenotype expression in various bone cell culture systems (Coelho and Fernandes, 1999). βGP induces mineralization because it is hydrolyzed by ALP to produce high levels of phosphate ions Pi, thus providing the required chemical conditions for mineral deposition (Bellows et al., 1991). The presence of dexamethasone results in increased proliferation and increased expression of ALP by osteoblasts (Coelho and Fernandes, 1999). In ROS17/2.8 osteoblastic cell line, dexamethasone induces mineralization. In these experiments, dexamethasone increased the transcriptional activity of *Runx2*, which in turn induced an increased expression of osteocalcin and osteopontin (Mikami et al., 2007). The presence of

AA promote synthesis of collagen. Data also suggest that the increased accumulation of extracellular matrix leads to a higher ALP activity and the ability to form mineralized matrix (Franceschi, 1992).

The bone differentiation process until its mineralization step will be investigated in this Thesis in cultured trunk NC cells.

1.4.2. Adipogenesis

Adipocytes store and mobilize fat in response to various hormones. Adipose tissues can be classified into two types: -white, whose main function is the energy stock of fat, and -brown, which burns fat to produce heat. Furthermore, adipocytes have a very important role due to their autocrine, paracrine and endocrine properties, such as secretion of leptin and adiponectin (War et al., 1998; Arner et al., 2011).

Adipocytes originate from mesenchymal progenitors, which, by still poorly understood mechanisms, differentiate into preadipocytes and subsequently into mature adipocytes that store lipids. This process is called adipogenesis and occurs during embryonic development and also later, in the postnatal period. In adulthood, approximately 10% of the adipocytes are renewed each year (Rosen and MacDougald, 2006; Spaldin et al., 2008).

In the trunk and limbs, the mesenchyme is of mesodermal origin, so it is assumed that the fatty tissues that develop in these locations are derived from the mesoderm, although few regions of adipose tissue had their progenitors yet identified during development (Billon et al., 2007). Experiments of NT transplantation in avian embryos revealed that the NC cells may be the source of adipocytes in some areas

of the face and neck (Le Lievre and Le Douarin, 1975), which was confirmed in mice for the adipocytic deposits near the salivary glands and the ears (Billon et al., 2007).

The commitment of mesenchymal cells to the adipocytic lineage is poorly understood, sinde most studies have been performed in vitro with preadipocyte cell lines. Pref-1 (pre-adipocyte factor 1 or DLK1, delta-like 1) is considered the first marker of preadipocytes. The expression of this transmembrane protein is expected to decrease during progression of the differentiation process and is absent in mature adipocytes. Indeed, constitutive expression of *Pref-1* gene inhibits differentiation of adipocytes, and its absence increases adipogenesis in vitro (Smas and Sul, 1993; Smas et al, 1999).

The differentiation of preadipocytes into adipocytes has been extensively studied in vitro (Otto and Lane, 2005; Rosen and MacDougald, 2006), mainly due to the establishment of cell lines of preadipocytes derived from the dissociated mouse embryos or from adult adipose tissue (Green and Kehinde, 1975, 1976, Green and Meuth, 1974; Negrel et al., 1978). This differentiation is driven by the sequential expression of a set of transcription factors (**Figure 10**). The accumulation of cAMP (cyclic adenosine monophosphate) in preadipocytes leads to increased phosphorylation of CREB (cAMP-responsive element linker). This, in turn, activates the transcription of C/EBPβ (CCAAT-enhancer element to the connector). C/EBPβ induces the expression of KLF5 (Kruppel-like factor 5), and both promote the expression of two key adipogenic transcription factors, C/EBPα and PPARγ2 (peroxisome proliferator-activated receptor). Moreover, the latter two factors mutually stimulate one another. C/EBPα and PPARγ2, along with SREBP1c (binding protein of the sterol regulatory element 1c), coordinate activation of genes responsible for

adipocyte maturation, involved in the metabolism of lipids and carbohydrates, such as FABP4 (fatty acid binding protein 4), GLUT4 (responsive glucose transporter insulin 4) and glycerolphosphate dehydrogenase (Rosen et al., 2000; Farmer, 2006). The terminal differentiation is accompanied by changes in cytoskeleton and extracellular matrix protein expression (Farmer and Spiegelman, 1982). Importantly, PPARγ is the only transcription factor necessary and sufficient to induce the maturation of adipocytes (MacDougald and Lane, 1995).

The preadipocytes differentiation can usually be stimulated in vitro by some extrinsic factors, which are usually most effective when combined into differentiation cocktails. Among these factors, IBMX (3-isobutyl-1-methylxantine) is a phosphodiesterase inhibitor, which inhibits cAMP degradation. Another component is dexamethasone, a glucocorticoid responsible for the expression of C/EBPδ, which acts with C/EBPβ to induce PPARγ2 and C/EBPα. Insulin is used at overphysiological concentrations to activate the receptor IGFR1 (insulin-like growth factor receptor type 1) and to induce SREBP1c (Smith et al., 1988). Finally, T3 (tri-iodo-thyronine) hormone acts on PPARγ expression through the nuclear receptor of thyroid hormone (Mishra et al., 2010). Recently, pharmacological agonists of PPARγ, such as rosiglitazone, have been used in the adipogenic differentiation cocktails (Albrektsen et al., 2002). Besides acting in preadipocyte differentiation, some of these factors also act on the commitment of mesenchymal cells to the adipocytic lineage. There are also some evidence for the function of dexamethasone, insulin and IBMX in preadipocytesinduction (Pantoja et al., 2008, Yang et al., 2008).

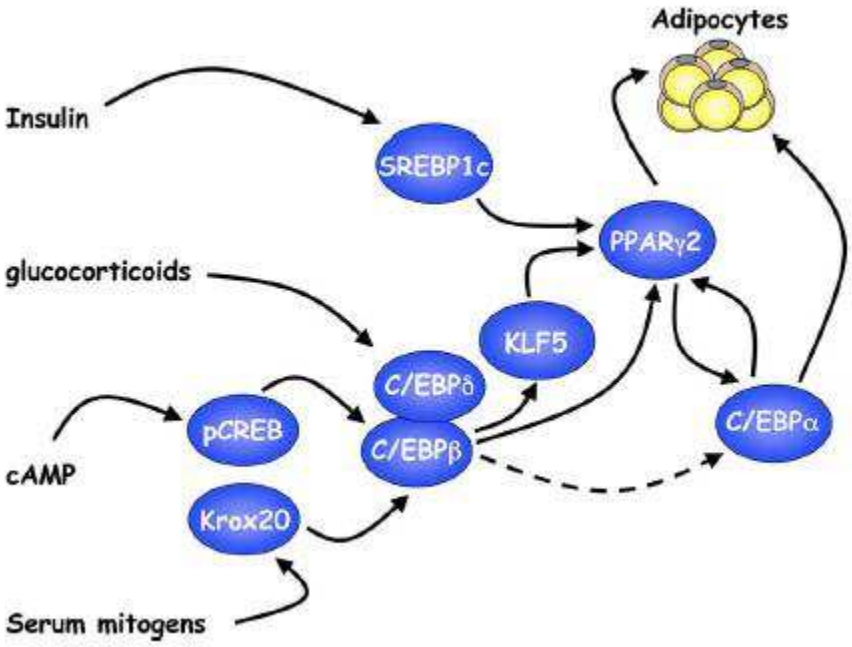

Figure 10 - Induction of adipogenesis by a cascade of transcription factors. Exposure of preadipocytes to adipogenic cocktail consisting of insulin, glucocorticoids, IBMX (agent which elevates cAMP) and fetal bovine serum. The cocktail activates the expression of several transcription factors that converge on PPARγ. PPARγ then induces the expression of C/EBPα. Together, these factors control terminal adipogenesis (Farmer, 2006).

The balance between bone and adipocyte differentiation is not known during the mesenchymal differentiation of the NC. In the bone marrow, differentiation of mesenchymal cell into adipocytes and osteoblasts is competitively balanced. Mechanisms that promote one cell fate, actively suppress the mechanisms that induce the other one. This occurs through cross-talk between different signaling pathways, and between transcription factors regulating the differentiation of osteoblasts and adipocytes, including Runx2 and PPARγ, respectively (Muruganandan et al., 2009). Thus, depending on the conditions, progenitors can undergo mesenchymal differentiation into either osteoblasts or adipocytes.

In this Thesis, we studied the in vitro differentiation potential of trunk NC into different mesenchymal phenotypes, including their differentiation into adipocytes.

1.4.3. Skeletogenic differentiation of the neural crest cells

During differentiation of the cephalic NC cells, cells that migrate to the ventral regions are intended to form ectomesenchymal derivatives, while cells that hold more close to the dorsal NT differentiate into PNS neurons and glial cells that form the cranial ganglia. Cells migrating later stop their migration in more dorsal positions, and thus form only non-mesenchymal cell types (Baker et al., 1997).

The mechanisms that segregate and induce these NC cell types begin to be unraveled. Blentic and coworkers showed that the NC cells assume a ectomesenchymal phenotype when they enter the BA and stop to express the early NC markers *Sox10* and *FoxD3*, to initiate *Dlx2* expression indicative of mesenchymal phenotypes. FGF is important in this process, since cells insensitive to FGF (eletroporated with a dominant negative FGFR1 construct) do not stop to express Sox10 and *FoxD3*, even after entering the BA, and do not start to express *Dlx2* (Blentic et al., 2008). Recently it was shown that FoxD3 acts to restrict the fate of the NC and that the loss of this transcription factor guides the NC cells to a mesenchymal fate (Mundell and Labosky, 2011). In vitro, FGF2 induces endochondral and membranous bone differentiation of the cephalic NC (Sarkar et al., 2001). Likewise, activating mutations of the receptors FGFR1 and FGFR2 induce chondrogenesis in these cells (Petiot et al., 2002).

The cells that migrate to the first BA give rise to most of the maxilla and mandible, to the side of the skull, the palate and middle ear bones. The formation of the jaw involves the growth factors BMP4, FGF8 and Shh. The presence of these molecules in the first BA ectoderm regulates differentiation of Meckel's cartilage. The conditional knockout for BMP4 in this region results in almost complete loss of the jaw (Liu et al., 2005). It has also been shown that the control of *FGF8* expression by BMP4 occurs in a concentration-dependent manner, with high levels of BMP4 repressing the expression of *FGF8* and low BMP4 promoting transcription of this gene (Liu et al., 2005). The balance between FGF8 and BMP4 levels is crucial for the formation and extent of Meckel's cartilage (Liu et al., 2005; Sttotman et al., 2001). Shh has two key roles in the formation of the jaw. This growth factor, initially expressed in the prechordal plate and pharyngeal endoderm, acts in the survival of NC cells that migrate to the first BA (Brito et al., 2006). Furthermore, Shh induces FGF8, BMP4 and its own expression in the ectoderm adjacent to the first BA. When a second source of Shh is supplied to the future first BA region, in early stages of chicken embryo development (HH stage 5-8), there is induction of an extra jaw positioned as a mirror-image relative to the other (Brito et al., 2008). Furthermore, it was shown recently that Shh promotes in vitro chondrogenesis by cephalic and trunk NC cells, promotes the development of avian NC progenitors endowed with both neural and skeletogenic potentials, and induces increased expression of *Runx2* in perichondrial cells around cartilage nodules formed by cultured cephalic NC cells (Calloni et al., 2007; Calloni et al., 2009).

Different growth factors also participate to membranous ossification of the skull. TGFβ molecules secreted by the duramater, for example, are involved in tissue

interactions between the meninges and the cranial sutures. TGFβ signaling is increased during the frontal suture melting, when compared to the still open sagittal suture (Most et al., 1998; Opperman et al., 2000). FGF2 is also involved in osteogenesis of the cranial sutures. Inhibition of FGF2 activity resulted in reduction of osteogenesis in sutures through the inhibition of cell proliferation and differentiation (Moore et al., 2002). BMP2 and BMP4 also positively regulate osteogenesis and are expressed in the osteogenic fronts. BMP4 is present in the mesenchyme of the sagittal suture and in the duramater (Kim et al., 1998). Noggin - antagonist of BMP - has a role in maintaining the skull suture. The levels of BMP are relatively equivalent between open and closing sutures of mice, but Noggin expression is mainly limited to the sagittal and coronal sutures, which are still open. Furthermore, the increased FGF signaling suppresses Noggin expression in rat osteoblasts in vitro (Warren et al., 2003a).

Wnt signaling also has a crucial role in skull bone formation. The deregulation of the canonical Wnt pathway in the frontal and sagittal sutures, leads to craniosynostosis, which is one of the most common human congenital craniofacial deformities (Yu et al., 2005). Individuals with craniosynostosis develop abnormal skull bones due to premature fusion of cranial sutures (Cohen and Maclean, 2000). Axin 2, is a negative regulator of the Wnt pathway (it promotes degradation of the βcatenin pathway effector), which controls the closing time of the suture. In mice knockout for *axin2* there is an increase of Runx2-positive osteoprogenitors stimulating the expression of downstrem osteogenic markers, increased extracellular matrix mineralization and premature closure of cranial sutures (Yu et al., 2005). Another study confirmed that gain-of-function of *βcatenin* in the mesenchyme of the cranial suture promotes the increase of osteoprogenitors, and enhances mineralization

(Mirando et al., 2010). These processes involve an increase in smad1, 5, 8 phosphorylation, and expression of FGF2 and its receptors FGFR1 and FGFR2. A recent study demonstrated that osteoblasts in the osteogenic frontal suture have a greater osteogenic potential than the osteoblasts of the sagittal suture, a fact that can be explained by the activated canonical Wnt signaling in the cells of the frontal sutures. The constitutive activation of the Wnt pathway in parietal osteoblasts confers them an increased osteogenic potential, as found in frontal osteoblasts. Furthermore, it was shown that FGF2 treatment of the suture cells induces phosphorylation of GSK3 by increasing the level of βcatenin into the nucleus, suggesting that the Wnt pathway activation could be mediated by FGF (Quarto et al., 2011).

Different transcription factors acting in bone differentiation from the NC are induced by the growth factors produced by adjacent tissues. The signals derived from the ectoderm induce the regionalized expression of transcription factors in the mandibular ectomesenchymal cells. BMP signaling in the ectoderm, in addition to induce *Runx2* expression, essential for the differentiation of osteoblasts (Otto et al., 1997), leads to the expression of the transcription factors *dhand, Msx1, Msx2* and different *Dlx* genes (Brito et al. 2008, Liu et al., 2005). The local expression of these transcription factors results in the correct patterning of the jaw. In addition, some of these genes are also involved in the morphogenesis of the skull. Each structure in the first BA must acquire their identity along the proximo-distal axis. The code of *Dlx* genes acts in the regional specification of these structures. *Dlx1/2* are initially expressed in early maxillary and mandibular primordia, while *Dlx5/6* are expressed only in the mandibular primordium. *Dlx3/4* are restricted to a small area of the mandibular primordium. Thus *Dlx1/2* regulate the development of the upper jaw,

while *Dlx5/6* confer the lower jaw phenotype (Depew et al., 2005; Jeong et al., 2008). In the cranial suture, it was shown that *Dlx5* is activated by BMPs in osteoblast precursors. Dlx5 then induces *Runx2* expression, and initiates the sequence of genes involved in skull osteogenesis, such as *osteopontin* and *alkaline phosphatase* (Holleville et al., 2003, 2007). *Msx* genes, which normally act as transcriptional repressors, are also involved in the craniofacial bone development (Bendall and Abate-Shen, 2000). In the first BA region, *dhand* induces the expression of *Msx1*, important also for the survival of the cells in this region (Thomas et al., 1998). In mice with a double mutation for *Msx1* and *Msx2,* no frontal cranial bone forms. In these embryos, the expression of *Dlx5* is blocked, and *Runx2* is not activated (Han et al., 2007). The *twist* gene is induced by FGF2 in cranial sutures and inhibits the activity of the transcription factor Runx2 (Rice et al., 2000, Bialek et al., 2004). During late steps of differentiation of the cranial sutures, *twist* expression is decreased. Mice with deleterious mutation in the *twist* gene have premature closure of cranial sutures (Paznecas et al., 1998).

Hox genes encode a family of homeodomain transcription factors known to play important roles in specifying rostrocaudal identity in many tissues (Pourquié and Iimura, 2007). Two domains can be distinguished in the NC cells that underlie the craniofacial skeletogenesis. Membranous bones arise from *Hox*-negative cells, while chondrocytes are originated from both *Hox*-positive and *Hox*-negative cells. It was demonstrated (Couly et al., 1998) that *Hox*-positive NC cells, when transplanted to the *Hox*-negative anterior domain, are unable to differentiate into cartilage and bone. Conversely, the NC cells of the *Hox*-negative domain transplanted posteriorly, can respond normally to local signals, participating in the hyoid cartilage formation, while

maintaining their *Hox*-negative status. One reason for this increased osteogenic capacity of the *Hox*-negative NC is its ability to respond to signals produced by the anterior endoderm. NC cells that express *Hox* do not respond to these signals. In the *Hox*-negative domain, NC from a fragment equivalent to one third of the neural fold is able to create a complete facial skeleton (Couly et al, 2002). The rostral *Hox*-negative domain of the NC (or FSNC to facial skeletogenic NC) therefore behaves as an "equivalence group". Thus, the cephalic NC potential is limited by *Hox* gene expression. In fact, in knockout mice for *Hoxa2*, the NC cells of the second BA behave like *Hox*-negative NC cells that fill the first BA, and form ectopic parts of the jaw skeleton (Rijli et al, 1993; Gendron-Maguire et al, 1993).

Furthermore, *Hox* genes have been associated with the lack of skeletogenic differentiation of the trunk NC cells. Chondrogenesis was demonstrated in long-term trunk NC cultures. Hoxa2 reduced and Hoxd10 completely inhibited chondrogenesis in cultures of mesencephalic NC cells. It was also observed that *Hoxb4* gene, expressed by pre-migratory trunk NC cells, is decreased after 14 days of culture, and few collagen2-positive chondrocytes showed expression of this gene. Furthermore, overexpression of *Hoxd10* also prevented trunk NC chondrogenesis in vitro (Abzanov et al., 2003). Another study noted that mouse trunk NC chondrogenesis is induced in vitro by FGF2, and that this process involves reduction of *Hoxd9* with increasing time of culture together with upreglation of *Id2*, a gene of the cephalic NC (Ido and Ito, 2006). In mesencephalic NC that normally does not express any *Hox* gene, It was shown that retinoic acid (RA) and Wnt signaling induce *Hoxa2* and *Hoxd9*, respectively, and that downregulation of *Hoxd9* decreases differentiation into collagen2-positive chondrocytes. RA and Wnt also promote maintenance of these *Hox* genes in the cultured trunk NC cells (Iwashita and Ito, 2009).

Therefore, different aspects of the cephalic NC differentiation into chondrocytes and osteoblasts are already described. However, little is known about the potential of trunk NC cells for skeletogenic as well as adipocytic differentiation. The understanding of how these cell types differentiate from the trunk NC cells and whether they can arise from mulipotent progenitors, will bring new informations on the specification and determination processes of the NC and will help in understanding the evolution of aminiotes Vertebrates.

2 - OBJECTIVES

The NC cells of amniote Vertebrates have an important role in the development of the head mesenchyme, including the craniofacial skeleton, but they do not contribute to the mesenchymal cell types in the trunk. Thus, the differentiation capacity of trunk NC cells has been assumed to be limited to the neural and melanocytic phenotypes that these cells generate in vivo.

To test whether the trunk NC cells possess some mesenchymal potential, it was needed to remove them from their normal in vivo context, and to challenge them with new environmental conditions.

In this regard, the general aim of this work was to uncover the possible mesenchymal differentiation of the trunk NC cells using an in vitro culture approach, and to identify putative trunk NC mesenchymal progenitors in single cell cultures.

2.1. Specific Objectives

2.1.1 – To investigate whether trunk NC cells can undergo in vitro differentiation into mesenchymal cell types.

With the goal of evaluating the competence of the trunk NC cell population to generate osteoblasts and adipocytes, cell culture methods were devised to provide the required conditions for the differentiation of trunk NC cells into mesenchymal derivatives. In these experiments, immunocytochemistry, in situ hybridization and RT-PCR were used to identify the differentiating cell types.

2.1.2 – To examine the developmental potential of individual trunk NC cells.

To identify the NC progenitors for mesenchymal cells, we employed in vitro cloning by micromanipulation of individual trunk NC cells soon after their migration from the neural tube. At this step we used different growing conditions, with special interest in favoring the differentiation into osteoblasts and adipocytes in NC cell clonal progeny. Colony analysis with markers specific for the distinct NC lineages allowed to define common progenitors for both neural-melanocytic cells and osteoblasts or adipocytes

2.1.3. – To test possible activity of different factors on the mesenchymal differentiation of trunk NC cells.

The addition of different molecules (growth factors and pharmacological agents) to our NC culture systems will be used to study how the adipogenic and osteogenic differentiation of the trunk NC cells is regulated. This analysis will focus on the effects of Wnt and FGF signaling pathways.

3 - RESULTS

The research work realized in this Thesis led to results presented in a manuscript that is actually in the final stage of writing, to be submitted to a high impact scientific journal.

During this work, we initially validated molecular markers and we established the appropriate culture conditions in order to be able to analyze mesenchymal lineage potentials of the trunk NC cells in vitro.

The Results chapter of this Thesis is thus divided in 3 sections:

3.1. Section1 – Investigations to settle the in vitro culture system appropriate to the mesenchymal differentiation from quail trunk NC cells

3.2. Section2 – "**Multipotent neural and skeletogenic-adipogenic progenitors in the avian trunk neural crest**" (Manuscript in preparation)

This section includes Materials and Methods

3.3. Section3 – Complementary analyses of trunk NC mesenchymal differentiation

3.1. (Section1)

Establishment of the culture conditions for mesenchymal differentiation from quail trunk NC cells

3.1.1. Expression pattern of the osteoblast marker gene *Runx2*

The expression of *Runx2* gene defines early osteoblasts and we have used this marker to identify the NC cells engaged in the osteoblastic differentiation in vitro. It was therefore important to previously verify the specifity of our chicken *Runx2* probe and determine the expression pattern of this gene in the quail embryo.

For this purpose, in situ hybridization experiments were performed in whole mount quail embryos of 2 days, 2.5 days, 3.5 days, 5 days and 7 days of incubation (**Figure 11**). We could identify *Runx2* expression from 2 day-old embryos, where *Runx2* was already present in the prosencephalon and in the branchial arches (BA) (**Figure 11A**). Other regions later were also positive for *Runx2*, like frontal bones in the region covering the eye (**Figure 11G**), the basal region of the limbs (**Figure 11E, H, I**), the face with the upper and lower jaw (**Figure 11J**) and in limb buds (**Figure 11H**) and in development of the fingers bone (**Figure 11K, L**).

These data are consistent with the expression pattern of *Runx2* described in developing mouse embryos (Ducy et al., 1997) and validate this gene as an early marker of osteoblasts in the quail embryo and in quail NC cell cultures.

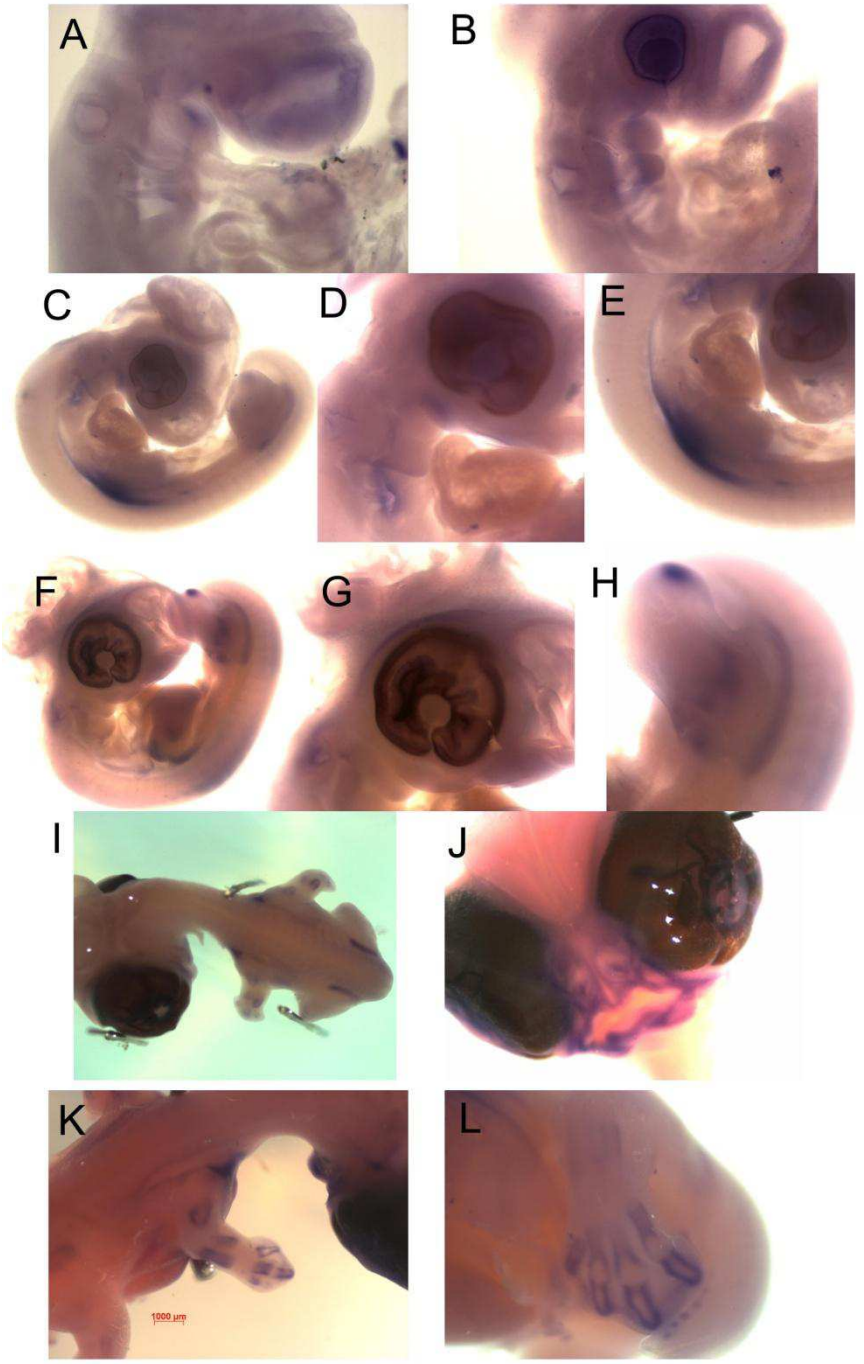

Figure 11 - Whole mount *Runx2* transcripts expression in quail embryos. (A) A 2 day-old embryo showing *Runx2* expression around the prosencephalon primordial and in the branchial arches (BA). (B) In 2,5 day-old embryo, expression around the eye can also be observed. (C-E) Embryos of 3,5 days (C) with *Runx2* expression in the BA (D) and the base of the limb (E). (F-H) 5days embryo with Runx2 expression in head over the eye (G), in the base of the limbs, and also in apical and medial regions of the limb (H). (I-L) Embryos of 7 days with *Runx2* expression overspread in different osteogenic regions, like the base of the limbs that will form the shoulder (I),

in the upper and lower jaw (J), in the digits and medial region of the hindlimbs (K) and forelimbs (L).

3.1.2. Establishment of culture conditions for trunk NC cell differentiation into osteoblasts

Having validated our molecular tools as markers to identify differentiating osteoblasts, the next step was to establish the culture conditions appropriate for the study of the mesenchymal potential of the NC cells, that is conditions in which these cells could efficiently develop into skeletal cell types.

- The first experiments were performed on 3T3 feeder-layers. The trunk NC cells were obtained after 15h of migration from the E2-quail neural tubes cultured in cloning medium (enriched in FCS, embryo extract, growth factors and hormones as described by Trentin et al, 2004). After detachement, NC cells were put in secondary cultures in DMEM with 10% FCS for 10 days on 3T3 feeder-layers, previously established as previously (Calloni et al., 2007). The presence of osteoprogenitors was investigated by in situ hybridization for detection of *Runx2* mRNA.

After this period of culture, we found numerous *Runx2*-positive cells. Many of them were scattered or in small groups (**Figure 12A**).

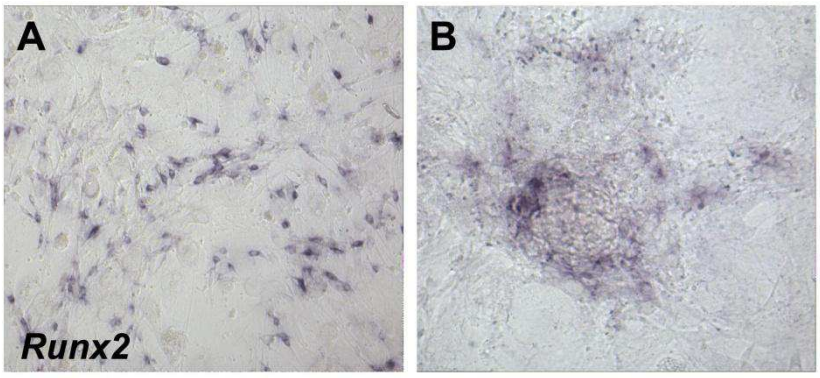

Figure 12 – *Runx2* mRNA detection in trunk NC secondary cultures of 10 days. **(A)** Scattered osteoblasts in cultures on 3T3 cells. **(B)** *Runx2*-positive cells around of a cartilage nodule.

This culture condition allowed us to also observe differentiated chondrocytes, as shown before (Calloni et al., 2007), and a subset of *Runx2*-positive osteoblasts were found located around cartilage nodules, forming the perichondrium (**Figure 12B**).

- To analyze if this trunk NC osteoblastic potential was maintained over time, we performed primary cultures of different durations, in order to isolate the NC cells after different times of migration. Cells that had migrated from the explanted neural tube for 15, 18, 24, 36 or 48 hours were replated in secondary cultures and grown for 10 days. We found that the NC cells derived from primary cultures of 15h, 18h and 24h hours gave rise to a large number of osteoblastic cells (**Figure 13A**), as compared to the osteoblasts obtained from the NC cells of 36h and 48h hours of migration (**Figure 13B**). Within the three earlier time points of primary culture analyzed, it was observed that the NC cells taken from 18h-primary cultures showed slightly more *Runx2*-positive cells after 10 days of secondary culture than those obtained after 15h and 24h.

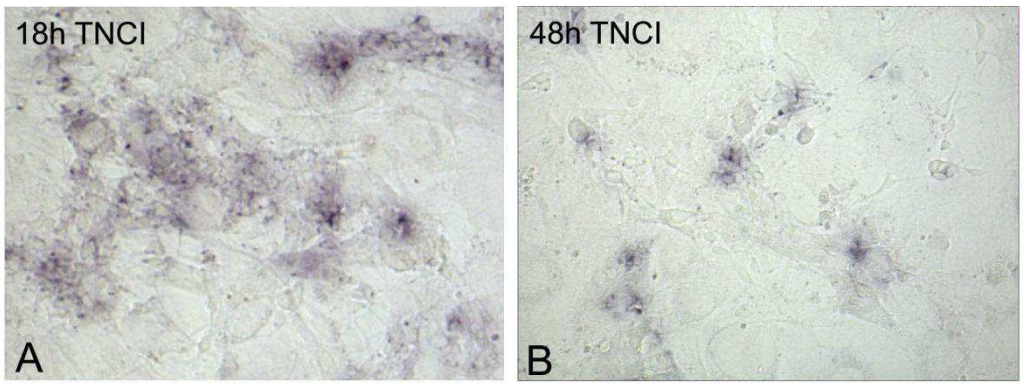

Figure 13 - ***Runx2* mRNA detection in trunk NC secondary cultures of 10 days** performed after 18h (A) and 48h (B) hours in primary culture (TNCI).

The *Runx2* expression was then analyzed at different times in secondary cultures of trunk NC cells prepared after 18h of migration in primary culture. After 4 days we found *Runx2* expression in rare cells (**Figure 14A**). After 7 days, the number of *Runx2*-positive cells had significantly increased, and after 10 days of cuture, we observed the highest production of *Runx2*-positive osteoblasts (**Figure 14B**). At 15 days, the number of these cells was already much lower (**Figure 14C**).

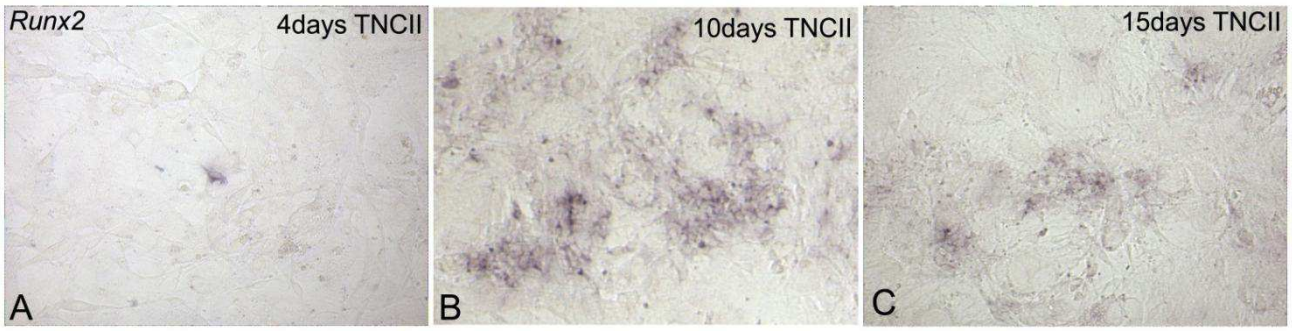

Figure 14 - *Runx2* expression at different secondary culture (TNCII) timepoints from trunk NC cells harvested after 18h of in vitro migration. Expression at 4 days (A), 10 days (B) and 15 days (C) of culture.

3.1.3. Establishment of culture conditions for trunk NC cell differentiation into adipocytes

- With the aim at obtaining osteoblasts, chondrocytes and adipocytes in the same culture condition, the first experiments for the analysis of adipocytic conditions were also performed on 3T3 feeder-layers with secondary culture performed after 15h.

Initially we used the the two adipogenic differentiation media previously employed by Billon and coworkers (Billon et al., 2007). The medium1 consists of

insulin (170nM) triiodothyronina (T3, 2nM) and roziglitazone (0.5μM), a synthetic ligand of the PPARγ nuclear receptor, an essential regulator of adipogenesis. In the treatment with medium2, the cells receive dexamethasone (1 μM) and 1-methyl-3-isobutylmethyl-xanthine (IBMX, 0.5 μM) for the first 2 days of secondary culture, together with medium1. Subsequently, we devised our "mesenchymal differentiation cocktail" that contain both adipogenic (insulin 85nM, triiodothyronine 1nM, and roziglitazone 0.5μM) and osteogenic factors (dexamethasone 0.05μM, ascorbic acid 25μg/ml and βglycerolphosphate 5mM), intended to favor both osteogenic and adipogenic differentiation in the same culture condition.

Adipocyte differentiation in these conditions was variable. A few cultures exhibited areas with adipocytes (**Figure 15**). In six different experiments, of 204 culture wells treated with either of the three adipogenic media, only 14 showed adipocytes, and we did not observe a clear difference in adipogenesis between these different media. We thus concluded that these conditions were not efficient for the differentiation of adipocytes from trunk NC cells. In addition, we faced a technical problem, due to the fact that the 3T3 fibroblasts of the feeder-layers were often responsive to the inductive factors present in the culture medium. Interestingly, we observed that 3T3 cells underwent efficient adipogenesis in the cultures treated with media containing dexamethasone.

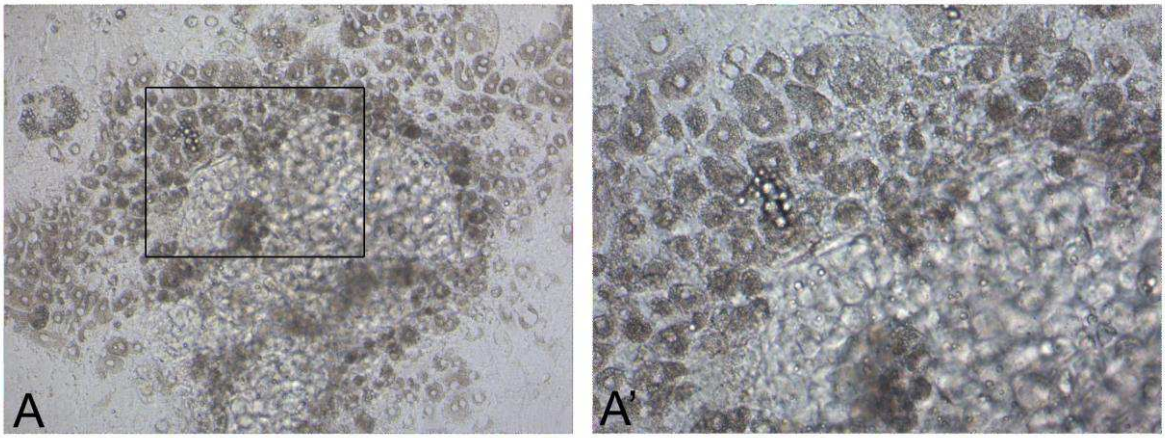

Figure 15 - Adipocytes around a cartilage nodule on 3T3 feeder-layer; same field at low (A) and higher (A') magnifications. Trunk NC culture of 14 days on 3T3 feeder-layer treated with medium2 from the seventh day.

- Since the culture system of trunk NC cells on 3T3 feeder-layer did not seem to be appropriate for the study of adipogenesis by NC cells, we sought to evaluate this differentiation potential by using other culture conditions. We thus plated the NC cells in culture wells coated with collagen1. NC cells obtained as described before, were seeded in a 10μl-drop deposited in the center of the culture well. After 4-5h the cultures were completed with culture medium. Initially, we used NC cells obtained from 48h-primary cultures following the protocol established by Billon and collaborators. Cultures were then treated with the adipogenic medium2 (Billon et al., 2007) from day-4 or with the mesenchymal differentiation cocktail as described above, given from day-6 onwards. At 15 days of culture, we observed that all 8 culture wells treated with the mesenchymal differentiation cocktail showed large areas of differentiated adipocytes. In the presence of medium2, only 25% of the culture wells (2 from 8) showed adipocytes (in smaller areas than those obtained

using the mesenchymal differentiation cocktail); furthermore, these cultures in medium2 showed much less cell density.

The next step was to test adipogenesis of the trunk NC cells obtained after only 15h of migration from the neural tube, in order to establish a culture condition in which we could efficiently obtain NC cell differentiation into osteocytes and adipocytes simultaneously. Thus, we treated the cells from 7 days of culture with the mesenchymal differentiation cocktail, which was more efficient for inducing adipogenesis and also favored the production by NC cells of *Runx2*-positive osteoblasts. The mesenchymal differentiation cocktail was very efficient to promote adipogenesis by the NC cells of 15h-migration: 90.5% of these treated cultures presented areas of differentiated adipocytes.

Therefore, under this latter culture condition, it was possible to obtain trunk NC cell differentiation into both adipocytes and osteoblasts. In addition, chondrocytes also differentiated with a significant frequency. In **Figure 16**, is shown such a trunk NC culture, observed from 14 to 21 days of culture, presenting large numbers of chondrocytes, osteoblasts and adipocytes.

The further analysis of the characterization of these mesenchymal phenotypic differentiation was performed and results are described in the scientific article prepared for publication as shown in section 2.

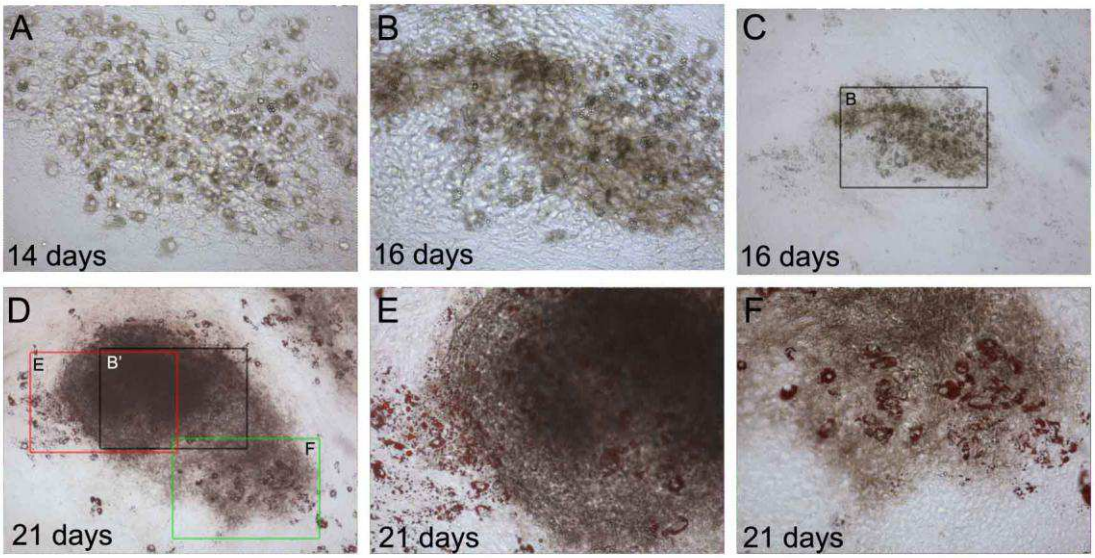

Figure 16 – Trunk NC culture observed from 14 to 21 days after treatment with the mesenchymal differentiation cocktail from day 7 onward. (A) 14 day-culture showing a zone of chondrocytes (round and refractile cells) together with adipocytes (cells between the chondrocytes, identifiable as darker in phase-contrast due to their lipid droplets). (B) The same region observed after 16 days showing beginning of mineralization in the center of the chondrocytic zone. (C) The same region as in B shown at lower magnification. The highlighted area is that indicated in B. (D-F) The same region at 21 days of culture, showing extensive mineralization. Adipocytes are stained with Oil Red O. The highlighted area B' is the field shown in (B) after more 5 days of culture. The other highlighted areas in (D) are shown in higher magnification in (E) and (F).

3.2. (Section2)

"Multipotent neural and skeletogenic-adipogenic progenitors in the avian trunk neural crest"

(Manuscript in preparation)

Multipotent neural and skeletogenic-adipogenic progenitors in the avian trunk neural crest

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Abbreviations: NC, neural crest; NCSC, neural crest stem cell; PNS, peripheral nervous system; DRG, dorsal root ganglia.

ABSTRACT

In the vertebrate embryo, the dorsal neuroepithelium forms the neural crest (NC), a transient structure that converts into migratory cells of various homing sites and very diversified developmental fates. Along the whole anterior-posterior axis, migratory NC cells condense to form the ganglia of the peripheral nervous system (PNS) and they yield Schwann cells lining the peripheral nerves and pigment cells in the skin. In addition to these neural-melanocytic derivatives, the cephalic NC provides a variety of mesenchymal cell types essential to head formation, including bones, cartilages, adipose tissue and vascular smooth muscle. In amniote Vertebrates, the mesenchymal derivatives of the NC are limited to the cephalic region, whereas in the trunk of amphibians and zebrafish, they are also present in the distal skeleton of the fins. These findings suggest that the trunk NC may be devoid of any ability to generate mesenchyme in Amniotes, and that the mesenchymal lineages of the cranial NC may arise from a specific NC cell population, distinct from the “bona fide” NC cells of neural and melanocytic fates. To address these issues, we investigated the capacity of trunk quail NC cells to adopt mesenchymal fates when challenged with appropriate in vitro culture conditions. We show that trunk NC cells efficiently undergo in vitro differentiation into mature adipocytes, chondrocytes and osteoblasts. Moreover, in vitro clonal analysis reveals that the large majority of clonogenic trunk NC cells generate osteoblasts in their progeny together with glial cells and neurons. Likewise, progenitors for adipocytes were identified as multipotent cells with adipogenic, glial, melanocytic and/or myofibroblastic potentials. These data show that mesenchymal developmental potentials have not been lost during evolution and are indeed present in the trunk NC of Amniotes; although dormant in vivo, they can be disclosed after in vitro culture.

INTRODUCTION

In Vertebrates, mesenchymal cell types such as chondrocytes, osteocytes, smooth muscle cells, adipocytes and connective tissue cells, have a dual embryonic origin. In Amniotes, mesenchymal cell types in the trunk are derived from the mesodermal germ layer, whereas in the head and neck, most of them originate from the neural crest (NC), an ectodermal multipotent structure that forms from the lateral edges of the neural plate during late gastrula-neurula stages (for reviews, Le Douarin and Kalcheim, 1999; Le Douarin et al., 2004; Sauka-Spengler and Bronner-Fraser, 2008). At the time of neural tube closure, the NC cells delaminate from the dorsal neuroepithelium to give rise to migratory progenitors that colonize diverse embryonic territories and eventually generate a wide array of differentiated cell types. In addition to their cephalic mesenchymal derivatives, the NC cells along the whole neural axis generate skin melanocytes, glial and neuronal ganglionic cells and Schwann cells in the peripheral nervous system (PNS) (Le Douarin and Kalcheim, 1999).

The NC origin of craniofacial mesenchymal cells has been extensively documented in avian and mouse species by using interspecific tissue transplantations and genetic fate mapping, respectively. The cranial NC, extending from the mid-diencephalon down to somite 4, plays an essential role in making up the vertebrate head, by contributing all of the cartilages and bones in facial and pharyngeal skeletons, and a large part of the cranial bones. In addition, it yields dentine, fat deposits, tendons, dermis and connective tissue cells as well as vascular smooth muscle cells and pericytes lining the blood vessels in brain and face (Le Lièvre et al., 1975; Couly et al., 1993, 1996; Chai et al., 2000; Jiang et al., 2000, 2002; Etchevers et al., 2001; Billon et al., 2007; Grenier et al., 2009). Cephalic NC cell patterning and coordinated

craniofacial morphogenesis rely on the complex interplay between NC cell intrinsic genetic programs and the action of extrinsic factors (reviewed by Le Douarin et al., 2004; Minoux and Rijli, 2010). The mechanisms whereby NC lineages become diversified during NC ontogeny and particularly those that underlie the emergence of mesenchymal progenitors remain poorly understood. The in vitro analysis of NC cell clonal progeny provided first evidence that early migratory cephalic NC cells exhibit heterogeneous developmental potentials ((Baroffio et al., 1988) and the identified progenitors encompass highly multipotent cells able to differentiating into both skeletogenic and neural cell types (Baroffio et al., 1988, 1991; Sieber-Blum et al., 1991; Trentin et al., 2004; Calloni et al., 2007, 2009). Investigation of the developmental fate of individual NC cells labeled in vivo further supports the multipotentiality of avian premigratory cranial NC cells (Bronner-Fraser and Fraser, 1988).

Following migration along several defined pathways patterned by the somites, the trunk NC cells give rise to skin pigment cells, to the peripheral neurons and glial cells (in sensory, parasympathetic and sympathetic ganglia and nerves) and to the catecholaminergic cells of the adrenal medulla (Le Douarin and Kalcheim, 1999). Though recent data suggest that trunk NC cell diversification in the chick embryo may obey a pre-determination model (Krispin et al., 2010), previous in situ lineage analysis of premigratory and migratory NC cells have shown that the avian and murine NC include multi-fated progenitors (Bronner-Fraser and Fraser, 1988, 1989; Fraser and Bronner-Fraser, 1991; Serbedzija et al., 1994). This was in line with the vitro clonal analysis performed in avian and mammalian species, which provided compelling evidence that at least a subset of early trunk NC cells are multipotent stem cells (Sieber-Blum and Cohen, 1980; Sieber-Blum, 1989, 1991; Ito et al., 1993;

Stemple and Anderson, 1992; Dupin and Le Douarin, 1995; Shah and Anderson, 1997; Lahav et al., 1998; Kim et al., 2003; Trentin et al., 2004; Kleber et al., 2005). Thus, autonomic neurons, glial cells, melanocytes and myofibroblasts were shown to arise in vitro from multipotent trunk NC cells isolated from the quail (Trentin et al., 2004).

In contrast to the cranial NC, the trunk NC in the avian and mouse embryos virtually does not generate mesenchymal derivatives in vivo. However in lower Vertebrates, the NC contributes mesenchymal cells in the trunk, specifically in the fins of developing xenopus, axolotl and zebrafish (Collazo et al., 1994; Smith et al., 1994; Epperlein et al., 2007). In zebrafish, trunk NC cells colonizing the caudal and dorsal fins later differentiate into the membrane bones of the distal rays (Smith et al., 1994). These findings therefore suggest that, during evolution of Vertebrates, the trunk NC has lost its ability for mesenchymal differentiation. Attempts to reveal a possible mesenchymal differentiation capacity of the trunk NC cells in Amniotes has been undertaken by challenging these cells with new environmental conditions in vivo and in vitro. Heterotopic grafting experiments between quail and chicken embryos showed that, when transplanted to the cephalic level, trunk NC cells were unable to form skeletal tissues but provided some connective cells, together the normal contingent of PNS cranial ganglia (Nakamura and Le Lièvre, 1982). Recently, it has been shown that trunk NC cells can undergo in vitro differentiation into chondrocytes, in long-term cultures (McGonnell and Graham, 2002; Abzhanov et al., 2003) or when stimulated by appropriate growth factors (Ido and Ito, 2006; Calloni et al., 2007). The avian trunk NC cells also differentiated into adipocytes in vitro, albeit with a lower frequency than the cephalic NC cells (Billon et al., 2007). Attempts to identify the in vitro chondrogenic progenitors in the avian trunk and cephalic NC revealed that

multipotent progenitors were abundant in the latter (43% of the mesencephalic NC clonal cultures) and very rare in the former (a single case out of 225 clones derived from trunk NC cells) (Calloni et al., 2007). The developmental potentials of the osteogenic and adipogenic trunk NC cells have not yet been investigated. Hence it is needed to explore whether multipotent neural-mesenchymal progenitors are present in the trunk NC.

This study was aimed at further evaluating the mesenchymal potentials of the avian trunk NC cells and characterizing the corresponding progenitors at the single cell level. We show definitively that, as their cephalic NC counterparts, the trunk NC cells of Amniotes have a significant capacity for mesenchymal differentiation in vitro and can undergo terminal differentiation into chondrocytes, mineralizing osteoblasts and adipocytes. Our cloning experiments reveal that most of the NC cells are multipotent, generating both neural cells and osteogenic or adipogenic cells. Therefore, even in the trunk of Amniotes, common progenitors for mesenchymal and neural lineages exist in the NC. These findings shed new light on the developmental repertoire of the NC cells along the A-P axis. They also suggest that, during evolution of Vertebrates, the expression of the mesenchymal potentials present in multipotent NC cells has been repressed in the trunk of Amniotes; however, the cryptic mesenchymal potentials of these cells can be disclosed upon in vitro culture.

MATERIALS AND METHODS

NC Cell Cultures:

Trunk NC cells were obtained in vitro from thoracic neural tubes of 18-25 somite stage (ss) quail embryos as described before (Calloni et al., 2007). After 15 h of primary culture, NC cells that had migrated around explanted neural tubes were harvested for secondary plating in “mass cultures” or in single cell cultures. Cells were plated either on plastic coated with collagen-1 (BD Biosciences) (3000 cells in 10ml in the center of 4-well culture plates) or on a feeder-layer of growth-inhibited 3T3 fibroblasts as described (Calloni et al., 2009) (2000 NC cells/well). Control culture medium was DMEM containing 10% FCS for NC “mass” cultures on 3T3 feeder-layers and DMEM 10% FCS supplemented with 80ng/ml chick embryo extract (CEE) for TNC mass cultures on collagen-coated wells and in clonal cultures.

In order to trigger skeletogenic and adipogenic cell differentiation, TNC cultures grown in control medium for 7 days were subsequently fed with control medium supplemented with a “mesenchymal differentiation cocktail” (treated cultures) for 2 days. From day-9 onwards, CEE was removed from the medium. The mesenchymal differentiation cocktail included a combination of factors previously described to promote adipogenesis and osteogenesis: 0.05 mM dexamethasone, 85 nM insulin, 1 nM tri-iodo-thyronine, 25 mg/ml ascorbic acid, 5 mM b-glycerol phosphate (all from Sigma) and 0.5 mM rosiglitazone, which belongs to the TZD family of synthetic ligands of the nuclear receptor PPARg. Cultures were maintained at 37℃ in a humidified 5% CO2/95% air atmosphere and culture medium was changed every three days.

Immunocytochemistry:

Cultures were fixed with 4% paraformaldehyde. Clonal cultures of quail trunk NC cells grown on 3T3 feeder-layers were analyzed at day-10 and the colonies were first analyzed by in situ hybridization to detect *Runx2*. The colonies were observed after nuclear staining with Hoeschst 33342 (Sigma) to distinguish quail NC cells from mouse 3T3 fibroblasts. Differentiation of chondrocytes in tridimensional nodules was assessed by phase-contrast microscopy. Bone matrix proteins were detected by using mouse Mab against osteocalcin (1:100; Abcam ab13420) and rabbit antisera against BSP/SPP1 (LF-84, LF-119; 1/100), osteonectin (LF-8, 1/1000), and collagen-1a1 (LF-67; 1/1000, gifts from LW. Fischer), with Tyramide Amplification System (Perkin-Elmer). Immunocytochemical detection of non-skeletal phenotypes was essentially as described (Trentin et al., 2004; Calloni et al., 2009) using the following antibodies: melanoblast/melanocyte early marker (MelEM) for melanocytic cells, HNK1 and SMP (Dulac et al., 1998) for glial cells, tyrosine hydroxylase for catecholaminergic neurons (all undiluted hybridoma supernatants) and α-smooth muscle actin (1:400 clone 1A4; Sigma) for myofibroblasts/smooth muscle cells. Secondary antibodies were purchased from Southern Biotechnology Associates. Fluorescence was observed under X70 Olympus microscope. Statistical analysis was performed using two-tailed Student's *t*-test (for cell numbers) or *chi*-square analysis (for clone frequency).

Histological stainings:

To identify triglyceride lipid accumulated in adipocytes, we employed the Oil Red O staining: 0.5% Oil Red O (Sigma O-0625) stock solution in isopropryl alcohol was applied to the cells for 10 min after dilution in H2O at 6:4 vol:vol ratio and filtration.

Alcian Blue staining revealed the presence of cartilaginous condensation

Mineralized bone was identified by staining with Alizarin Red S

In situ hybridization:

Expression of gene transcripts in the trunk NC cells was detected by *in situ* hybridization with a digoxygenin-labeled probe according to already described procedure (Calloni et al., 2009). Plasmids for prob preparation were gifts from T. Jaffredo (chicken *Runx2*), P. Scotting (chicken *Sox9*); and A.H. Monsoro-Burq (chicken *alkaline phosphatase*).

RT-PCR experiments:

RNA was extracted from cultured cells using RNeasy mini kit (QIAGEN) according to the manufacturer's instructions. cDNAs were synthezised using M-MLV Reverse Transcriptase (Invitrogen) according to the supplier's instructions and were used as templates for the polymerase chain reaction (PCR). All primer sequences are detailed in Table x. PCR parameters were 94℃ for 30 seconds (first cycle 2.5 minutes) for the denaturing step, 60℃ for 30 seconds for the annealing step and 72℃ for 30 seconds for the elongation step (last cycle 2.5minutes). Primers were validated using quail embryonic tissues as controls (E2 embryos; E7 and E10 face, hindlimb and forelimb; E15 adipose tissue and muscle)

RESULTS

1 - Skeletogenic differentiation of trunk NC cells

Trunk NC cells were obtained from trunk neural tubes isolated from E2-quail embryos and cultured as explants on collagen as described (Lahav et al., 1998; Trentin et al., 2004). The NC cells were harvested after 15h of migration from the neural tube explants and replated at high density on collagen in FCS-enriched control medium (see M.&M.). In these conditions, trunk NC cells gave rise after 10 days of culture to diverse NC-derived phenotypes: autonomic neurons, glial cells, melanocytes and myofibroblasts (**Figure 1A-C**). Cells engaged in osteoblast differentiation were identified through expression of the early marker of osteoprogenitors *Runx2*, a transcription factor required for bone differentiation in vivo (Kimori et al., 1997; Ducy et al., 1997). *Runx2* transcripts were first detected by in situ hybridization after 5 days of culture and, at day-7 about 10% of the TNC cells expressed this gene. *Runx2*-positive cells were found in all the cultures at day-10 either isolated or as groups of cells located between the other differentiated cell types (**Figure 1B,C**). Expression of *Runx2* later decreased and only a few cells showed weak expression of this gene in day-18 cultures, consistent with previously reported down-regulation of *Runx2* in maturing osteoblasts (for references, Karsenty et al., 2009) (**Figure 1E,F).**

To investigate whether TNC cells can advance towards bone differentiation in vitro, the cultures were supplemented from day-7 with a “mesenchymal differentiation cocktail” that we devised in order to promote both in vitro osteogenesis and adipogenesis by quail NC cells (see M&M). Among other factors, this cocktail contains ascorbic acid, dexamethasone and β-glycerol phosphate, known as

necessary components for the in vitro differentiation of osteoprogenitors into mature osteoblasts. Three days after the onset of supplementation (10-day cultures), we observed collagen1a1-immunoreactivity in areas of high cell density in the center of the cultures, which formed a net of filaments covering the cells **(Figure 2A-B)**. Expression of collagen1a1 was not observed in cultures grown in the absence of the mesenchymal differentiation cocktail, although these cultures contained *Runx-2* positive cells (not shown). From culture day-17, we observed a gradual mineralization process in dense areas of the cultures supplemented with the differentiation cocktail. Calcification of mineralized regions appeared as plates or fibers covering the cells and was assessed by staining of the calcium deposits with Alizarin Red S (**Figure 2C,D**). In these regions, the skeletogenic cells exhibited expression of *alkaline phosphatase*, a gene with essential role in mineralization (**Figure 2E,F**) as well as vesicles immunoreactive to the osteocalcin protein, a marker of calcifiying osteoblasts (**Figure 2G,H**).

Analysis of chondrocyte markers was also performed at different times of TNC culture on collagen. Day-12 cultures supplemented with the differentiation cocktail displayed groups of cells expressing *Sox9* transcription factor, which is activated in chondrocyte progenitors and is essential for chondrogenesis (Bi et al., 1999; Akiyama et al., 2002, 2005; Mori-Akiyama et al., 2003) (**Figure 3A**). *Sox9*-positive cells were rarely seen in the absence of the differentiation cocktail (not shown). In day-15 cultures, chondrocytic cell clusters were identified by Alcian blue staining of the chondrocytic matrix (**Figure 3B**) and later were also immunoreactive to the cartilagenous matrix protein chondroitin sulfate (**Figure 3C,D**). In day-23 cultures, Alcian blue-stained cartilaginous nodules could co-localize with Alizarin Red-positive

mineralized bone areas (**Figure 3E-F**) while other mineralized regions stained with Alizarin Red but did not show Alcian blue staining (**Figure 3G,H**).

We previously reported that a large subset of cranial NC cells undergo chondrocyte and osteoblast differentiation when they are cultured in vitro on feeder-layers of 3T3 fibroblasts (Calloni et al., 2007, 2009). We therefore sought to investigate whether, in similar culture conditions, the trunk NC cells also display the ability to yield skeletogenic cells. We found that trunk NC cells grown on 3T3 fibroblasts generated numerous *Runx2*-expressing cells after 10 days of culture in conventional medium (see also §3). When supplemented with the mesenchymal differentiation cocktail from day-7 onwards, the cultures at day-15 exhibited cells immunoreactive to collagen1a1, localized in the perichondrial region surrounding chondrocyte nodules (**Figure 3I,J**).

The in vitro development of skeletal cell lineages was further characterized by semi-quantitative RT-PCR analysis of trunk NC cultures grown on collagen with and without the mesenchymal differentiation cocktail for 11, 15 and 21 days of culture (**Figure 4**). At all the time points analyzed, trunk NC cultures, either treated or untreated with the differentiation medium, showed expression of the early osteoblast marker *Runx2*, thus confirming the results of in situ hybridization. In similar conditions, we also identified mRNA expression of *Sox9*, a master regulator of chondrogenesis expressed in osteochondro-progenitors (Akiyama et al 2003). Other markers of chondrogenesis, such as the glycoprotein of cartilage matrix *Aggrecan* and the hypertrophic chondrocyte marker gene *collagen10* were also detected in the cultures at day-11, -15 and -21. The cultures also expressed osteogenic genes encoding the bone cell matrix proteins *collagen1a1* (which was detected by immunocytochemistry in the treated cultures) and *bone sialoprotein* (*SPP1)*. Treated

and untreated cultured cells at all analyzed times also showed the presence of transcripts for *Phospho1,* a phosphatase recently described to act in initial steps of mineralization of cartilage and bone (Houston et al., 2004; Roberts et al., 2007). By contrast, in the presence of the mesenchymal differentiation cocktail, we found a high expression of *alkaline phosphatase,* which catalyses production of inorganic phosphate required for bone mineralization (Narisawa et al., 1997), (**Figure 4**), thus confirming the expression pattern observed by in situ hybridization and the role of the differentiation cocktail to promote bone mineralization in the NC cultures.

Taken together, these data show that cultured trunk NC cells are able to give rise to osteochondro-progenitors that differentiate into chondrocytes and mineralizing bone cells. Although trunk NC cells grown in control medium expressed several genes of skeletogenesis, the terminal differentiation of skeletogenic trunk NC cells up to the mineralization stage only occurred upon in vitro exposure to the mesenchymal differentiation cocktail.

2 - Differentiation of trunk NC cells into Adipocytes

The adipocytic differentiation capacity of trunk NC cells was investigated in cultures prepared as described above for the analysis of skeletogenesis (see M&M). After 7 days of growth on collagen, the trunk NC cultures were treated by addition of the mesenchymal differentiation cocktail, which, in addition to previously described osteogenic components, contains adipogenesis-promoting factors, namely insulin, T3, dexamethasone (that also acts as an osteogenic factor) and roziglitazone, a synthetic agonist of peroxisome proliferator-activated receptor gamma (PPARγ), a nuclear receptor with an essential role in adipocyte differentiation (Rosen and

McDougald, 2006). After about 3 and 6 days of treatment according to the culture series (corresponding to day-10 and day-13 cultures, respectively), more than 90% of the cultures showed the presence of cells that displayed small cytoplasmic lipid droplets (**Figure 5A,B**). These adipocytic cells later were grouped in large areas and exhibited larger droplets of lipids that were stained with Oil Red O (**Figure 5A-C**). From day-17 in the presence of the mesenchymal differentiation cocktail, when mineralized bone areas were already present, we could often observe skeletogenic cells and adipocytes in the same regions of the cultures (**Figure 5D-F**). Adipocytes did not differentiate in trunk NC cell cultures grown in the absence of the mesenchymal differentiation cocktail (not shown).

The time-course of adipocytic differentiation was followed by semi-quantitative RT-PCR analysis of trunk NC cell cultures grown in control and cocktail-supplemented medium for 11, 15 and 21 days (**Figure 5G**). We investigated the expression of *PPARγ*, an indispensable gene for adipogenesis, which is expressed from the pre-adipocyte stage (Hu et al, 1995), of *cEBPα*, another crucial transcriptional regulator of adipogenesis, and of *FABP4*, a characteristic gene of mature adipocytes (for references, Rosen and McDougald, 2006). All these three genes were expressed at all analyzed times of culture in treated and untreated cells. Both *PPARγ* and *FABP4* showed a stronger expression in treated cells compared with cells that did not receive the mesenchymal differentiation cocktail (**Figure 5G**).

These data show that quail trunk NC cells can differentiate into adipocytes in vitro, thus supporting previous results (Billon et al., 2007). Trunk NC cells were able to acquiring expression of adipogenic gene transcripts in long-term cultures in the absence of the mesenchymal differentiation medium. However, the adipogenic trunk

NC cells required the stimulation by adipogenesis-promoting factors in order to undergo morphological adipocytic differentiation into *bona fide* lipid-storing adipocytes.

3 - Characterization of mesenchymal progenitors in single trunk NC cell cultures

3.1. Clonal analysis of osteogenic trunk NC cells

As trunk NC cells in mass cultures exhibited a significant capacity for bone cell differentiation, we sought to analyze the osteogenic potentialities of individual trunk NC cells in clonal cultures. Trunk NC cells were obtained from neural primordium explant cultures as described before, then seeded as single cells by micromanipulation to ensure clonality of the plating. Single trunk NC cells were grown on feeder-layers of growth-arrested 3T3 fibroblasts, as performed previously to characterize the osteoblast progenitors in the avian cranial NC (Calloni et al., 2009).

In contrast to single NC cells plated on collagen-1 (see below §3.2), a high yield of colony formation and of neuronal differentiation could be obtained in these culture conditions: after 10 days in the presence of control cloning medium (see M&M), 57.5 ± 5,37% of the plated NC cells gave rise to a clone (**Table 1**). Colonies (n=420 from three series of experiments) were phenotypically analyzed using markers specific of various NC-derived phenotypes to identify sympathetic-like neurons (immunoreactive to TH), glial cells (HNK1-positive), myofibroblasts (expressing aSMA) in addition to cells of the osteoblast lineage, as assessed by detection of *Runx2* transcripts. Trunk NC cell colonies under these conditions also included cells expressing the gllial marker Schwann cell myelin protein (SMP) (Dulac et al., 1989) and melanoblasts positive for the MelEM antigen (Nataf et al., 1993) (not shown); due to technical

limitations, these markers however could not studied in the cultures analyzed for the presence of osteoblasts by in situ hybridization for *Runx2*.

The percentages of clones containing neurons (N), glial cells (G) and osteoblasts (O) are shown in **Table 1**. More than 76% of trunk NC cells (76,29 ± 4,19%; n=328) generated clones containing *Runx2*-expressing cells. Nearly all of these clones also contained neural cells, thus revealing that osteoblasts arise from multipotent (GNO) and bipotent (GO) progenitors (**Figure 6**).

Myofibroblasts (αSMA-positive) rarely differentiated in these culture conditions. The presence of this cell type was investigated in one series of trunk NC clonal cultures. From 184 clones analyzed, only 6 (3,26%) contained myofibroblasts (F). Most of these colonies were mixed mesenchymal-neural clones of the GOF (n=2), GNF (n=1) and GF (n=2) types. One colony included myofibroblasts but no other identified phenotype (F clone; n=1) (not shown).

In sum, the presence of osteoblasts, neurons, glial cells and myofibroblasts in the progeny of single trunk NC cells grown on feeder-layers, led us to identify ten different types of progenitors in which a large proportion were multipotent NC cells endowed with both neural and osteogenic potentials.

3.2. Clonal analysis of adipogenic trunk NC cells

The developmental potentials of trunk NC progenitors with adipogenic differentiation capacity were evaluated using cultures on collagen-1 substrate. In fact, trunk NC cells inconsistently differentiated into adipocytes when cultured on 3T3 feeder-layers, and addition of adipogenesis-promoting factors in the culture medium appeared to trigger adipogenesis in the growth-arrested 3T3 fibroblasts; hence this compromised subsequent analysis of adipogenesis by NC cells in those conditions. Clonal cultures on collagen were performed after isolation of trunk NC cells as described before.

Adipogenic differentiation was analyzed in cultures that received the mesenchymal differentiation cocktail for a minimum period of nine days of treatment beginning from day-7 (day-15 cultures).

Individual trunk NC cells showed a low survival and proliferation rate in these cultures. After 7 days, the cloning efficiency of trunk NC cells ranged between 5,2% and 28,1% (average of 14,8%) in five different experiments where a total of 83 clones from 560 plated cells could be recorded. Furthermore, the total number of cells in the majority of the clones was low (less than 40 cells in 43% of the clones and about 100 cells in 34% of the colonies); the largest clones, which comprised between 500 and 800 cells, accounted for only 3%. Following addition of the differentiation cocktail from day-7 onwards, we observed in day-15 cultures that about 50% of the colonies exhibited a decreased number of cells compared to their cellular amount at day-7. These clones were not further considered.

The remaining day-15 clones, wherein cells had shown continuous growth (n=34), were analyzed for the presence of neurons, glial cells, pigment cells and unpigmented melanoblasts, and myofibroblasts as described before. Mature adipocytes were identified using morphological criteria and the lipid dye Oil red O. We found that adipocytes differentiated in 35.3% (n=9) of the clones, which all also included glial cells (G), melanocytes (M) and/or myofibroblasts (F) but no neurons. Specifically, adipocytes derived from multipotent trunk NC cells belonging to the GMFA (11.8%), GFA (5.9%) and FA (17.6%) types of progenitors (**Figure 7**). The clones deprived of adipocytes contained mostly myofibroblasts: 29.4% of the presented this cell phenotype only (F clones). The other clones types were GMF (17.6%), GF (2.9%), MF (17.6%) and M (5.9%) clones.

DISCUSSION

Tracing the migration pathways and fate of the NC cells in the avian and mammalian embryo led to document significant differences between trunk and cephalic NC cell with respect to their migratory behaviors and lineage potentials in vivo (for reviews, Le Douarin and Kalcheim, 1999; Le Douarin et al., 2004; Harris and Erickson 2008). One of the major features of the trunk NC of amniote Vertebrates is that it is virtually devoid of mesenchymal cell derivatives, as opposed to the widespread contribution of the cranial NC to the head mesenchyme, where much of the cranial and facial cartilages and bones, smooth muscle cells, adipocytes and connective tissue cells thus originate from the NC (Couly et al., 1993; Chai et al., Jiang et al, 2000). In the trunk, the axial and appendicular skeletons originate from the somites and lateral plate and the embryonic origin of adipocytes, although poorly experimentally ascertained, is also generally believed to be mesodermal (Tang et al 2008, Rodehoffer et al., 2008; see Billon and Dani, 2008). The contribution of the NC to the formation of fat tissues in the face and neck however has been demonstrated by isotopic quail-chick transplantations of the cephalic NC (le Lièvre and le Le Douarin, 1975) and by genetic fate mapping of NC cells in postnatal transgenic mice (Billon et al., 2007). The latter experiments further indicated that adipocytes found in the trunk of mammals do not originate from the NC.

Therefore, the derivatives of the trunk NC cells in Amniotes appear limited to skin melanocytes, PNS glial and neuronal ganglionic cells and Schwann cells along the peripheral nerves (Le Douarin and Kalcheim, 1999). In this respect, it is unclear whether cells of the early trunk NC are totally devoid of mesenchymal potentials. If they exist, these potentials must be strongly inhibited during in vivo development, which puts forward the hypothesis that removing trunk NC cells from their normal

inhibitory context and challenging them with new environmental conditions favorable to mesenchymal differentiation, might enable to unmask their ability to differentiate into mesenchymal cells. In order to investigate this possibility, we have examined the capacity of quail trunk NC cells to adopt mesenchymal lineage pathways when exposed to appropriate in vitro culture conditions. Our analysis of the expression of a number of mesenchymal markers in mass and clonal cultures indicate that trunk NC cells do have a robust potential to yield differentiated osteoblasts, chondrocytes and adipocytes. Moreover, these mesenchymal cell types originate in vitro from the same multipotent trunk NC progenitors as the neural-melanocytic cells. These data, by highlighting the ability of avian trunk NC cells to undergo skeletogenesis and adipogenesis, have significant implications related to the embryonic origin, the in vivo function and the evolution of the mesenchymal progenitors in amniote vertebrates.

Trunk NC skeletogenic progenitors differentiate in vitro

In the present work, we could evidence efficient in vitro mesenchymal cell differentiation by trunk NC cells, under culture conditions that also allowed the production of the different phenotypes (neural cells and melanocytes) derived in vivo from these cells and within a time-course similar to in vivo skeletal cell development. Our data from in situ hybridization, immunocytochemistry, cytochemical staining and RT-PCR experiments thus support the notion that the skeletogenic differentiation in these trunk NC cell cultures recapitulates the major steps involved in osteogenesis and chondrogenesis. Immature osteoblasts were defined by expression of the gene of the Runt family of transcription factors *Runx2*, which is expressed early in osteogenic cells and is required for the formation of all the bones (Komori et al., 1997; Ducy et al., 1997). We verified in preliminary experiments that our probe for the avian *Runx2* gene efficiently labeled developing bones in the jaw, axial skeleton and

limb buds of quail embryos by E5 to E8, according to bone locations (not shown). The first *Runx2*-positive cells were detected at 5 days of culture, a time point when melanocytes and catecholaminergic cells (expressing tyrosine hydroxylase), are being specified in vitro, which indicates that engagement to the osteoblastic pathway is not a late event in developing trunk NC cells. With exposed from day-7 of culture to the mesenchymal differentiation cocktail (consisting of both osteogenic and adipogenic factors), trunk NC cells were able to advance along the bone differentiation process and exhibited *alkaline phosphatase* transcripts, collagen-1 protein, calcium deposition reacting with Alizarin Red staining and acquired a mineralized extracellular matrix presenting immunoreactivity to osteocalcin, a late marker of osteogenesis. Progression along the bone cell lineage was confirmed by RT-PCR analysis of the cultures at incremental stages, which showed that expression of *Runx2*, *osteopontin/SPP1*, *collagen-1a1* and *alkaline phosphatase* genes. Noticeably, although most of these markers were detected by RT-PCR in cultures either in the absence or in the presence of the differentiation cocktail, *alkaline phosphatase*, encoding an enzyme essential for the mineralization process (Karsenty, 2005), showed a significant increase of expression by RT-PCR in the treated cultures, where it was also detected by in situ hybridization only in the calcified dense areas.

The trunk NC cells developing in the cultures also underwent chondrogenesis, as identified by using Alcian blue staining and by the expression of the chondrocyte markers *aggrecan* and *collagen10*, that were found by RT-PCR at 11, 15 and 21 days of culture in treated and untreated cells. *Sox9*, the early gene of the chondrocytic lineage, required in vivo for cartilage differentiation (Akiyama et al, 2003), could be detected by RT-PCR already at 3 days of culture.

The osteogenesis by the cephalic NC cells is either of the membranous type, as in the frontal bone where osteoblasts differentiate directly from the condensed mesenchyme, or endochondral, as in the jaw wherein osteoblasts need a cartilage template in order to differentiate and mineralize. In 21 day-cultures supplemented with the differentiation cocktail, we observed that most of the Alizarin Red-stained mineralized regions partly overlapped with, or surrounded chondrocyte clusters, thus indicating endochondral ossification of perichondrial regions. Other regions of these cultures stained with Alizarin Red but were negative for Alcian blue staining, suggesting formation of membranous bones, independently of any interaction with chondrocytes. Although this point will need further clarification, our data suggest that trunk NC might be able of both types of ossification in vitro.

Only the trunk NC cells treated with osteogenic factors underwent bone matrix mineralization in vitro, despite the fast that some of the skeletogenic gene transcripts could be expressed in absence of these factors. This suggests that extrinsic factors such as those provided in vitro by the differentiation cocktail, are necessary to enable mesenchymal trunk NC progenitors to progress along the skeletogenic differentiation pathway. Attempts to provide the trunk NC cells with an environment favorable to mesenchymal differentiation in vivo, by transplanting them to the locations of embryonic premigratory or migratory cranial NC cells did not succeed in eliciting significant skeletogenesis by engrafted trunk NC cells (Nakamura and Le Lièvre, 1982; McGonnell and Graham, 2002). Unilateral replacement of premigratory chick cephalic NC cells by quail trunk NC cells, led to the production by the grafted cells of some connective fibroblasts and vascular mural cells but no skeleton, resulting in strong head structure deficiencies (Nakamura and Le Lièvre, 1982). In addition, quail trunk NC cells transplanted into the first branchial arch of E2-chick embryos, after

being cultured in vitro for one day, could migrate to the Meckel's cartilage and the sclera of the hosts, albeit at low frequency. These cells were indeed very rare and their expression of skeletal markers was not documented (McGonnell and Graham, 2002). Therefore, if some trunk NC cells can survive when placed in these in vivo mesenchymal environments, it is very unlikely that they adopt a skeletogenic fate.

Altogether, these data suggest that the trunk NC cells do not differentiate into mesenchymal cells in vivo because they have their intrinsic mesenchymal potential still inhibited when they settle in permissive environments. Previous studies have shown that forced expression of anterior *Hox* genes in the *Hox*-negative, rostral cranial NC prevents its skeletal differentiation (Creuzet et al., 2002, 2004...). It has also been suggested that *Hox* gene activity decreases during chondrogenesis by avian and mouse NC cells in vitro (Abzhanov et al., 2003; Ido and Ito, 2006). Down-regulation of *Hox* genes in the trunk NC cells undergoing skeletogenic differentiation possibly also occurs in our culture system, and further experiments will investigate this possibility. Several growth factors intervene to promote skeletogenesis by cranial NC cells (reviewed by Santagati and Rijli, 2003). Among those, Sonic Hedgehog (Shh) was shown to act in endochondral ossification by the cephalic NC cells in the developing jaw (Brito et al., 2006, 2008...). In vitro, Shh promoted the chondrogenic differentiation of cephalic and trunk quail NC cells (Calloni et al., 2007) and increased the number of *Runx2*-positive cephalic NC cells developing in perichondrial regions (Calloni et al., 2009). Moreover, when applied to cultures of single NC cells, Shh also increased the number of multipotent progenitors endowed with both chondrogenic and neural capacities (Calloni et al., 2007, 2009). However, our preliminary experiments investigating the effect of the addition of Shh to trunk NC cells in our

culture conditions did not show any significant changes in their differentiation into osteoblasts (not shown).

Trunk NC differentiation into adipocytes in vitro

Our data show that trunk NC cells have also a great adipogenic differentiation potential in vitro. We previously had shown that quail trunk NC cells were able to differentiate into adipocytes (Billon et al., 2007). However, this phenotype was obtained in only 40% of the cultures, compared with 90% of the cultures treated with the mesenchymal differentiation cocktail in our present work. We used secondary cultures of trunk NC cells taken after 15h of primary culture, as opposed to 48 h of primary culture in the previous study; however, we found that trunk NC cells harvested after both 15 and 48 hr of migration gave a similar high yield of adipocyte differentiation (not shown). A likely explanation for the high adipogenic differentiation efficiency is the constant presence of the glucocorticoid hormone dexamethasone in our mesenchymal differentiation cocktail, whereas in previous work, this hormone was added to NC cells only during the first 2 days of cultures (Billon et al., 2007). Besides acting on osteogenic differentiation, dexamethasone has also an important role in adipogenesis, by indirectly inducing the essential adipogenic transcription factors CEBPα and PPARγ (Pantoja et al., 2008).

The adipogenic differentiation of trunk NC cells was detected morphologically and using the Oil Red O histological staining. In addition, the expression of genes of adipogenesis was monitored by semi-quantitative RT-PCR analysis of the developing trunk NC cultures. These experiments showed that *PPARγ, CEBPα* and *FABP4* are already expressed from 11 days, even in untreated cultures, with a tendency towards a higher expression level in cells stimulated by the differentiation medium. In

addition, the transcription factor *Pbx1* was present from 3 days of culture and throughout the analyzed period. This factor was recently identified as necessary for the early steps of adipogenesis by neuralized mouse ES cells and it inhibited adipocyte final differentiation (Monteiro et al., 2011). The data thereore suggest that, independently of the provided differentiation medium, the trunk NC cells can generate in vitro an immature cell type engaged in the adipocyte differentiation pathway; the mesenchymal differentiation factors thus play a role only to trigger the terminal differentiation of these trunk NC "pre-adipocytes" into morphologically differentiated and lipid-storing adipocytes, as observed in pre-adipocytic and mesenchymal stem cell lines widely used for the study of in vitro adipogenesis (McDougald and Rosen, 2006).

Another important characteristic of our in vitro system is that osteogenesis and adipogenesis occur concomitantly under the same culture conditions. These two differentiation pathways are known to be competitively regulated in the bone marrow and in mesenchymal stem cells, and a number of factors that favor one of these two phenotypes inhibit the other one (Chamberlaim et al., 2007). The signaling molecules that regulate the choice by NC cells between adipocyte and bone cell development are not known. Our culture system might be a useful model to investigate how the balance between osteogenesis and adipogenesis is regulated in differentiating NC cells.

Osteogenic and adipogenic progenitors of the trunk NC are multipotent

Given the fact that the trunk NC of higher vertebrates does not yield mesenchymal derivatives, a longstanding idea proposed that the mesenchymal potentials encountered in the cranial NC likely originate from distinct progenitors,

independent of those giving rise to “bona fide” NC phenotypes of PNS cells and melanocytes. This idea however has been refuted since in vitro analyses carried out by us and others have shown that the cranial NC cells comprise common progenitors for neural cells-melanocytes and at least some mesenchymal cells, i.e., chondrocytes and smooth muscle cells (Baroffio et al., 1988, 1991; Dupin et al., 1990; Trentin et al., 2004; Ito et al., 1991, 1993; Calloni et al., 2007) and osteoblasts (Calloni et al., 2009). The issue of whether the mesenchymal potentials displayed by the trunk NC cell population in vitro are similarly expressed by multipotent progenitors began to be answered only recently. Calloni and colleagues provided first evidence for multipotent trunk NC cells with chondrogenic differentiation capacity, although their frequency in was very low (about 1 in 225) when compared to those of the cephalic NC (Calloni et al., 2007).

The clonal analyses we provided here further highlight the cellular origin of the mesenchymal trunk NC cells able to differentiate in vitro into osteoblasts, chondrocytes and adipocytes. We have first aimed at identifying osteogenic progenitors derived from trunk NC cells developing on a 3T3 feeder-layer, according to the previous procedure described for the cephalic NC (Calloni et al., 2009). Our data show that more than 76% of the clonogenic trunk NC cells generate both *Runx2*-positive osteoblasts and neural cells. In these experiments in which the cell clonogenicity was high (average of 57.5%), osteoblasts derived from multipotent progenitors GNO (for glial cells, neurons and osteoblasts) and GO (for glial cells and osteoblasts). Although technical limitations impaired to identify melanoblasts by immunocytochemistry in the clones already processed for *Runx2* mRNA detection, we could evidence in independent cultures a high percentage (91%) of clones including melanoblastic cells (not shown), thus suggesting that most of the

osteogenic multiphenotypic clones likely also contain melanoblasts. In contrast, myofibroblasts barely differentiated in cultures and were not further considered for the analysis of the clones.

When adipocyte progenitors were investigated, the cloning efficiency of the trunk NC cells cultured on collagen was much lower than that found on 3T3 fibroblasts. As described previously for mass cultures, the clones received the mesenchymal differentiation cocktail from day 7 and were phenotypically analyzed after two to three weeks of culture. During this culture period, most of the clones exhibited a low cell proliferative activity and significant cell death, which resulted in a reduced number of colonies suitable for the recording of adipocyte terminal differentiation. After treatment with the mesenchymal factors, adipocytes were found in 35.3% of the clones (n=35), which retrospectively identified three types of adipocyte progenitors: FA, GFA and GMFA, according to the presence of glial cells (G), melanocytes (M), myofibroblasts (F) and adipocytes (A). Thus all the clonogenic cells that produced adipocytes, also generated myofibroblasts. Furthermore, about half of these adipocytic clones also included melanocytes. Neuronal cells were not detected in these experiments, but glial cells were identified in 17.6% of the clones with adipocytes. By showing for the first time that adipocytes can originate from multipotent NC cells, these data provide new insights into the origin and commitment of adipogenic progenitors, which remained poorly understood, mainly because markers specific of adipocyte progenitors are still lacking. Our results also suggest the possibility is the NC cells could participate to the formation of fat depots in the trunk; this hypothesis yet received no support from current data of genetic fate mapping of NC cells in transgenic mice (Billon et al., 2007). The second, more likely, possibility is that adipogenic progenitors present in the early trunk NC have this

potential inhibited during development in vivo, in the same way as the differentiation capacity of osteogenic progenitors is locked in the trunk NC of developing Amniotes.

In sum, our in vitro studies of the osteogenic and adipogenic potentials of single trunk NC cells provide new evidence for the multipotency of the trunk NC cells. We show that the neural-melanogenic and the mesenchymal lineages are not segregated into distinct progenitors, but are present in several types of multipotent trunk NC cells (**Figure 8**). These data also suggest that most of the trunk NC cells adopting neural and melanocytic phenotypes in vivo are likely derived from progenitors that also possessed dormant osteogenic and/or adipogenic potentials.

Conclusion

The present work has several implications related to the role of the NC during evolution of Vertebrates and the ontogeny of the mesenchymal cells. Our findings suggest that the NC cells along the whole anterior-posterior axis possess the ability to make bone. Early in evolution, the skeletogenic NC is thought to have produced the mineralized dermal armor covering the head and trunk of extinct fishes designated as Ostracoderms, as this fossilized armor was found to contain dentine, a mineral exclusively of NC origin (Janvier, 1976; Hall and Smith, 1990; Smith et al, 1991). Later in the evolution of vertebrates, this trunk exoskeleton has been lost and replaced by an internal skeleton of mesoderm origin, and, in the amniote vertebrates, only the cephalic NC still contributes to the skeleton in the head. Teleostean fishes, like zebrafish, however present remnants of this ancestral trunk NC-derived skeleton in the distal bones of dorsal and caudal fins (Smith et al, 1994). Our data therefore suggest that, even if amniote vertebrates do not present NC-derived mesenchymal derivatives in the trunk, their NC progenitors of the trunk level have kept dormant

skeletogenic potentials that are prevented to being realized in vivo, although these progenitors are still able to differentiate into osteoblasts, chondrocytes and adipocytes when challenged with appropriate conditions in vitro.

The finding that the early trunk NC has a great differentiation potential for mesenchymal phenotypes in vitro, also allows us to consider the possibility that NC-derived stem cells recently found at postmigratory stages and in a variety of adult tissues (for references, Shakova and Sommer, 2010; Dupin and Sommer, 2012) could be similarly capable to yield mesenchymal cells. Production of osteoblasts, chondrocytes and adipocytes was indeed demonstrated in the progeny of cultured NC-derived cells isolated from the facial skin of adult transgenic mice using *Wnt1*- or *P0*-Cre lines wherein the NC cells can be genetically traced until adulthood (Sieber-Blum et al., 2004; Wong et al., 2006; Jinno et al., 2010). However, such extended lineage repertoire could be expected in these facial adult NCSC-like cells, as their ancestors in the early cranial NC are skeletogenic and adipogenic. In contrast, it is noteworthy that some adult progenitors of trunk NC origin also have been shown recently to generate skeletal cells and adipocytes in vitro. This is the case of adult NC-derived stem cells found in the bulge region of human and mouse hair follicles in the dorsal skin (Wong et al., 2006; Clewes et al., 2011) and in those that populate the bone marrow of tibia and femur where they settle close to the bone surface (Nagoshi et al., 2008). These cells could differentiate in vitro not only into neurons, glial cells and myofibroblasts, but also into bone and fat cells (Nagoshi et al., 2008; Glezer et al., 2010), supporting new evidence suggesting a neurectodermal/NC origin of a subset of mesenchymal stem cells in the bone marrow stroma (Takishima et al., 2007; Morikawa et al., 2009). Although these data needs to be confirmed, the

identification of NC-derived stem cells with mesenchymal potential in adult tissues raises new issues on the origin and function of mesenchymal progenitors in vivo.

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Table 1 : PCR primer sequences

Gene	Forward primer	Reverse primer	Insert (bp)
Runx2	ACAGGACTTCCAGCCATCAC	GCTTGTGAACTGCCTGGGAT	90
Sox9	GCCATCTTCAAGGCGCTGC	GAGCTGCTGATGCCGTAGG	377
Sox10	TGAAGACCACCACTGCCTCT	CTTGCTGCTCCTTCTTGACC	110
col1a1	ATGTCCACCGAGGCCACCCA	CAGGTTGCCGGTGTCGTGGT	81
col10	TTGCCAGGGATGAAGGGACACA	AAGCCATTCTCACCAGGCAAGC	164
SPP1	AGAACGACAGCCGGCCAAGA	AGCGCTCTCTAGCGTCTGGT	137
Aggrecan	GCTGGTCTGATGGACACTCGCT	CGTCGTTCCATTCGCCTTGCT	123
ALPL	GTGTGGACTTCCTGCTGGGTCT	TTTGCGGTCCACGTCGCTCT	380
FABP4	GAGTTTGATGAGACCACAGC	AACAGTCTCTTTGCCATCCC	105
CEBPα	TTCTACGAGGTCGATTCCCGGC	TCGTACAGCGGGTCGAGCTT	404
PPARγ	TACATAAAGTCCTTCCCGCTGACC	TCCAGTGCGTTGAACTTCACAGC	470
GAPDH	TATCTTCCAGGAGCGTGACC	TAACACGCTTAGCACCACCC	134

Table 2: Quantification of clone types after analysis of osteoblastic differentiation

Colony types	Number	%
GNO	239	56.90
GN	21	5.00
GO	88	20.95
G	56	13.33
O	1	0.24
Negative	5	1.19
Total	420	100,00

Distribution of trunk NC clone types according to cell phenotypes identified. Glial (G), neuronal (N) and osteoblastic (O) cells were evidenced in day-10 trunk NC clonal cultures. Data were obtained from three series of experiments (n=420). “Negative” clones showed absence of O, N and G cell types.

Figure 1

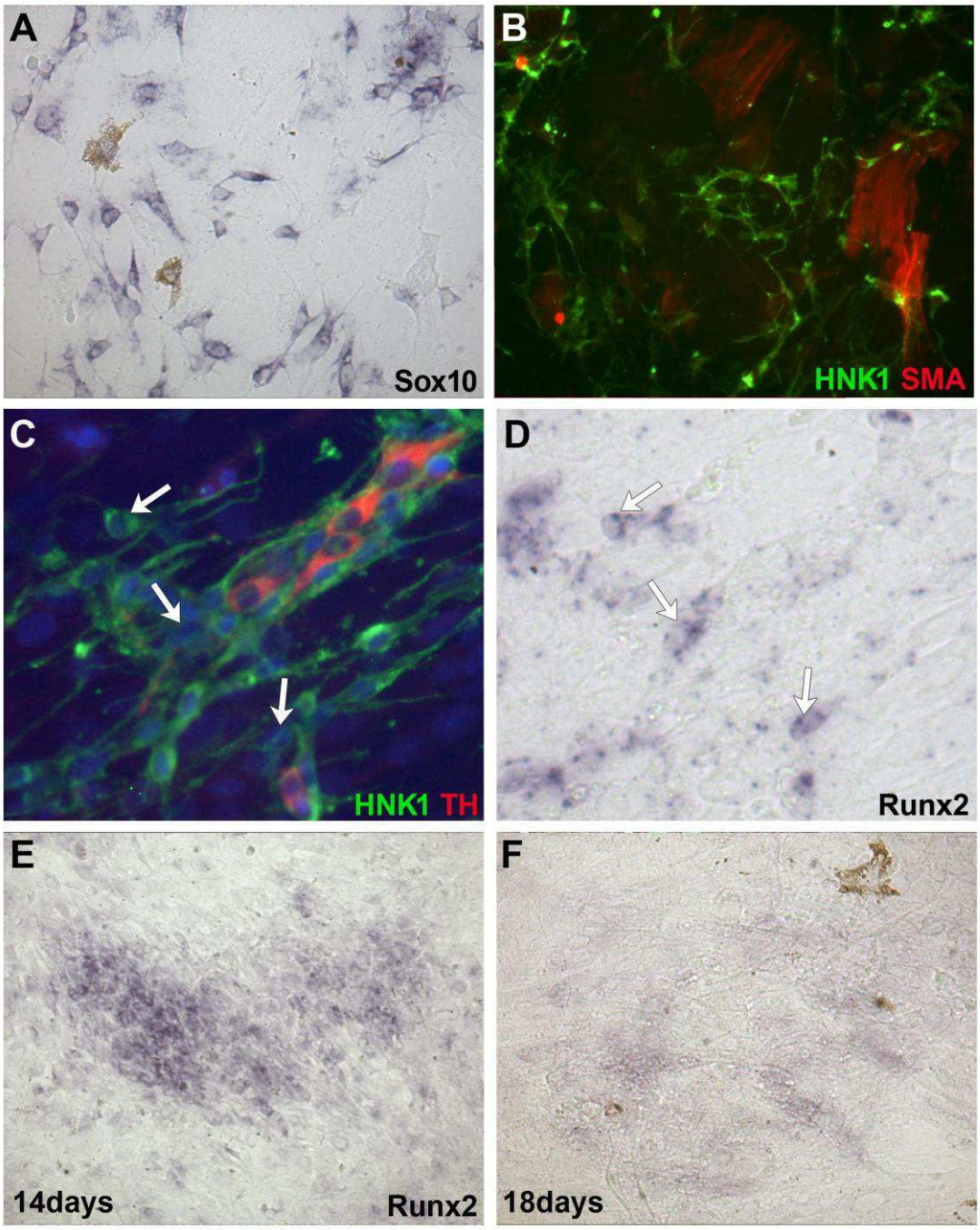

Figure 1. In vitro differentiation of trunk NC cells into diverse phenotypes. (A,B) Trunk CN culture at day-10 with the presence of pigmented melanocytes and *Sox10*-positive cells identified by in situ hybridization (A), as well as αSMA-positive smooth muscle cells and HNK1-positive cells identified by immunocytochemistry (B). (C,D) Field of day-10 culture with HNK1-positive cells and adrenergic neurons expressing TH (C), together with osteoblasts identified by *Runx2* transcripts (D). (E,F) Expression of *Runx2* mRNA in day-14 (E) and day-18 (F) cultures.

Figure 2

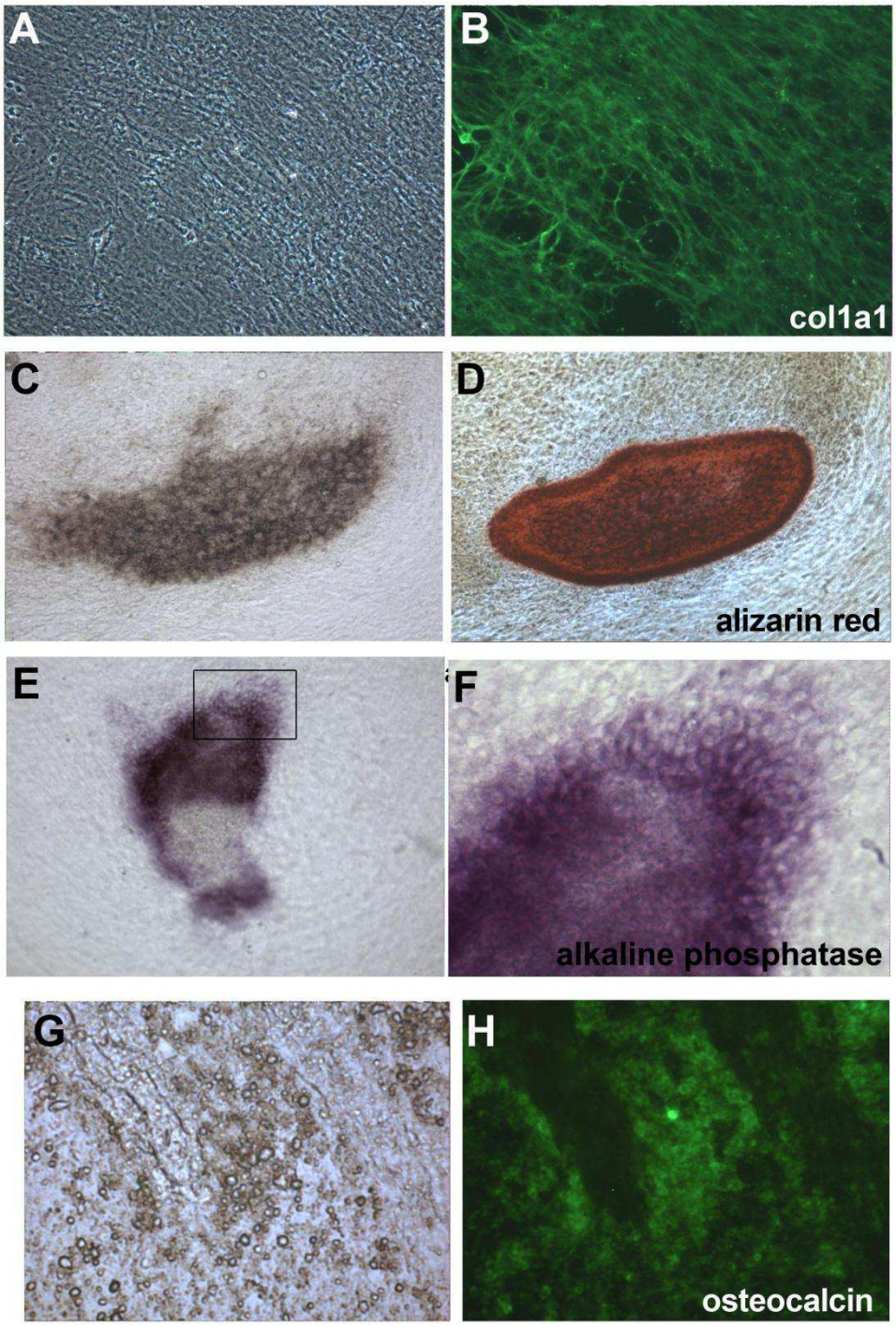

Figure 2. Characterization of trunk NC osteoblastic differentiation in vitro. (A,B) Collagen1a1 protein expression after 15 days of culture. (C,D) Mineralized region in day-21 culture. The deposited mineral was observed by phase microscopy (C) and characterized as calcified bone matrix by Alizarin redS staining (D). (E,F) *Alkaline phosphatase* gene expression in mineralized region at day-21 of culture. (G,H) Day-23 culture under phase-contrast (G) and immunodetection of osteocalcin (H).

Figure 3

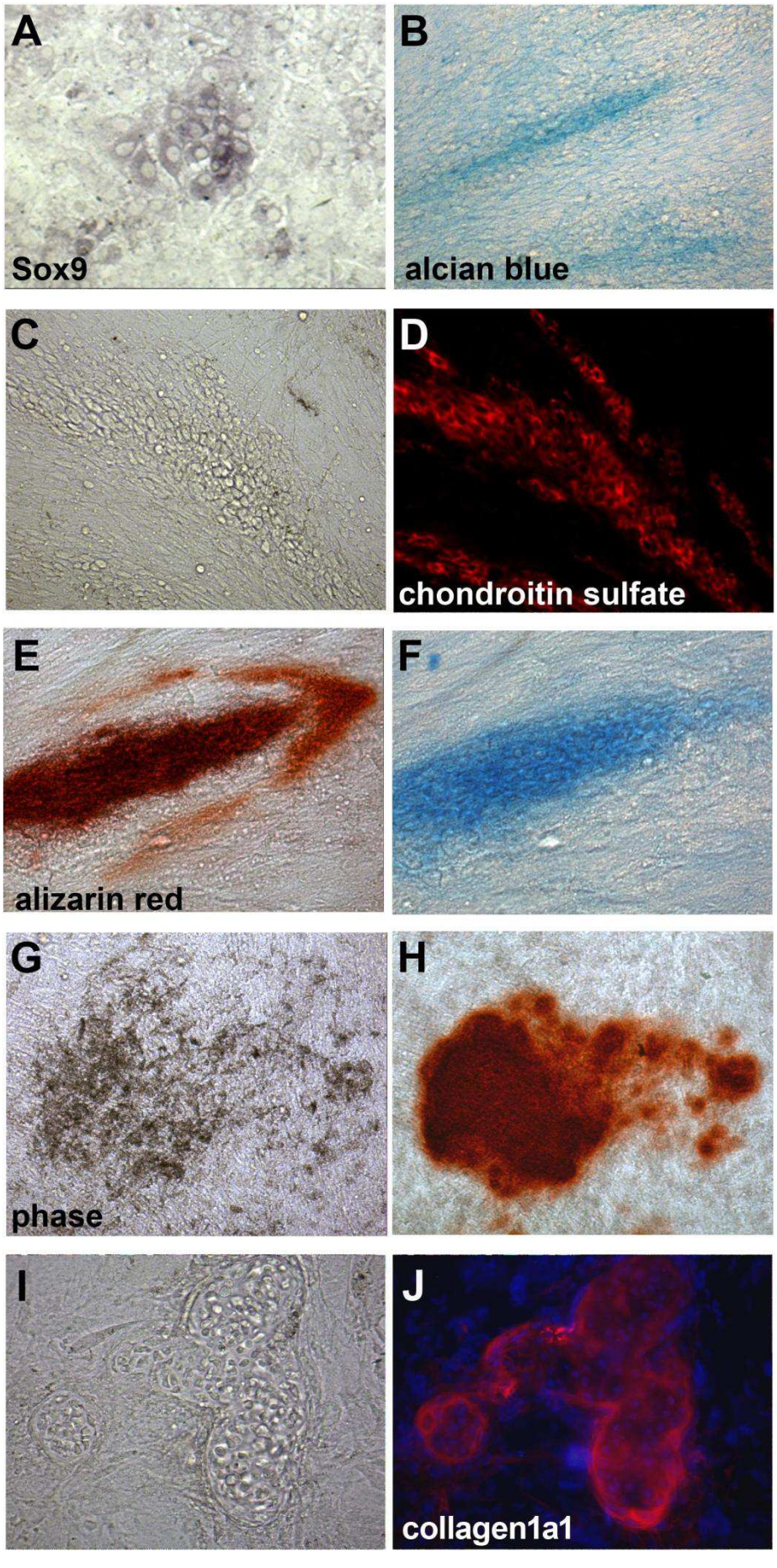
A
Sox9
B
alcian blue
C
D
chondroitin sulfate
E
alizarin red
F
G
phase
H
I
J
collagen1a1

Figure 3. Chondrocytic differentiation of trunk NC cells in vitro. (A) Detection of the *Sox9* mRNA in day-12 culture. (B) Alcian blue staining of a cartilage nodule in day-15 trunk NC culture. (C,D) Clustered chondrocytes (phase-contrast) (C) showing expression of the extracellular matrix protein chondroitin sulfate (D) in trunk NC cell cultures in control medium for 21 days. (E,F) Mineralization region in a day-23 culture with calcified extracellular matrix stained with Alizarin red (E) located on chondrocytic nodule identified using Alcian blue stain (F). (G,H) day-23 culture with a region of bone matrix mineralization (G, bright-field) stained with Alizarin red (H), which differentiated in absence of chondrocytes. (I,J) Trunk NC cells cultured for 15 days on a 3T3 feeder layer, after treatment with the mesenchymal differentiation cocktail from day-7 to day-15 showing chondrocytic nodules (I, phase-contrast) and expression of collagen1a1 in the perichondrium (J).

Figure 4

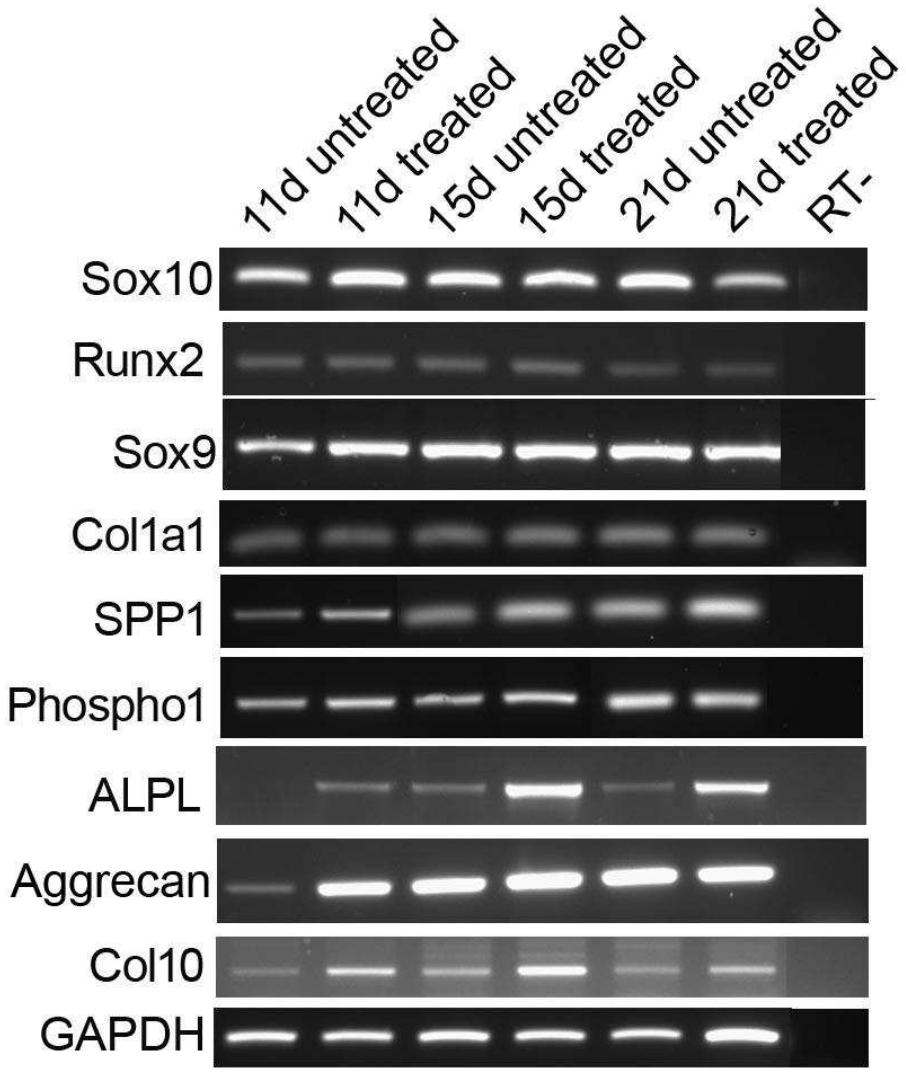

Figure 4. Semi-quantitative RT-PCR analysis of untreated and treated cultures at day-11, -15 and –21 for the expression of *Sox10* NC marker gene and genes involved in the osteoblasts and chondrocytes differentiation process. *GAPDH* was used as a housekeeping gene.

Figure 5

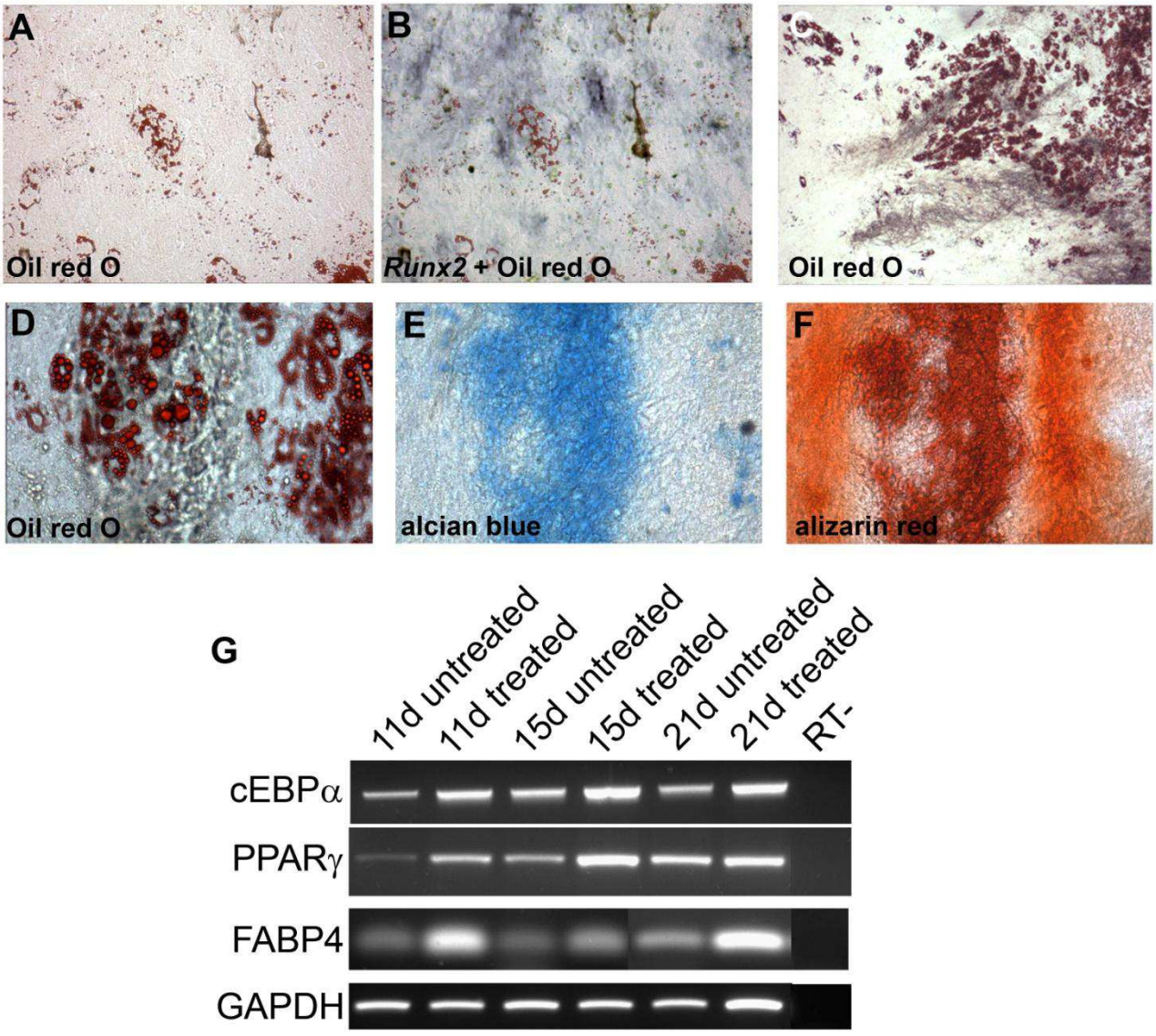

Figure 5. Adipocytic differentiation of trunk NC cells in vitro. (A,B) Trunk NC day-12 culture with adipocytes stained with Oil redO (A) and osteoblasts expressing *Runx2* (B). (C) At day-23, trunk NC cell cultures exhibit large mineralized regions and numerous adipocytes stained with Oil RedO. (D-F) Field of a 23-day culture with Oil redO-stained adipocytes (D), chondrocytes stained with Alcian blue (E) and mineralized extracellular matrix stained with Alizarin red (F). (G) Semi-quantitative RT-PCR analysis of untreated and treated cultures at day-11, -15 and –21 for the expression of genes involved in the adipocyte differentiation process; *GAPDH* was used as a housekeeping gene.

Figure 6

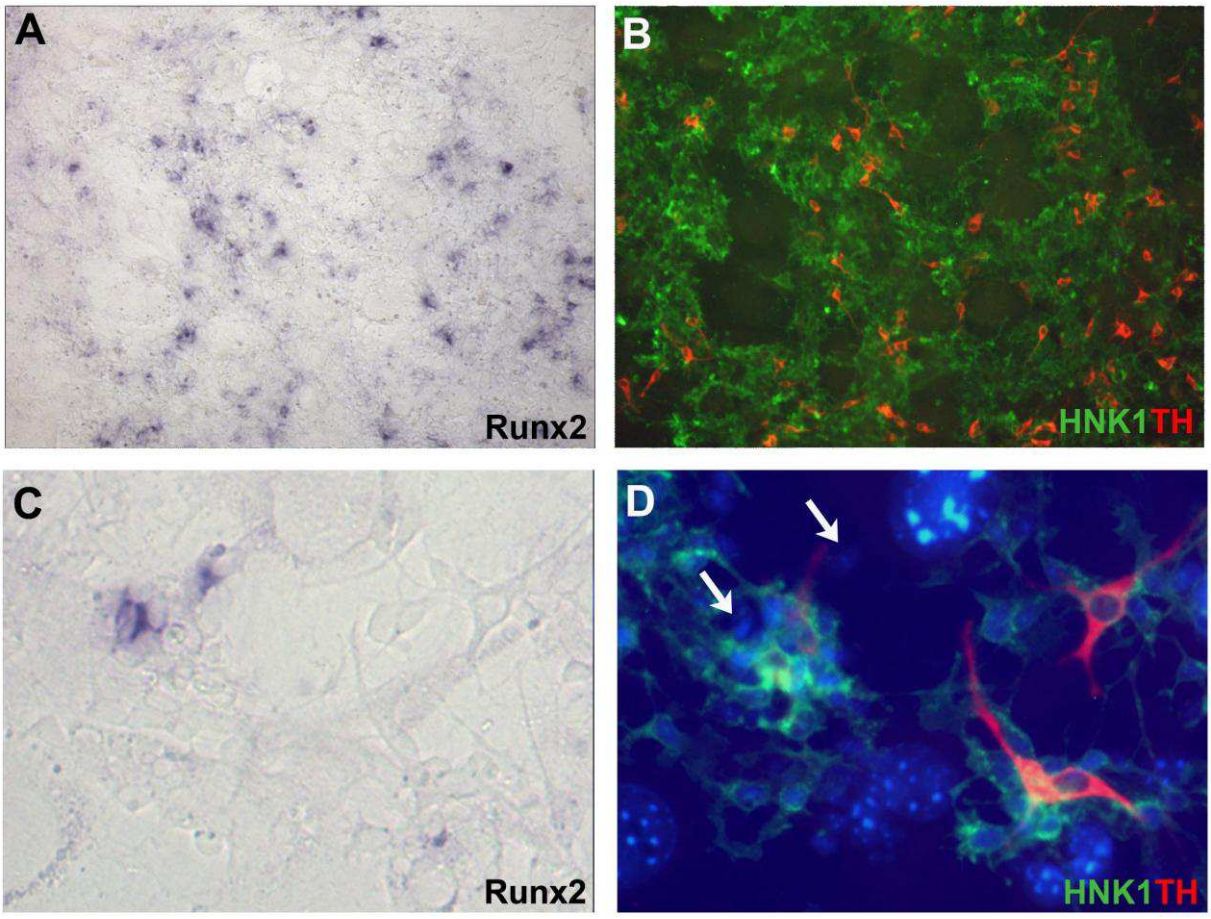

Figure 6. Mixed neural-osteoblast clones (GNO) derived from trunk NC cells. Day-10 clonal cultures were analyzed for the presence of glial cells (identified using HNK1 Mab), neurons (labeled with anti-TH) and osteoblasts identified by in situ hybridization for *Runx2*. (A,B) Global view of a clone showing both *Runx2*-positive cells (A) and neurons and glial cells (B). (C,D) High magnification of a GNO clone showing two Runx2-positive cells (C, arrows in D), in the vicinity of TH-positive neurons and HNK1-positive glial cells (D); cell nuclei were stained using by the blue dye Hoechst.

Figure 7

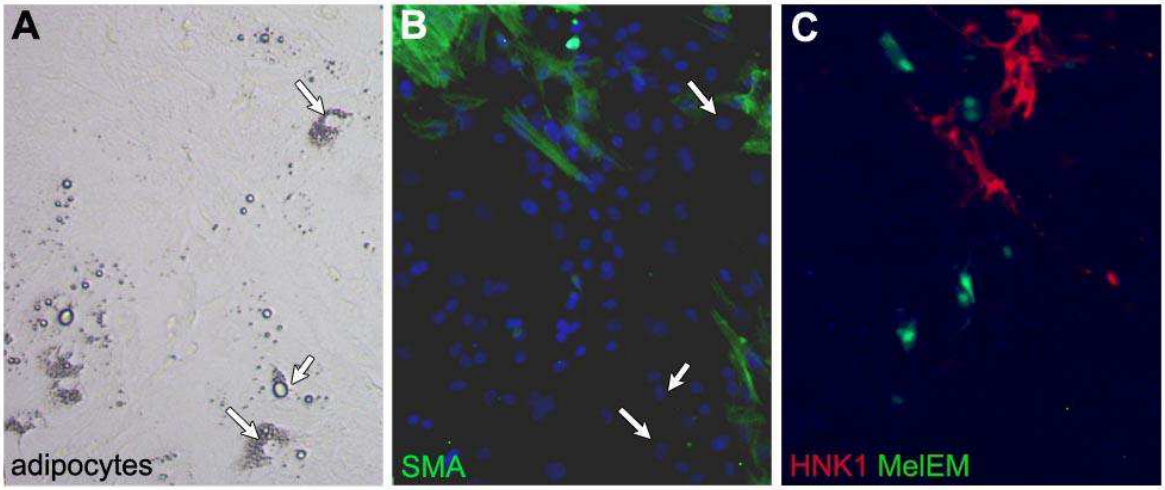

Figure 7. Multiphenotypic clone (GMFA) of TNC cells cultivated for 16 days on collagen. (A) Adipocytes were morphologically identified by their cytoplasmic lipid droplets under phase-contrast microscopy. (B) Same microscopic field showing myofibroblasts immunoreactive to αSMA; the nuclei (Hoechst blue staining) of some adipocytes are shown by arrows. (C) Same field after immunocytochemistry for HNK1 and MelEM Mab to identify glial cells and unpigmented melanocytes, respectively.

Figure 8

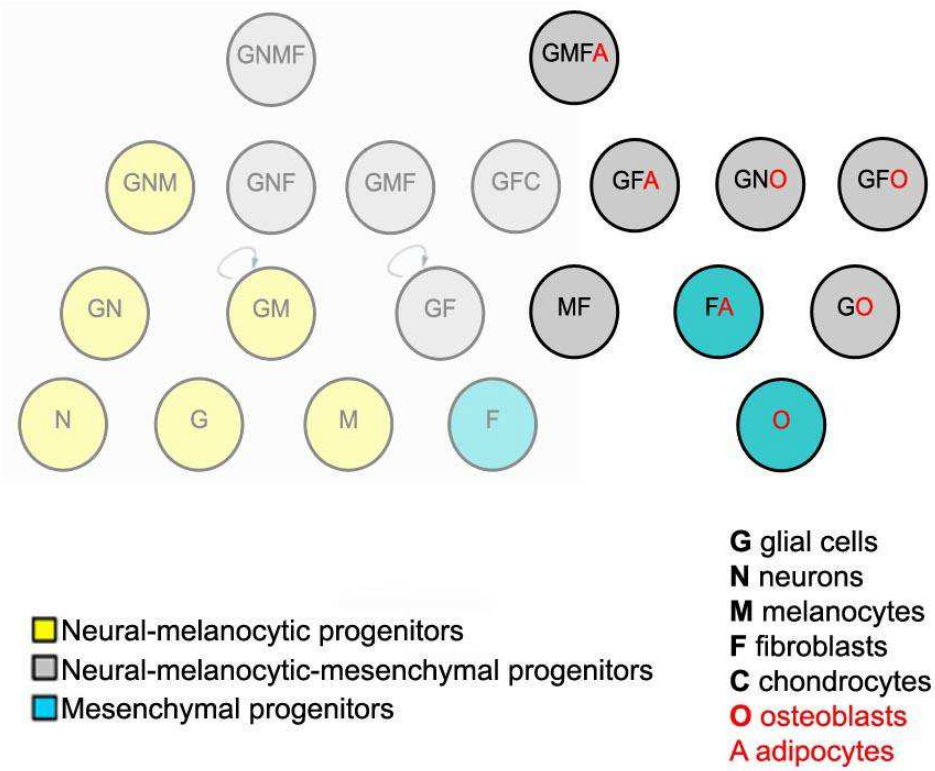

Figure 8: Summary of the distinct types of progenitors identified by in vitro clonal analyses of trunk quail NC cells. On the left, previously known progenitors, those for the classical phenotypes of trunk NC cells, i.e., (glial and neuronal) neural cells and mélanocytes, are shown in yellow and those yielding myofibroblasts (F) in gray, including bipotent GM and GF cells with self-renewal activity, as deduced from previous analysis by Trentin et al. (2004). In addition, a rare progenitor (GFC) for the glial, myofibroblastic and chondrocytic (C) lineages was recently evidenced in single trunk NC cells grown on 3T3 fibroblasts and exposed to Shh treatment (Calloni et al., 2007). On the right side, is shown a number of additional mesenchymal trunk NC progenitors described in the present study (in bold). They comprise of common progenitors for osteoblasts and neural cells (GNO and GO), which differentiated at high frequencies from trunk NC cells clonally seeded on 3T3 feeder-layers, and multipotent progenitors for adipocytes, glial cells and/or melanocytic-fibroblastic cells (GMFA and GFA), the existence of which could be assessed in clonal cultures performed on collagen. These data underline the existence of several types of multipotent trunk NC cells endowed with both neural-melanocytic and mesenchymal potentials (in gray).

3.3. (Section3)

Complementary data of the in vitro trunk NC cell mesenchymal differentiation

3.3.1. Influence of Wnt signalling on trunk NC mesenchymal differentition

Wnt signalling pathway was reported to act in the development of osteoblasts, adipocytes and chondrocytes. Some studies have shown that Wnt proteins inhibit adipocytic differentiation of mesenchymal stem cells (Bennett et al., 2002; Takada et al., 2009). It also influences skeletogenesis in long bones by inhibiting chondrocyte differentiation, while favoring osteoblast differentiation of osteochondro-progenitors (Day et al., 2005; Hill et al., 2005). In the mouse NC, a recent report suggested a regulation of the in vitro skeletogenic properties of the NC cells by Wnt signaling (Iwashita et al., 2009). On one hand, the activation of the canonical Wnt pathway induced expression of *Hoxd9*, normally only activated in the trunk, which led to inhibit chondrogenesis (Iwashita et al., 2009). On the other hand, the trunk NC cells maintained *Hoxd9* in vitro expression after activation of the canonical Wnt pathway resulting from treatment with BIO (6-bromoindirubin-3'-oxime), a glycogen synthase kinase-3 (GSK-3)-specific inhibitor (Iwashita et al., 2009).

We investigated whether the Wnt pathway might play a role in the regulation of the mesenchymal potentialities of the avian trunk NC cells. First, we treated cultures of quail trunk NC cells by addition of recombinant mouse Wnt3a (R&D System), but we could not observe any effect. Second, we sought to directly activate the Wnt signaling pathway and we thus tested the action of BIO at 1μM and 5μM. Preliminary results showed that untreated and treated trunk NC cultures were very similar morphologically until the 7th day of culture, showing large amount of pigmented cells

and similar cell density. All the cultures were thereafter exposed to the mesenchymal differentiation cocktail from day-7. At day 9, we could observe an increase in the number of small cells in cultures treated with BIO 5μM that, as compared to the untreated cultures and to those treated with 1μM BIO. Later, adipocytes with lipid droplets were identified in control cultures from day-11 and, at day-13, all of these control cultures presented adipocytes. In contrast, cultures treated with BIO 5μM showed very rare adipocytes at 15 days of culture. Some cultures treated with BIO 1μM had some areas of adipocytes, although smaller than in control cultures. The cultures with supplemented BIO also showed an increase in melanocytes, with cells treated with 5μM showing a greater effect than that treated with 1μM BIO (**Figure 17B-E'**). At 17 days the control cells started to show mineralization, while BIO-supplemented NC cells never showed mineralized regions. Moreover pigmented cells were less numerous in all conditions and the cultures showed increased fibroblastic cells.

These results suggest that BIO treatment of the cultures can decrease the in vitro mesenchymal differentiation of trunk NC cells.

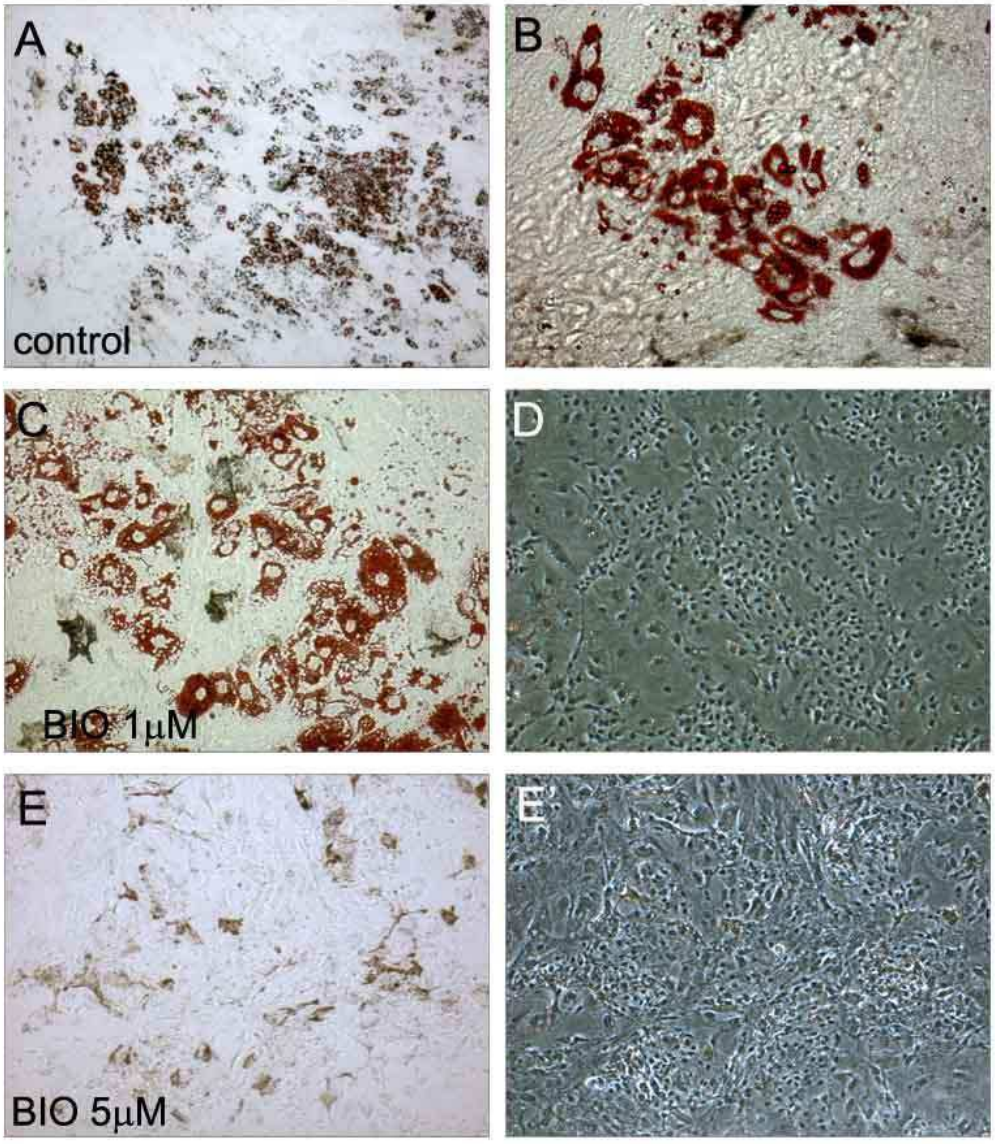

Figure 17 – Trunk NC cultures treated with BIO for 15 days. (A-B) Control cultures showing large adipocytes areas (A) and some chondrocytes nodules (B). (C-D) Trunk NC cells supplemented with BIO 1μM, where we could found some smaller areas of adipocytes (C) and many small, melanoblasts-like, cells (D). (E-E') Same region of a culture after BIO 5μM, showin very rare adipocytes, but some treated with

3.3.2. Effect of FGF signaling on trunk NC cell mesenchymal differentiation

The FGF signaling is important for skeletogenic differentiation of cephalic NC cells in vitro (Sarkar et al., 2002; Ido and Ito, 2006). We thus tested the possible effect of FGF2 on quail trunk NC differentiation. We performed culture experiments with a FGF2 (10ng/ml) supplement and thereafter analyze *Runx2* expression and other derived NC phenotypes.

The first observed result was the great increase in NC cell proliferation. When 10.000 cells obtained from 15h of primary culture where supplemented with FGF2 for 4 days of secondary cultures, the number of NC cells was three-fold (about 180.000 cells) that obtained in control conditions (about 60.000 cells).

Next we performed experiments where trunk NC cells were supplemented with FGF2 during primary and secondary cultures. After 12 days, the cells were subcultured for 3 days either in absence or presence of FGF2. These cultures were analyzed for adrenergic neurons (anti-TH), glial and undifferentiated cells (anti-HNK1), osteoblasts (in situ hybridization for *Runx2*) and chondrocyte precursors (in situ hybridization for *Sox9*). We found that HNK1-positive cells, *Runx2*-expressing osteoblasts and *Sox9*-positive cells showed increased numbers in cultures continuously maintained with FGF2 for 15 days, when compared to the cultures switched to control medium between day-12 and day-15. By contrast, TH-positive neurons were present in a similar way in both conditions (**Figure 18**).

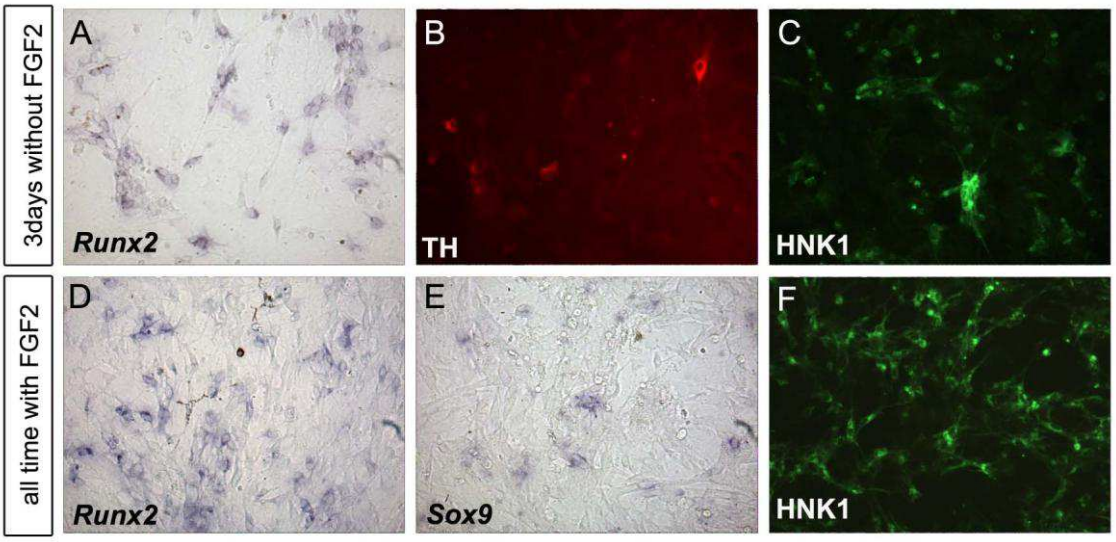

Figure 18 – Trunk NC cells supplemented with FGF2 10ng/ml during 12 days and subcultured for more 3 days in presence or absence of FGF2. (A-C) Cells without FGF2 for 3 days showed a decreased expression of *Runx2* transcripts (A) and HNK1 marker (C), and TH-positive cells were also present in these cultures (B). (D-F) Cells maintained in FGF2 showed increased expression of *Runx2* (D), *Sox9* (E) and HNK1 (F).

4- DISCUSSÃO

A diferenciação celular é determinada pelo potencial intrínseco da célula e pelos fatores ambientais extrínsecos. No caso da CN, as células passam por diferentes contextos moleculares ambientais ao longo de sua migração até seu destino final, e, desta forma, o sistema multifatorial que dirige a diferenciação celular é dinâmico e ainda mais complexo. O sistema de cultura *in vitro* nos permite estudar o potencial de diferenciação celular em condições controladas e sem a influência do meio em que as células se encontram *in vivo*. Neste trabalho mostramos que as células da CN truncal, que não adquirem fenótipos mesenquimais *in vivo* em vertebrados amniotas, possuem um grande potencial de diferenciação mesenquimal.

Neste contexto, foi possível mostrar que as células da CN truncal de codorna se diferenciam em osteoblastos, condrócitos e adipócitos *in vitro*.Estas células se diferenciam até a fase terminal, onde ocorre o armazenamento de lipídeos pelos adipócitos, e a fabricação de matriz extracelular calcificada pelos osteoblastos associados ou não com condrócitos. Além disso, estes fenótipos mesenquimais são encontrados juntamente com os outros fenótipos derivados da CN truncal, como neurônios, células gliais e melanócitos.

Assim como foi previamente demonstrado para a CN cefálica (Calloni et al., 2009), experimentos de culturas clonais mostraram que os osteoblastos são derivados de progenitores multipotentes da CN truncal que dão origem à pelo menos um fenótipo neural (glia ou glia+neurônio), além de alguns darem origem também à melanócitos e miofibroblastos. Também reportamos nesta tese que adipócitos também puderam ser obtidos à partir progenitores multipotentes da CN truncal que dão origem à adipócitos, miofibroblastos e/ou células gliais e melanócitos.

Estes resultados, que mostram a capacidade das células da CN truncal de realizar esqueletogênese e adipogênese nos amniotas, trazem novas percepções sobre a ontogenia das células mesenquimais derivadas da CN e sobre o entendimento da evolução das células da CN.

4.1. Diferenciação da CN truncal em osteoblastos e condrócitos *in vitro*

Neste trabalho, células mesenquimais foram obtidas num meio de cultura enriquecido com soro fetal bovino e extrato de embrião de galinha, usado previamente para o cultivo das células da CN e que possibilita a diferenciação nos diferentes fenótipos encontrados durante o desenvolvimento destas células *in vivo.* Osteoblastos imaturos foram definidos através da expressão do fator de transcrição Runx2, que é expresso inicialmente nas células osteogênicas e é necessário para a formação de todos os ossos (Otto et al, 1997; Ducy et al., 1997). Os primeiros osteoblastos Runx2-positivos foram encontrados à 5 dias de cultura, período em que outros fenótipos da CN já foram especificados, como os melanócitos por exemplo, que começam a se apresentar pigmentados à 4 dias, e os neurônios adrenérgicos que já expressam a proteína TH à 3 dias de cultura. A 7 dias, aproximadamente 10% das células presentes na cultura expressam o mRNA de Runx2.

Com a administração do coquetel de diferenciação mesenquimal que consiste de fatores osteogênicos e adipogênicos no sétimo dia, os osteoblastos Runx2-positivos foram capazes de avançar no processo de diferenciação até a fase de mineralização de sua matriz extracelular. Experimentos de hibridização *in situ*, imunocitoquímica, RT-PCR e coloração citoquímica mostraram que a diferenciação óssea nestas culturas de células da CN truncal recapitula todas as principais etapas envolvidas no processo de osteogênese e condrogênese. Os primeiros genes

envolvidos da osteogênese e condrogênese, Runx2 e Sox9 respectivamente, já puderam ser detectado à 3 dias de cultura através de RT-PCR. A proteína colágeno1 foi detectada a partir de 10 dias de cultura por imunocitoquímica, embora o RNAm de pro-colageno1 já tenha sido detectado à 3 dias. Além destes o RNAm de osteopontina, aggrecan e colageno10 também puderam ser encontrados à 11, 15 e 21 dias de cultura em células tratadas e não-tratadas com o coquetel de diferenciação mesenquimal. O RNAm de fosfatase alcalina, enzima indispensável para o processo de mineralização, mostrou um aumento significativo nas células tratadas com o coquetel de diferenciação mesenquimal. Por hibridização *in situ*, transcritos desta enzima foram encontrados somente em regiões muito bem delimitadas que apresentaram o processo de mineralização. A proteína osteocalcina foi detectada por imunocitoquímica apenas em culturas de células que apresentaram mineralização de sua matriz extracelular. Desta forma, o sistema de cultura celular estabelecido por esta tese é um bom modelo de estudo para o comprometimento de células multipotentes com o fenótipo mesenquimal e do processo de diferenciação em osteoblastos e condrócitos.

A diferenciação óssea realizada pelas células da CN pode ser do tipo intramenbranosa, com osteoblastos se diferenciando diretamente a partir do mesênquima condensado, como acontece nos ossos frontais do crânio, por exemplo, ou endocondral, onde os osteoblastos utilizam o molde cartilaginoso para a deposição de na matriz extracelular que se torna mineralizada, como acontece na mandíbula, por exemplo. Nossos experimentos sugerem que os dois tipos de ossificação ocorrem em nosso sistema de cultura. A mineralização da matriz extracelular foi confirmada com alizarina red, que colore o cálcio depositado. Os condrócitos puderam ser identificados por alcian blue em culturas de 15 e 21 dias

tratadas e não-tratadas com o coquetel de diferenciação mesenquimal. A 21 dias pudemos observar que a maior parte da coloração alizarin red se encontra co-localizada com a coloração alcian blue, indicando a ocorrência de ossificação endocondral nestas culturas. Algumas áreas coloridas apenas com alizarin red também foram observadas. Muitas destas áreas se localizam próximas das áreas que são marcadas também com alcian blue, e se assemelham à pericôndrios. Outras regiões marcadas apenas com alizarin red não possuem qualquer interação com os condrócitos identificados por alcian blue e sugerem a ocorrência de ossificação intramembranosa.

McGonnel e Graham mostraram em 2002 que células da CN truncal secretam, após 4 semanas de cultura, matriz extracelular típica de osteoblastos e condrócitos, como as proteínas colágeno1a1 e colágeno2a1, além de coloração com alcian blue e alizarin red (McGonnell and Graham, 2002). Foi mostrado também que células da CN truncal transplantadas no primeiro AB de embriões de 2 dias, após serem cultivadas in vitro por um dia, estão presentes na cartilagem de Meckel da futura mandíbula e na cartilagem esclerótica que envolve o olho. Entretanto, se algumas células da CN truncal podem sobreviver nestes ambientes mesenquimais *in vivo*, ainda é desconhecido se podem adotar um fenótipo esqueletogênico (McGonnell and Graham, 2002).

Observamos que, apesar da maior parte dos genes expressos na osteogênese e condrogênese estarem presentes nas células tratadas e também nas não tratadas com o coquetel de diferenciação mesenquimal desde etapas precoces do cultivo celular, o processo de mineralização da matriz óssea é observado somente nas células tratadas. Estes resultados sugerem que este meio de diferenciação atua possibilitando que os progenitores mesenquimais já presentes

possam avançar no processo de diferenciação. Além disso, os transplantes heterotópicos da CN cefálica de codorna na região truncal de embrião de galinha resultaram em cartilagens ectópicas, o que indica que o ambiente de migração ventral da CN truncal é permissivo à diferenciação mesenquimal esquelética. Podemos então pensar que as células da CN truncal não realizam diferenciação mesenquimal *in vivo* pois quando se encontram em ambientes permissivos já tem este potencial inibido.

Alguns trabalhos já relacionaram os genes *hox* com a ausência de diferenciação esqueletogênica pelas células da CN truncal. Foi mostrado em embriões de galinha e camundongo que a expressão dos genes *hox* diminui ao longo da condrogênese da CN truncal *in vitro*,e que a manutenção da expressão do gene *hoxd10* impede este processo (Abzanov et al., 2003; Ido and Ito, 2006). Talvez esta diminuição de genes hox também aconteça em nosso sistema de cultivo, permitindo a diferenciação esqueletogênica da CN truncal. Um dos fatores de crescimento que podem estar envolvidos em nossa cultura favorecendo a diferenciação em condrocitos e osteoblastos é Shh. Este fator de crescimento atua no processo de ossificação endocondral a partir das células da CN cefálica (Brito et al., 2008). *In vitro*, Shh aumenta o potencial de condrogênese das células da CN cefálica e também truncal (Calloni et al., 2007, 2009). Além de aumentar os nódulos de cartilagem encontrados em cultura de massa, este fator também aumenta o numero de progenitores multipotentes com capacidade neural e mesenquimal, e aumenta o numero de osteoblastos Runx2-positivos ao redor dos condrócitos na região do pericôndrio. Entretanto, nossos testes preliminares com a adição deste fator em nosso sistema de cultura não mostraram alterações no processo de diferenciação ósseo e adipocitica da CN truncal.

Num trabalho recente, John e colaboradores mostraram que é possível induzir diferenciação óssea da CN truncal de camundongos através do curto tratamento de 4h com TGFβ1 (John et al., 2011). Estas células tratadas com TGFβ1 são então convertidas em progenitores mesenquimais, e perdem a sua capacidade neurogênica. Além disso, o tratamento com TGFβ1 induz a perda de expressão de Sox10 e o ganho de Sox9 em todas as células. A perda de Sox10 é essencial para a aquisição da capacidade mesenquimal das células da CN (Blentic et al., 2008). Em nosso trabalho, o meio de cultura estabelecido oferece às células da CN de codorna as condições de diferenciação em diversos fenótipos derivados da CN, inclusive nos derivados mesenquimais normalmente não encontrados no tronco *in vivo*. Além disso, células Sox10-positivas são encontradas neste sistema em culturas de longo período (três semanas), concomitantemente com a mineralização das células ósseas. Desta forma, nosso método de cultivo permite estudar os eventos e fatores envolvidos na diferenciação de células multipotentes em diferentes tipos celulares.

4.2. Diferenciação da CN truncal em adipócitos *in vitro*

Esta tese também revelou que as células da CN truncal possuem um grande potencial de diferenciação adipocitica. Trabalhos anteriores já haviam mostrado que a CN truncal é capaz de se diferenciar em adipócitos (Bilon et al., 2007). Entretanto, este fenótipo foi obtido somente em 40% das culturas. Neste trabalho, os experimentos realizados para o estabelecimento das melhores condições de cultivo nos permitiram observar que mais de 90% das culturas tratadas com o coquetel de diferenciação mesenquimal apresentaram grandes agrupamentos de adipócitos. Estes resultados foram obtidos em culturas secundárias realizadas após 15h de

cultura primária em presença do TN, embora culturas realizadas após 48h de cultura primária também tenham apresentado alto rendimento. O coquetel de diferenciação mesenquimal que contém fatores adipogênicos e osteogênicos foi mais eficiente para a adipogênese que os dois diferentes meios adipogênicos utilizados no trabalho precedente (Billon et al., 2007). Uma provável explicação para a maior eficácia do meio de diferenciação mesenquimal é a presença do glucocorticóide dexametasona durante todo o tempo de aplicação do coquetel. A dexametasona também esta presente em 1 dos meios adipogênicos testado, mas é acrescido neste meio apenas nos 2 primeiros dias de contato com as células. Além de atuar na diferenciação osteogênica, a dexametasona tem também um papel importante na adipogênese, onde induz indiretamente PPARγ e CEBPα, dois fatores de transcrição essenciais para a adipogênese (Pantoja et al., 2008).

A diferenciação adipogênica foi observada morfologicamente e através do corante oil red o. Além disso, a expressão de genes implicados neste processo foi acompanhada por experimentos de RT-PCR semi-quantitativo em células tratadas e não-tratadas com o coquetel de diferenciação mesenquimal. Estes experimentos mostraram que a partir de 11 dias de cultura os genes PPARγ, CEBPα e FABP4 já estão expressos, mesmo em culturas não tratadas, com tendência à um maior nível de expressão nas células estimuladas com o meio de diferenciação. Além destes, o fator de transcrição Pbx1 esteve presente em nossas culturas desde 3 dias e durante todo o tempo analisado. Este fator foi recentemente identificados como necessário para as etapas iniciais de adipogênese de células-tronco embrionárias de camundongos neuralizadas, e parece inibir a diferenciação final dos adipócitos (Monteiro et al., 2011). Estes dados sugerem que a CN truncal gera *in vitro* um tipo celular avançado no processo de diferenciação em adipócitos, independente do

meio de diferenciação mesenquimal fornecido, o que nunca foi anteriormente mostrado. Neste caso, podemos afirmar que o coquetel de diferenciação mesenquimal tem um papel apenas na diferenciação final dos “pré-adipócitos” da CN truncal em adipócitos que estocam lipídeos, como observado nas linhagens celulares de pré-adipócitos e células-tronco mesenquimais comumente utilizadas para o estudo da adipogênese (Rosen and McDougald, 2006).

Outra característica importante do nosso sistema de cultura é que osteogênese e adipogênese ocorrem em conjunto sob as mesmas condições de cultura. Estes dois eventos são competitivamente regulados na medula óssea e nas células-tronco mesenquimais *in vitro*, e condições que favorecem um fenótipo inibem o outro (Chamberlaim et al., 2007). O balanço entre diferenciação óssea e adipocítica não é conhecido durante o processo de diferenciação mesenquimal da CN. Nosso sistema de cultura fornece as condições necessárias para o estudo deste balanço entre osteogênese e adipogênese.

4.3. Progenitores mesenquimais multipotentes da CN truncal

Nossos resultados colocaram em evidência o potencial mesenquimal da CN truncal, que é capaz de se diferenciar *in vitro* em osteoblastos, condrócitos e adipócitos. Entretanto, experimentos de clonagem se fazem necessários para estudarmos a origem deste potencial de diferenciação. O fato de a CN truncal não dar origem a células mesenquimais *in vivo* levou à hipótese de que a população celular de CN cefálica possui progenitores com potenciais mesenquimais independentes dos progenitores neurais e/ou melanociticos. Entretanto, Baroffio e colaboradores mostraram através de clonagem que alguns progenitores da CN

cefálica do mesencéfalo geram uma progênie diversa, que possuí ao mesmo tempo capacidades neurais e condrogênicas (Baroffio et al., 1991). Recentemente Calloni e colaboradores mostraram que a CN truncal também compreende progenitores com capacidade condrogênica, embora progenitores com esta capacidade sejam muito raros (em torno de 1 em 200) quando comparamos à CN cefálica (Calloni et al., 2007).

Após verificarmos que a CN truncal de codorna também é capaz de realizar diferenciação óssea, realizamos os experimentos de clonagem para observação dos progenitores com capacidade osteogênica, como já realizado para a CN cefálica (Calloni et al., 2009). Culturas clonais também foram realizadas para observação dos clones com adipócitos em sua progênie, embora em condições de menor eficiência, sem a camanada de fibroblastos 3T3 como substrato.

Nos experimentos de clonagem sobre 3T3, onde uma média de 57,5% das células gerou clones, mais de 76% dos clones geraram osteoblastos Runx2-positivos, e quase todos estes clones também apresentaram células neurais. Assim, osteoblastos se diferenciaram a partir de progenitores multipotentes GNO (gliais HNK1-positivos, neuronais TH-positivos e osteoblásticos Runx2-positivos) e GO (gliais e osteoblásticos). Aproximadamente 91% dos clones investigados nestas condições apresentaram melanoblastos MelEM-positivos. Embora limitações técnicas impeçam a detecção de melanoblastos por imunocitoquímica em clones já processados para detecção do RNAm de Runx2, esta alta porcentagem de clones com melanoblastos sugere que grande parte dos clones com potencial osteoblástico possue também melanoblastos em sua progênie. Apenas 3,26% dos clones apresentaram miofibroblastos (F) nesta condição.

A eficiência de clonagem obtida quando células individuais da CN truncal foram cultivadas sobre a placa de cultura revestida com colágeno1 foi bem menor que a encontrada nas células cultivadas sobre 3T3, e variou entre 5,2% e 28,1%. Estes clones receberam o coquetel de diferenciação mesenquimal a partir do 7° dia de cultura e foram fenotipicamente analisados após duas a quatro semanas de cultura. Encontramos adipócitos em 35,3% dos clones que foram classificados como progenitores FA (17,64%), GFA (5,88%) e GMFA (11,76%), de acordo com a presença de células gliais (G), melanócitos (M), miofibroblastos (F) e adipócitos (A). Podemos observar então que todas as células que deram origem à adipócitos geraram também miofibroblastos (clones totais com miofibroblastos compreendem 94,1%). Além disso, aproximadamente a metade dos clones com adipócitos apresentaram também melanócitos. Células neuronais não foram encontradas neste sistema, mas células gliais foram identificadas em 17,6% dos clones com adipócitos. Desta forma, pudemos mostrar pela primeira vez que adipócitos também podem ser obtidos a partir de células multipotentes da CN truncal.

Juntos, os estudos com experimentos de clonagem vêm então reforçar o aspecto multipotente das células da CN truncal no inicio de sua migração (**Figura 19)** – sumário dos progenitores encontrados). Estes resultados mostram que o potencial de diferenciação nas linhagens neural, melanogênica e mesenquimal não se encontra segregado em progenitores diferentes, mas estão presentes em uma única célula multipotente capaz de gerar os três fenótipos. A heterogeneidade destes progenitores multipotentes identificados sugerem um modelo de segregação hierárquica, no qual uma única célula multipotente, através de restrições progressivas, gera células-filhas com potenciais intermediários e eventualmente precursores unipotentes.

Estes dados sugerem que grande parte das células que adotam fenótipos neurais e melanociticos *in vivo* é derivada de progenitores que possuíam também potencial osteogênico e/ou adipogênico. Desta forma, estes dados reforçam a idéia de que as influências ambientais encontradas pelas células da CN truncal *in vivo* especificam e determinam estas células para fenótipos neuronais, gliais e melanociticos antes que se encontrem em ambientes favoráveis e possam responder aos sinais que levam a diferenciação mesenquimal.

4.4. Aspectos evolutivos e perspectivas

Os resultados deste trabalho de tese têm implicações relacionadas ao papel da CN durante a evolução dos vertebrados e a ontogenia de fenótipos mesenquimais.

Pouco se conhece quanto à origem e comprometimento dos progenitores adipogênicos, já que marcadores específicos de estágios iniciais da adipogênese ainda são desconhecidos. A origem dês adipócitos da cabeça a partir da CN cefálica pode ser traçada em aves (Le Lièvre and Le Douarin, 1975) e camundongos (Billon et al., 2007). Assume-se que, no tronco dos vertebrados, tecidos adiposos são originados por progenitores mesodermais, embora poucos tenham tido sua origem embrionária revelada (Tang et al., 2008). Uma possibilidade é a de que alguns depósitos de gordura do tronco possam ser derivados da CN truncal, embora esta hipótese não receba suporte dos estudos de mapeamento genético do destino das células da CN em cammundongos trangênicos (Billon et al., 2007). A segunda possibilidade, mais provável, é a de que progenitores para adipocitos presentes na

CN truncal tem seu potencial inibido in vivo, da mesma forma que os progenitores de osteoblastos.

Estes resultados sugerem que, uma vez que as células da CN ganharam a capacidade de fazer osso em todo o eixo ântero-posterior, este potencial não foi mais perdido durante a evolução, embora não se realize no tronco dos amniotas. No inicio da evolução, a CN esqueletogênica produzia armadura dermal mineralizada que cobria a cabeça e o tronco dos extintos peixes ostracodermes. Mais tarde, este esqueleto truncal foi perdido nos vertebrados superiores amniotas, e substituído por um esqueleto interno dde origem mesodérmica. Nossos dados mostraram que mesmo se os vertebrados amniotas que não apresentam derivados mesenquimais derivados da CN no tronco in vivo, os progenitores da CN truncal ainda são capazes de se diferenciar em osteoblastos, condrocitos e adipocitos quando se encontram em condições apropriadas in vitro.

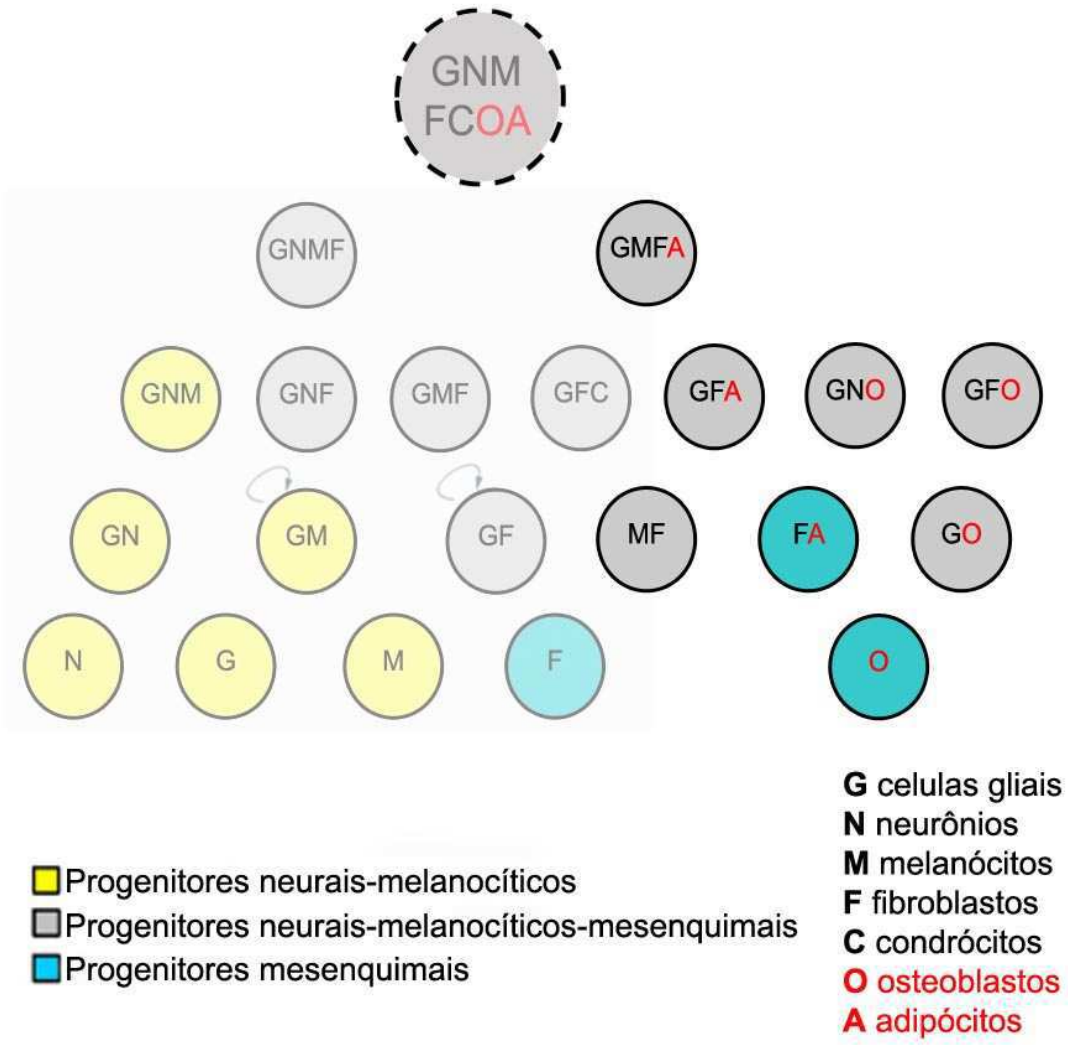

Figura 19 - Síntese dos diferentes tipos de progenitores identificados pela análises clonal in vitro de células da CN truncal de codorna. Progenitores neurais-melanociticos sao mostrados em amarelo, progenitores mesenquimais em azul e progenitores com ambos os potenciais em cinza. Células mais claras foram identificadas a partir de analises anteriores realizadas por Trentin et al. (2004), com exceção do raro progenitor GFC para glia (G), miofibroblastos (F) e condrocitos (C), que foi recentemente encontrado em células da CN truncal cultivadas em fibroblastos 3T3 e expostas a um tratamento Shh (Calloni et al., 2007). Células bipotentes GM e GF possuem capacidade de auto-renovação (Trentin et al., 2004). Neste trabalho, um número adicional de progenitores mesenquimais da CN truncal têm sido evidenciados (células com cores mais fortes) (Coelho et al, em preparação, ver também seção Resultados). Eles incluem progenitores comuns para osteoblastos e células neurais (GNO e GO), que se diferenciaram em altas freqüências a partir de células da CN trunncal semeadas clonalmente sobre monocamadas de fibroblatos 3T3, e progenitores multipotentes para adipócitos, fibroblastos e/ou células gliais e melanocitos (células GMFA , GFA, FA), a existência das quais foi avaliada em culturas clonais realizados sobre colageno. Todos este progenitores da CN truncal seriam derivados de um progenitor hipotético altamente multipotente GNMFCOA.

4 - DISCUSSION

Differentiation of a cell is determined by its intrinsic potential and by the influence of extrinsic environmental factors. In the case of the NC, the cells need going through different molecular environments along their migration paths to their final fate, and thus the multifactorial system that drives cell differentiation is even more complex and very dynamic. The in vitro culture paradigm allowed us to study the differentiation potential of NC cells outside of their in vivo environment and under controlled conditions. In this study, we show that the TNC cells, which do not acquire mesenchymal phenotypes in vivo in amniote vertebrates, in fact have a great potential for mesenchymal differentiation.

In this context, it was shown that the quail TNC cells can robustly differentiate into osteoblasts, chondrocytes and adipocytes in vitro. The progression along these phenotypes reached the terminal phase, until lipid storage by adipocytes and calcified extracellular matrix production by osteoblasts, associated or not with chondrocytes. Moreover, these differentiated mesenchymal cell types were found together with the other TNC derivatives produced in vivo, such as neurons, glial cells and melanocytes.

As previously shown for the quail cephalic NC (Calloni et al., 2009), our clonal experiments revealed that osteoblasts are derived from trunck NC multipotent progenitors that gave rise to at least one neural phenotype (glia or both glia and neurons), some of them also generating myofibroblasts. We also report here the first clonal study of adipocyte progenitors, which provides evidence for the existence of multipotent trunk NC cells producing adipocytes, myofibroblasts and /or glial cells and melanocytes.

These results, by sheding new light on the ability of trunk NC cells in amniotes to undergo skeletogenesis and adipogenesis, provide significant insights in the ontogeny of NC mesenchymal fate and the understanding of NC cell evolution.

4.1. Trunk NC differentiation into osteoblasts and chondrocytes in vitro

In this work, mesenchymal cells were obtained in a medium enriched in FCS and embryo extract, which was previously used for the cultivation of the avian NC cell and allows differentiation into the different phenotypes found during the in vivo development of these cells. Immature osteoblasts were defined by expression of the gene of the Runt family of transcription factors *Runx2*, which is expressed early in osteogenic cells and is required for the formation of all the bones (Otto et al., 1997; Ducy et al., 1997). The first *Runx2*-positive osteoblasts were detected at 5 days of culture, a time point when other NC phenotypes have already been specified, as pigmented melanocytes were present from 4 days and adrenergic neurons expressed the TH enzyme from the third day of culture. At 7 days, approximately 10% of the cells present in NC cultures expressed *Runx2* mRNA.

With stimulation by the mesenchymal differentiation cocktail consisting of both osteogenic and adipogenic factors, given from day-7 of culture, the *Runx2*-positive osteoblasts were able to advance along the bone differentiation process until mineralization of their extracellular matrix. In situ hybridization, immunocytochemistry; cytochemical staining and RT-PCR experiments showed that the skeletogenic differentiation in these trunk NC cell cultures recapitulates all major steps involved in osteogenesis and chondrogenesis. The osteogenesis and chondrogenesis early genes, *Runx2* and *Sox9*; respectively, could be detected already at 3 days of culture by RT-PCR. The protein collagen1a1 was evidenced after 10 days of culture by

immunocytochemistry, although mRNA of *pro-collagen1* was detected from 3 days. In addition, mRNAs of *osteopontin* and of the chondrocyte markers *aggrecan* and *collagen10*, could also be found at 11, 15 and 21 days of culture in cells treated and untreated with the mesenchymal differentiation cocktail. Transcripts of *alkaline phosphatase*, an essential enzyme for the mineralization process, showed a significant increase in treated cells. By in situ hybridization, this gene was found expressed only in defined dense areas of TNC cultures, which exhibited mineralization. Osteocalcin protein, a late marker of osteogenesis, was detected by immunocytochemistry also only in the cultures that showed extracellular matrix mineralization. Thus, the cell culture system established in the present work is a good model to study the commitment of multipotent cells toward mesenchymal phenotypes and the process of NC cell differentiation into osteoblasts and chondrocytes.

The osteogenesis by NC cells may be either of the membranous type, where osteoblasts differentiate directly from the condensated mesenchyme, as in the frontal bone of the skull, or endochondral, where osteoblasts use a cartilage template to deposit extracellular matrix that becomes mineralized, as in the jaw. Our experiments suggest that both types of ossifications occur in TNC cultures. The extracellular matrix mineralization was confirmed with Alizarin Red staining of calcium deposits. Chondrocytes were identified by Alcian Blue in 15- and 21-days treated and untreated cultures. At 21 days, we could observe that most of the Alizarin Red stained regions co-localized with Alcian blue staining, thus indicating endochondral ossification in these cultures. Other areas stained only with Alizarin Red: most of these were located close to or surrounding the Alcian Blue-labeled ones, and thus are similar to the perichondrium. Other regions were stained only with Alizarin Red

without interaction with chondrocytes, suggesting the occurrence of membranous ossification.

McGonnell and Graham previously showed that the avian trunk NC cells secrete collagen1 and collagen2 after 4 weeks of culture, and show Alcian Blue and Alizarin Red staining (McGonnell and Graham, 2002). In addition, quail trunk NC cells transplanted in the first branchial arch of 2 day-chick embryos, after being cultured in vitro for one day, could engraft in the Meckel's cartilage and in the eye sclera. However, the quail cells found in the developing skeleton of the hosts were very rare and their expression of skeletal markers was not documented. Therefore, if some trunk NC cells can survive in these in vivo mesenchymal environments, it is still unknown whether they also can adopt a skeletogenic fate (McGonnell and Graham, 2002).

We found that, although most of the osteogenesis and chondrogenesis genes were expressed both in differentiation cocktail-treated and untreated cells from early culture stages, only the treated ones underwent bone matrix mineralization. These results suggest that the differentiating factors act by enabling already present mesenchymal progenitors to advance in the bone differentiation process. In addition, heterotopic transplantations of quail cephalic NC to the trunk region of the chick embryo resulted in ectopic cartilage formation, which indicates that the ventral trunk NC migratory environment is permissive to skeletal differentiation. Altogether, these data suggest that the trunk NC cells do not differentiate to mesenchymal cells in vivo because they have their mesenchymal potential already inhibited when they settle in permissive environments.

Previous studies have related *Hox* gene expression with the absence of skeletogenic differentiation by the trunk NC cells. It has been shown in chicken and

mice that *Hox* gene activity decreases during chondrogenesis of trunk NC cells in vitro, and even that *Hoxd10* maintenance prevents this process (Abzhanov et al., 2003; Ido and Ito, 2006). Down-regulation of *Hox* genes possibly occurs also in our culture system, allowing skeletal differentiation of the trunk NC cells. One of the growth factors that may be involved to promote trunk NC cell differentiation into chondrocytes and osteoblasts is Sonic Hedgehog (Shh). This morphogen acts in the endochondral ossification process by the cephalic NC cells in the developing jaw (Brito et al., 2008). In vitro, Shh favored the chondrogenic potentiality of cephalic and trunk NC cultured cells (Calloni et al., 2007, 2009). Besides enhancing the production of cartilage nodules found in mass culture, Shh also increased the number of multipotent progenitors with both mesenchymal and neural capacities and of *Runx2*-positive osteoblasts developing in the perichondrial regions. However, our preliminary experiments investigating the effect of the addition of Shh to trunk NC cultures in our conditions, did not show any significant changes in their differentiation into osteoblasts and adipocytes.

In a recent work, John and coworkers showed that it is possible to induce mesenchymal differentiation of mouse trunk NC cells in vitro following a 4hr-initial treatment with TGFβ1 (John et al., 2011). TGFβ1-treated trunk NC are then converted to mesenchymal progenitors, and they loose their neurogenic ability. In addition, TGFβ1 induced the loss of Sox10 expression and acquisition of Sox9 in all cells. Down-regulation of Sox10 is essential for the acquisition of the mesenchymal differentiation capacity (Blentic et al., 2008). In our study, the culture conditions enable the quail NC cells to differentiate into the various NC-derived phenotypes, including mesenchymal ones not found in the trunk in vivo. In addition, *Sox10*-expressing cells were found to be present until extended culture periods (three

weeks), concomitantly with bone cell mineralization. Thus, our culture system allows studying the events and factors involved in the differentiation of multipotent cells into a large variety of cell types.

4.2. Trunk NC differentiation into adipocytes in vitro

This thesis work revealed that trunk NC cells have a great adipogenic differentiation potential. Previous work had already shown that quail trunk NC cells were able to differentiate into adipocytes (Billon et al., 2007). However, this phenotype was obtained in only 40% of the cultures. In our present work, the experiments performed to establish the best culture conditions allowed us to observe that more than 90% of the cultures treated with the mesenchymal differentiation cocktail exhibited large clusters of adipocytes. These results were obtained in secondary cultures of trunk NC cells taken after 15h of primary culture, although the cells isolated after 48 h of primary culture also showed a high yield of adipocytes. Our mesenchymal differentiation cocktail containing adipogenic and osteogenic factors was more efficient that the two different media used in the previous work (Billon et al., 2007). A likely explanation for the greater efficiency of the mesenchymal differentiation cocktail is the constant presence of the glucocorticoid dexamethasone, whereas in previous work this hormone was added to NC cells only during the first 2days of cutures. Besides acting on osteogenic differentiation, dexamethasone has also an important role in adipogenesis, where it indirectly induces the essential adipogenic transcription factors, CEBPα and PPARγ (Pantoja et al., 2008).

The adipogenic differentiation was observed morphologically and using the Oil Red O histological marker. In addition, the expression of adipogenesis genes was monitored by semiquantitative RT-PCR in trunk NC cultures, treated and untreated

with the mesenchymal differentiation cocktail. These experiments showed that *PPARγ, CEBPα* and *FABP4* are already expressed from 11 days of culture, even in untreated cultures, with a tendency towards a higher expression level in cells stimulated by the differentiation medium. In addition, the transcription factor *Pbx1* was present at 3 days of culture and throughout the analyzed period. This factor was recently identified as necessary for the early steps of adipogenesis by neuralized mouse ES cells, and seems to inhibit adipocyte final differentiation (Monteiro et al., 2011). These data suggest that, independently of the provided differentiation medium, the trunk NC can generate in vitro a cell type engaged in the adipocyte differentiation pathway, which has never been shown previously. The mesenchymal differentiation cocktail plays a role only to trigger the final differentiation of trunk NC "pre-adipocytes" into lipid-storing adipocytes, as observed in pre-adipocytic and mesenchymal stem cell lines commonly used for the study of adipogenesis (McDougald and Rosen, 2006).

Another important characteristic of our system is that osteogenesis and adipogenesis occur concomitantly under the same culture conditions. These two differentiation pathways are known to be competitively regulated in the bone marrow and in mesenchymal stem cells in vitro, and conditions that favor one of these two phenotypes inhibit the other one (Chamberlaim et al., 2007). The factors that regulate the choice by NC cells between adipocyte and bone cell development is not known. Our culture system thus provides the necessary conditions for the study of the balance between osteogenesis and adipogenesis in differentiating NC cells.

4.3. Multipotent mesenchymal progenitors of the trunk NC

Our results highlighted the mesenchymal potentials of the trunk NC cells, which were able to differentiate into osteoblasts, chondrocytes and adipocytes in vitro. However, single cell experiments were needed to study the cellular origin of these differentiation potentials. The fact that the avian trunk NC does not give rise to mesenchymal cells in vivo led to the hypothesis that the cephalic NC cell population might comprise mesenchymal progenitors that are independent of the neural and/or melanocytic ones. However, Baroffio and collaborators first demonstrated by in vitro cellular cloning that some of the midbrain quail NC progenitors generated a diverse progeny of both neural cells and chondrocytes (Baroffio et al., 1991). Recently Calloni and colleagues further showed that the trunk NC also comprises progenitors with chondrogenic capacity, although they were very rare (about 1 in 200) when compared to the cephalic NC (Calloni et al., 2007). It appeared therefore that mesenchymal lineages are not completely segregated from the neural ones in the early avian NC.

After having shown that the quail trunk NC is able to undergo bone cell differentiation, we have performed in vitro cloning experiments to identify osteogenic progenitors, using a 3T3 feeder-layer as already described for the cephalic NC (Calloni et al., 2009). Clonal cultures were also performed although at lower efficiency condition, without a feeder-layer substrate, to analyze the presence of adipocytes in trunk NC cell progeny.

In cloning experiments on 3T3 cells, where an average of 57.5% of the cells generated clones, more than 76% of the trunk NC cells generated *Runx2*-positive osteoblasts and almost all of them generated also neural cells. Thus, osteoblasts differentiated from multipotent progenitors GNO (for HNK1-positive glial cells, TH-

positive neurons and *Runx2*-positive osteoblasts) and GO (for glial cells and osteoblasts). Approximately 91% the clones presented MelEM-positive melanocytes. Although technical limitations impaired to identify melanoblasts by immunocytochemistry in the clones already processed for *Runx2* mRNA detection, we could evidence in independent experimental series a high percentage of melanoblastic clones, thus suggesting that most of the osteoblastic clones likely also contain melanoblasts. In contrast, only 3.26% of the clones comprised myofibroblasts in this condition.

The cloning efficiency obtained when the trunk individual NC cells were cultured on collagen-coated culture plates was much lower than that found in cells cultured on 3T3 fibroblasts, and ranged between 5.2% and 28.1%. These clones received the mesenchymal differentiation cocktail from day 7 and were phenotypically analyzed after two to three weeks of culture. Adipocytes were found in 35.3% of the clones, which retrospectively identified three types of progenitors: FA (17.64%), GFA (5.88%) and GMFA (11.76%), according to the presence of glial cells (G), melanocytes (M), myofobroblasts (F) and adipocytes (A). All the clonogenic cells that gave rise to adipocytes, also generated myofibroblasts (the total number of clones with myofibroblasts was 94.1%). Furthermore, about half of the adipocytic clones also included melanocytes. Neuronal cells were not detected in these experiments, but glial cells were identified in 17.6% of the clones with adipocytes. Therefore, we could show for the first time that adipocytes can originate from multipotent trunk NC cells.

Altogether, our in vitro studies of the osteogenic and adipogenic properties of single trunk NC cells provide new evidence for the multipotency of the trunk NC cells at the beginning of their migration (**Figure 19)**: summary of all the progenitors). The results show that the potentials for neural, mesenchymal and melanogenic lineages

are not segregated into distinct progenitors, but are present in multipotent trunk NC cells. The heterogeneity of the identified multipotent progenitors suggests a hierarchical model in which a single multipotent cell, through progressive restrictions, generates daughter cells with intermediate potentials and eventually unipotent precursors.

These data also suggest that most of the trunk NC cells adopting neural and melanocytic phenotypes in vivo are likely derived from progenitors that also possessed dormant osteogenic and/or adipogenic potentials. Thus, our findings put forward the idea that environmental factors encountered by trunk NC cells in vivo specify and determine these cells to the neuronal, glial and melanocytic phenotypes at early stages, before they could reach favorable environments and respond to signals, which lead to mesenchymal differentiation.

4.4. Evolutionary aspects and perspectives

The results of this thesis work have several implications related to the role of the NC during evolution of Vertebrates and the ontogeny of the mesenchymes.

Little is known about the origin and commitment of adipogenic progenitors as specific markers of the early stages of adipogenesis are still lacking. The origin of head adipocytes from the cranial NC could be traced in avian (Le Lièvre and Le Douarin, 1975) and mouse species (Billon et al., 2007). It is assumed that, in the trunk of vertebrates, fat tissues arise from mesodermal progenitors, although few have their embryonic origin unveiled (Tang et al., 2008). One possibility is that some fat depots in the trunk could be derived from the NC; yet this hypothesis did not receive support from data of genetic fate mapping of NC cells in transgenic mice (Billon et al., 2007). The second, more likely, possibility is that adipogenic

progenitors, which we have shown to be present in the early trunk NC, have this potential inhibited in vivo, in the same way as the differentiation of osteogenic progenitors is locked in the trunk NC of developing Amniotes.

Our results suggest that the NC cells along the whole anterior-posterior axis, i.e., including trunk NC cells, gained the ability to make bone, and later during evolution, this potential has not been lost, although it is not accomplished in the trunk of Amniotes. Early in evolution, the skeletogenic NC is thought to have produced the mineralized dermal armor covering the head and trunk of extinct fishes Ostracoderms; later this trunk exoskeleton has been lost and replaced by an internal skeleton of mesoderm origin, and only the cephalic NC contributes to the skeleton in higher vertebrates. Importantly, our data demonstrate that, even if amniote vertebrates do not present NC-derived mesenchymal derivatives throughout the trunk in vivo, their trunk NC progenitors are still able to differentiate into osteoblasts, chondrocytes and adipocytes when challenged with appropriate conditions in vitro.

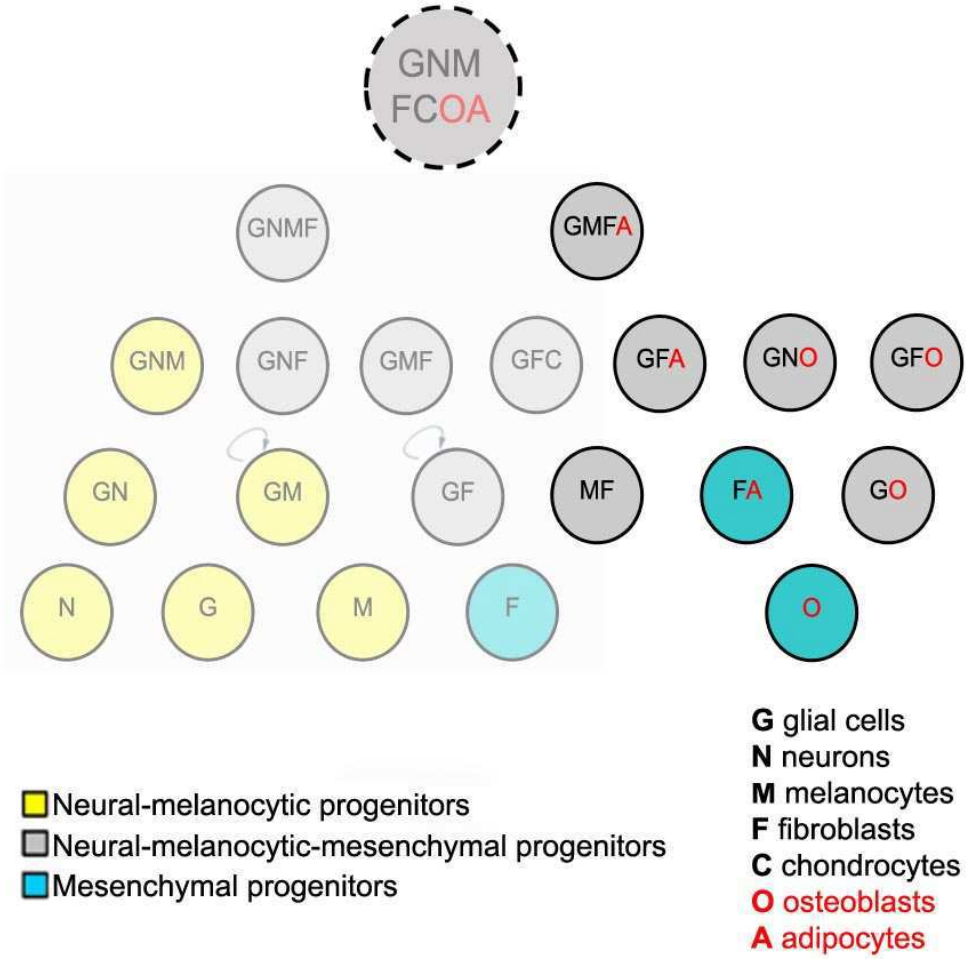

Figure 19 – Summary of the distinct types of progenitors identified by the in vitro clonal analyses of trunk quail NC cells. Neural-melanocytic progenitors are shown in yellow, those yielding mesenchymal cells are in blue, and progenitors for both potential are signalized in grey. Progenitors with lighter colors were deduced from previous analysis by Trentin et al. (2004), with exception for a rare progenitor (GFC) for the glial (G), myofibroblastic (F) and chondrocytic (C) lineages, that had been recently found in single trunk NC cells grown on 3T3 fibroblasts and exposed to Shh treatment (Calloni et al., 2007). The bipotent GM and GF cells showed self-renewing activity (Trentin et al., 2004). In this work, a number of additional mesenchymal trunk NC progenitors have been evidenced (highlighted progenitors) (Coelho-Aguiar et al, in preparation; see also Results section). They comprise common progenitors for osteoblasts and neural cells (GNO and GO), which differentiated at high frequencies from trunk NC cells clonally seeded on 3T3 feeder-layers, and multipotent progenitors for adipocytes, fibroblasts and/or glial cells and melanocytic cells (GMFA, GFA, FA), the existence of which could be assessed in clonal cultures performed on collagen. All these trunk NC progenitores would be derived from a hypothetical highly multipotent progenitor GNMFCOA.

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Annexe1

Submission to CYTOMETRY A

« Isolation and differentiation properties of neural crest stem cells »

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Isolation and differentiation properties of neural crest stem cells

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Abbreviations: NC, neural crest; NCC, neural crest cell; NCSC, neural crest stem cell; PNS, peripheral nervous system; DRG, dorsal root ganglia; SKP, skin-derived precursor.

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Abstract

A wide array of neural and non-neural cell types arises from the neural crest during vertebrate embryogenesis. The neural crest forms transiently in the dorsal neural primordium to yield migratory cells that will invade nearly all tissues and later, differentiate into bones and cartilages, neurons and glia of the peripheral nervous system, endocrine cells, vascular smooth muscle cells and melanocytes. Due to the amazingly diversified array of cell types they generate, the neural crest cells represent an attractive model in the stem cell field. We review here in vivo and in vitro studies of individual cells, which led to discovery and characterization of neural crest progenitors endowed with multipotency and stem cell properties. We also present an overview of the diverse types, marker expression and locations of the neural crest-derived stem cells identified in the vertebrate body, with emphasis on those discovered recently in mammalian adult tissues.

Introduction

The existence of stem cells in the nervous system has become a reality only in the recent years. An extensive body of work is currently devoted to the understand the role of neural progenitors in constructing the embryonic brain and spinal cord as well as to characterize the neural stem cells (NSCs) that are present in discrete regions of the adult brain (for references, 1). Besides those stem cells in the CNS, other types of neural progenitors/stem cells have been discovered in the peripheral nervous system (PNS), which originate from the neural crest (NC) during embryogenesis. The progenitors and stem cells derived from the NC are, in all Vertebrates, the source of a wide variety of cell types beyond the neural cells of the PNS. Multipotent NCCs have been identified at different stages of embryogenesis, and some persist in many adult organs and tissues, in animals and in humans. We review here the current knowledge concerning the isolation, specific markers and differentiation properties of the distinct NC stem cells (NCSCs) and discuss their potential role in development and repair.

1) The NCCs, definition and general properties

The NC is an embryonic structure characteristic of the Vertebrates, which is located at the margins of the neural primordium, forming an area intermediate between the presumptive CNS and epidermis (2). The induction of the early NC territory depends largely upon the coordinated action of a set of signaling molecules and transcription factors during gastrulation and neurulation stages. Current knowledge of the regulatory gene network involved in formation and specification of the NC is reviewed elsewhere (3).

The NC *per se* is only transient, since its component cells rapidly delaminate from the neural epithelium and migrate extensively to colonize many embryonic tissues, where they eventually undergo differentiation. The emergence of migratory NCCs from the dorsal

neuroepithelium involves an epithelial-to-mesenchymal transition (4,5 for reviews). Recent cell imaging techniques led to addressing the migration behavior and motile properties of the NCCs in vivo and began to uncover the genetic determinants of the EMT in NCCs, which represents a model system for a key step in dissemination of tumor cells towards metastasis (reviewed in 6).

A plethora of neural and non-neural cell types originate from the NCCs (Table 1). The NC gives rise to the PNS neurons and their associated glial cells (in sympathetic, parasympathetic, enteric and, partly, sensory ganglia) as well as to the Schwann cells lining the peripheral nerves (Figure 1). The NCCs are at the origin of the pigment cells in the skin (melanocytes) and of some neuro-endocrine cells (in the carotid body, adrenal medulla and thyroid gland). In addition, the NCCs that populate the head and neck structures are the source of diverse mesenchymal cell types, which in the trunk instead derive from the mesodermal germ layer. The cranial NCCs thus contribute to craniofacial bones, cartilages and adipose tissue, and they yield brain pericytes and vascular smooth muscle cells of the aorticopulmonary cardiac septum (5) (Table 1). The NC origin of cranial mesenchymal tissues was first established in quail-chick chimeras (2) and, later, confirmed in mammals by genetic fate mapping in the mouse using the *Cre-Lox* system associated with early NC-specific gene promoters (e.g., *Wnt1, P0* and *Sox10*) (for reviews, 7,8).

Thus, the NC is a highly pluripotent structure which, in this respect, can be compared to the hematopoïetic system generating the large diversity of blood cells. How do the immature NCCs become phenotypically diversified? Do all the NC-derived cell types arise from one single type of multipotent stem cell or is the premigratory NC a heterogenous collection of distinct committed cells? These issues have attracted developmental biologists for long and begun to be answered since methods were devised to trace the fate of single cells.

2) Discovery of multipotent progenitors in the early NC

In vivo lineage tracing studies were performed to monitor the fate of early NCCs in the avian embryo, from the beginning of their migration from the neural tube, using microinjection of a vital fluorescent dye at premigratory stage (9,10). These studies showed that the clonal descendants of trunk NCCs populate different types of NC derivatives, producing after 2 days several combinations of neurons (in DRG and/or sympathetic ganglia), nerve glial cells, melanocytes and adrenomedulary cells. Similar dye labeling experiments in other species also revealed the presence of multipotent progenitors in the early NC in vivo. However, recent data rather supported early determination of premigratory avian NCCs (11). Evaluating the extent and temporal changes of the potentials of early NCCs in vivo, will therefore need further investigations and new methods to permanently trace the fate of NCCs.

Following colony-forming unit assays developed to identify progenitors at the origin of hemopoietic cell diversity, a number of in vitro cloning experiments have permitted to analyze the repertoire of phenotypes that a single NCC is able to produce. These experiments, which requires previous isolation of the NCCs as a pure cell population, were initially devised by Cohen and Konigsberg (1975), who found that quail NCCs migrate from neural tube explants cultured in vitro; after removal of the intact neural epithelium explant, the NCCs can be harvested for subsequent replating and clonal assays (Figure 2). This method was a mean to first demonstrate that trunk NCCs are heterogeneous in their potential and can give rise to unpigmented, pigmented or mixed progeny (12,13).

Since this pioneer work, improvements in culture techniques and differentiation markers allowed more extensive characterization of NC progenitors, isolated at different stages and from different rostro-caudal levels of the avian NC (for references, 5,14). For example, it was shown that unpigmented and mixed (with unpigmented and pigmented cells) clones contained several hundred of sensory and adrenergic neuroblasts (15).

Altogether, data supported heterogeneity of the NCCs regarding their proliferation and differentiation potentials. Most of them generated different combinations of two or more cell types (16-19). The analysis of colonies resulted in a hierarchical model for cephalic and trunk NC lineage diversification (5,20). The cephalic NC model includes recent results showing the existence of multipotent precursors capable to yield cell types belonging to all the major NC lineages, i.e., neural, melanocytic and mesenchymal (21,22). Even if they do not contribute to the formation of mesenchymal tissues in vivo, avian trunk NCCs are able to differentiate into chondrocytes in vitro (21).

Using technical approaches similar to that devised for the avian NC, clonal assays were performed with the NCCs of rats and mice (23,24). Stemple and Anderson (1992) were the first to identify mammalian multipotent NCCs generating glia, autonomic neurons and myofibroblasts, and able to self-renew. These NCSCs were isolated by FACS through the high expression of the neurotrophin receptor p75 (23). In the avian NC, bipotent progenitors that differentiate into glia and melanocytes or into glia and myofibroblasts were also identified as self-renewing stem cells (19).

Efforts have been made to characterize the differentiation and stem properties of early NCSCs (for references, 8). While Wnt and BMP signalings promote neurogenesis along sensory and autonomic phenotypes, respectively, together these two pathways play a key role in the self-renewal and the maintenance of multipotency in mammalian NCSCs (25). The transcription factor Sox10 expressed by premigratory and migratory NCCs (Figure 1), functions in stem/progenitor cell maintenance and inhibits neuronal differentiation in the early NCCs both in vitro and in vivo (26). The differentiation of multiple NC derivatives also depends upon the activity of *FoxD3* regulatory gene. After *FoxD3* gene ablation in the NC, mice die perinatally with catastrophic loss of NC-derived craniofacial structures, nerves, DRG and enteric NCCs (27).

3) NCSCs at postmigratory stages and in adult tissues

After leaving the neural primordium, the early-migratory NCCs are modified during migration in response to the distinct environmental cues they received during their homing to their final sites of differentiation. Although some become rapidly specified, the migratory NCCs en route to, or arriving at, their embryonic target tissues still comprise a subpopulation of progenitors that retain multiple differentiation options. Because migrating NCCs are mixed with cells of the surrounding tissues, their identification was greatly improved through the use of NC-specific markers combined with flow cytometry. Multifated progenitors thus could be evidenced in the NCCs colonizing the branchial arches during early jaw development (28,29) and in those transiently located in the mesentery, which express the tyrosine-kinase receptor Ret while homing to the gut (30).

Anderson and collaborators were the first to demonstrate the persistence of NCSCs in NC derivatives, isolating subpopulations of multipotent NCSCs in the peripheral nerve, DRG and gut of rodent embryos (Table 2). FACS experiments led to identify NCSCs in the embryonic rat sciatic nerve, which were positive for p75 and negative for myelin protein P0 (31). These nerve NCSCs exhibited trilineage potential in vitro, similar to early NCSCs. However, they displayed distinct responsiveness to BMP2 and gave rise to few neuronal types, when carefully compared to the NCSCs of the rat early NC (32). Likewise, enriched NCSC populations could be sorted from the rat gut at fetal or postnatal stages, by using p75/α4-integrin or p75 alone, respectively (33,34).

Amazingly, even adult NC-derived organs and other adult tissues appeared to contain some stem cells of NC origin, in addition to their expected differentiated cell types. As shown in Table 2, NCSCs were found in diverse tissue locations in several mammalian species. The NC-origin of these adult stem cells could be proven in transgenic mice using the *Cre/Lox*

system and NC-specific promoters driving expression of reporter genes like GFP, resulting in permanent labeling of the progeny of early NCCs. NC-derived mutipotent progenitors thus persist in adult rodent DRG and bone marrow (35,36), gut (34), carotid body (37), heart (38,39) and several craniofacial tissues including the cornea (40,41), iris (42), dental pulp (43), hard palate (44) and oral mucosa (45,46) (Table 2). After isolation and in vitro culture, these NC-derived progenitors differentiate into classical NC derivatives like neurons, glial cells and myofibroblasts, and in some cases, also into mesenchymal cell types.

The function of these NCSC-like cells in vivo remains elusive, except for rodent NCSCs of the carotid body, which offer a unique example clearly demonstrating a physiological role of adult NC progenitors in vivo (37). During adaptation to hypoxia, these cells undergo proliferation and eventually lead to de novo differentiation of dopaminergic chemosensory neurons that activate the brainstem respiratory center to increase ventilation. The carotid body is therefore a niche for adult neurogenic stem cells in the PNS. These stem cells could be isolated by flow cytometry through the expression of GFAP and the absence of p75 and HNK1 (37) (Table 2).

The dental pulp is formed by the cranial NC; it contains NC-derived precursors that differentiate into odontoblasts and are responsible for dentinal repair in adults. Shi and collaborators have isolated highly proliferative clonogenic stem cells in humans from the dental pulp (of adult molar and exfoliated deciduous teeth) and from the periodontal ligament (47-49) (Table 2). These progenitors can be expanded ex vivo and reconstitute dentin and bone in transplantation models, thus offering promise for mesenchymal tissue engineering and tooth regeneration (50).

The adult skin is another source where NC-derived stem cells can be isolated with minimally invasive procedures. The multipotent NCC populations recently identified in the murine skin

are located in several epidermal (51,52) and facial dermal structures (53,54) (Table 2). These multipotent NCCs differ from the melanocyte stem cell identified in the permanent lower portion (bulge) of the hair follicle, which replenishes melanocytes during the hair cycle (55) and from the epidermal stem cells that ensure continuous renewal of skin keratinocytes (for a review, 56). In the back skin, trunk NC-derived multipotent cells are present in the hair bulge and share early markers of glial and melanocytic cell lineages (52). These NCSCs isolated in rodent and human skin display neural and mesenchymal differentiation properties and can be long-term maintained in vitro.

Expanded skin-derived NC progenitors were efficient to repair nerve tissue when tested in murine models of spinal cord and nerve injury (56-60). The skeletogenic potential of human skin-derived NC progenitors has also been exploited to repair bone fracture after transplantation in mice (61). The NCSCs of the adult skin are accessible and share some characteristics with pluripotent stem cells without being tumorigenic. They constitute a potentially attractive source of adult somatic stem cells for autologous transplantations and future cell replacement therapy in a variety of human tissues.

4) Towards the characterization of NCCs in human

The NC is of particular clinical significance, as its aberrant development results in a large group of human pathologies collectively known as neurocristopathies (see 62 for references). Theses NC-related pathologies encompass congenital heart defects (mainly in the outflow tract), Hirschsprung disease (with abnormal innervation of the posterior gut) and Waardenburg syndromes, labio-palatine clefting and other craniofacial malformations, and several cancers such as melanoma and neuroblastoma. It is thus of crucial importance to

develop experimental approaches suitable for the dissection of the cellular and genetic features of the human NCCs (hNCCs).

Compared to extensive investigations on NCCs in many animal models, almost nothing is known about the endogenous characteristics of human hNCCs. Histological analysis and spatio-temporal expression patterns of early NC markers (63) indicated that the premigratory/migratory hNCCs develop toward the end of the first month of pregnancy. Recently, hNCC lines have been derived from the NCCs that emigrated in vitro from human embryonic neural tube cultures (64). Transcriptome profiling revealed that hNCCs exhibitit a NC-specific gene signature while sharing expression of many stem cell genes with human embryonic stem cells (hESCs). These hNCCs could be maintained and expanded in vitro for months, thus offering an extended source for further characterization of hNCCs; however, the in vitro generation of NC derivatives by these cell lines was so far unsuccessful.

To bypass the difficulties linked to working on the human embryo, new in vitro strategies have been developed in the recent years to derive NCCs from human pluripotent cells. Populations of hNCCs have been derived from neuralized hESCs according to various induction protocols that produce neural cells of both CNS and NC types in variable amounts (Table 3). Most of these protocols involved the use of stromal feeder-layers (65,66), and, more recently, the technique of neural rosettes, developed by Studer and collaborators (67). These epithelial structures can mimic the embryonic neuroepithelium in vitro and yield both NCCs and CNS progenitors. These procedures, which are quite long and of low efficiency, were combined with flow cytometry to get enrichment for hNCCs using CD57 and CD271 (67,68). Expression of Cadherin-11 and Frizzled-3 was also exploited by Zhou et al. (2008) to sort cranial NC-like progenitors from preparations of human embryoid bodies (69).

Recently, defined culture conditions could be devised for deriving NCCs from hESCs under controlled procedures. One culture system is based on the action of two inhibitors of Smad,

which promote NC fate in human neural rosettes by counteracting BMP and TGF/activin signalings (70). Another approach uses the generation of neurospheres in defined medium supplemented with neural inducers FGF2 and EGF; in this assay, NCCs are produced by migration from adherent neurospheres in less than 10 days (71).

The availability of unlimited numbers of hNCCs in fully defined conditions, presents a valuable tool for investigating features of human NC development and for modeling NC-related diseases. Thus, the role of the chromatin remodeler CHD7 in the generation of premigratory hNCCs and CHARGE syndrome was recently evidenced thanks to neurosphere-mediated derivation of hNCCs fom hESCs (72). Using similar approaches, Cimadamore et al. (73) explored the role of the transcription factor Sox2 in hNCC migration and sensory neurogenesis.

Strategies to model and treat NC-related disorders are likely to emerge also from experiments using human induced pluripotent stem cells (hiPSCs) for the production of early NCCs and their various differentiated derivatives (74). This was recently illustrated by the generation of iPSCs from Familial Dysautonomia (FD) patients, who carry *IKBKAP* gene mutation and show PNS neuron degeneration (75). The NCCs differentiated from FD-iPSCs, exhibited *IKBKAP* gene splicing and in vitro neuronal differentiation defects, hence could be used for screening drugs for therapeutic design.

Concluding remarks

A remarkable diversity of vertebrate cell types arises from the NC in the developing embryo. The NCCs are very invasive cells, not only giving rise to multiple NC derivatives but also colonizing virtually all tissues and organs when they differentiate into Schwann cells accompanying the peripheral nerves or into melanocytes homing to the entire surface of the body. Several lines of evidence demonstrated the multipotency and self-renewal capacity of

various subsets of NC progenitors in avian and mammalian species. These conclusions were mostly based upon the analysis of the developmental repertoire that individual NCCs displayed in vitro. Due to technical limitations, a small number of in vivo lineage tracing experiments have so far attempted to understand lineage determination in the NC. New methods to permanently marking NCC progeny in living organisms should help to determine more precisely what roles the NCSCs play in development and tissue homeostasis.

A significant advance towards deciphering the lineage mechanisms in the early NC was to evidence that the neural-melanocytic and mesenchymal NC derivatives can originate from common multipotent progenitors in the cephalic NC. Although they still require to be supported by in vivo experiments, these findings argue that individual NCSCs are endowed of an amazingly wide repertoire of differentiation options, which is unique among the other somatic stem cells found in amniote vertebrates. It was thus proposed that the NC might be the fourth germ layer, producing cell types belonging to derivatives of both mesoderm and ectoderm (76).

Nowadays other NC derivatives emerged, including the adult NCSCs discovered in many tissues, which strengthens the importance of this cell population in vertebrate physiology. The compiling finding of NCSCs that share multipotency with their embryonic counterparts and are located in easily accessible adult body sites like the skin and the teeth, has recently attracted a growing interest in the potential of these cells for replacement therapy. Much remains however to be known with respect to the molecular regulation of stem cell properties and differentiation, of the various putative progenitors present in many of NC target tissues. It is very likely that developmental and therapeutic potentials of adult NCSCs are presumably influenced by intrinsic characteristics imposed by origin and spatiotemporal cues. These issues are of clinical importance to understand the alterations in NC cell number and function that occur in human neurocristopathies.

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Figure legends

Figure 1: Markers of migratory NCCs.

(A) Expression of HNK1 (whole-mount immunocytochemistry) in a 3 day-quail embryo. (B) Expression of *Sox10* transcripts (whole-mount in situ hybridization) in a 3.5-day-quail embryo. Both markers are expressed in NCCs migrating to PNS nerves and ganglia. (DRG, dorsal root ganglia; SG, sympathetic ganglia; TG, trigeminal ganglia).

Figure 2: Method of avian NCC isolation and clonal culture.

(A) 2-day-old quail embryo showing the delimited trunk region used for enzymatic isolation of the neural tube including the premigratory NC. (B) After 15h of culture, the neural tube gives rise to an outgrowth of NCCs migrating onto the dish. (C) Neural tube explant is then removed, leaving behind the adherent NCCs. (D) Following detachment of the NCCs, single cell seeding is carried out by micromanipulation. (NT, neural tube; NCCs, neural crest cells).

Table 1. Derivatives of the NC in avian and mammalian species

	NEURAL CREST-DERIVED CELL TYPES			
	Neurons and Glial cells	**Pigment cells**	**Endocrine cells**	**Mesenchymal cells**
CRANIAL	PNS Neurons Sensory cranial ganglia, Parasympathetic (ciliary) and Enteric ganglia Satellite glial cells in PNS ganglia Schwann cells Ensheating Olfactory Cells	Skin melanocytes Innear ear pigment cells	Carotid body cells C cells (thyroid)	Craniofacial Osteocytes and Chondrocytes Smooth muscle cells (vascular and heart conotruncus) Pericytes (brain) Odontoblasts, Adipocytes Dermal cells Corneal cells (endothelium and stroma)
TRUNK	PNS Neurons Sensory ganglia (DRG), Sympathetic and Parasympathetic ganglia Satellite glial cells in PNS ganglia Schwann cells	Skin melanocytes	Adrenal-medullary cells	Endoneurial fibroblasts (mouse sciatic nerve)

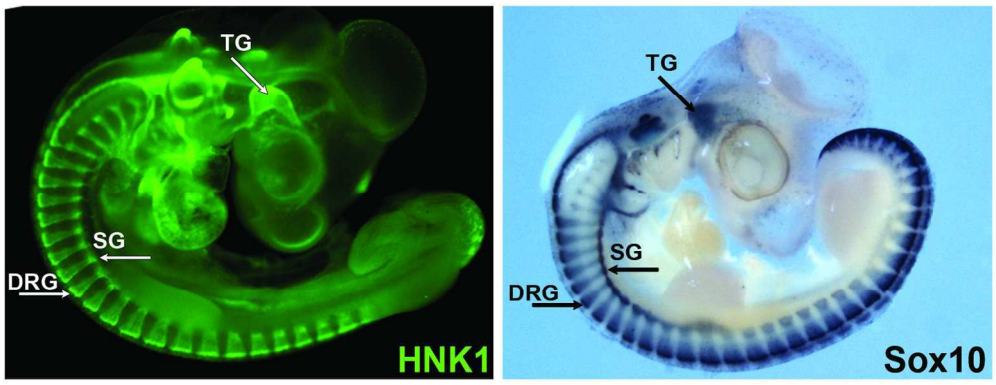
Table 2. Postmigratory NC-derived multipotent cells identified in fetal and adult tissues

Tissue	Species	Location	Cell designation	Markers	References
Sciatic Nerve	Rat fetal	n.d.	NCSCs	($p75^+P_o^-$) α4	(31)
Gut	Rat fetal, adult	n.d.	NCSCs	($p75^+α4^+$) ($p75^+$)	(33) (34)
DRG	Rat fetal Mouse adult	n.d.	NCSCs	p75, P0, PMP22, Sox10, Nestin, p75 Mushashi	(77) (34) (35,36)
Bone Marrow	Mouse adult	n.d.	BM-NCSCs	p75, Sox10, Slug, Snail	(36)
Carotid Body	Mouse adult	n.d.	NCSCs	($HNK1^-$ $p75^-$) ($GFAP^+$)	(37)
Cornea	Mouse adult, juvenile	Corneal limbus	COPs	Sca1, CD34	(40,41)
Iris	Mouse adult	Iris stroma	NCSCs	p75, Sox10	(42)
Heart	Mouse adult Rat adult		NCSCs	Nestin, Musashi	(38,39)
Palate	Rat adult	Palatal ridges	pNCSCs	p75, Nestin	(44)
Oral mucosa	Human	Lamina propria	OMLP-SCs OMSCs	CD44, CD90, CD105, CD106 CD29, CD73, CD166	(44) (45)
Skin	Mouse adult	Face and Trunk Facial Dermis Whisker follicle	NCSCs SKPs Epi-NCSCs	p75, Sox10 Nestin Nestin, Sox10	(52)(36) (53,54) (51)
	Human neonatal, adult	HF dermal papillae HF bulge (scalp) HF bulge	SKPs HFSCs Epi-NCSCs	p75, Sox10 Nestin, Sox9 Nestin, Sox10	(78) (79) (80)
Teeth	Mouse adult Human	Dental pulp Dental pulp Periodontal ligament	DPSCs DPSCs, SHED PDLSCs	CD44 Nestin, p75 Nestin, SSEA4 p75, HNK1	(43) (47,48) (49) (81)

Markers indicated in brackets were used in FACS experiments. For details, see the main text.

Table 3. Cell surface markers of human NCCs derived from pluripotent stem cells

Stem cell source	Derivation protocol	Surface Markers	References
hESCs	Stromal feeder-layers		(65,66)
		p75/CD271	(68)
	Neural rosettes	p75/CD271, HNK1 /CD57	(67)
		α4/CD49d	(82)
	Dual Smad inhibitors		(70)
	Neurospheres	CD73,	(68)
		HNK1 /CD57	(71)
	Embryoid bodies	Cadherin-11 Frizzled-3	(69)
hiPSCs	Fibroblast reprogramming	p75/CD271, HNK1 /CD57	(70,74,75)



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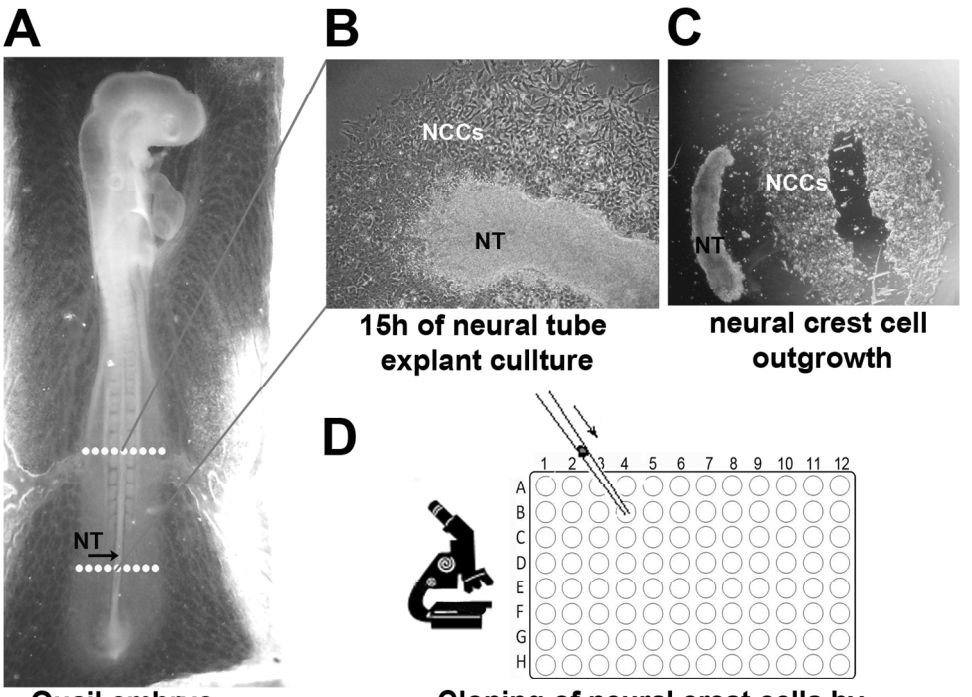

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