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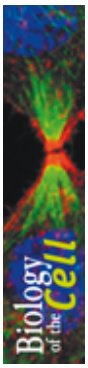
Review



Developmental origin of adipocytes: new insights into a pending question

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The current epidemic of obesity has caused a surge of interest in the study of adipose tissue formation. Much progress has been made in defining the transcriptional networks controlling the terminal differentiation of preadipocytes into mature adipocytes. However, the mechanisms that direct MSCs (mesenchymal stem cells) down the adipocyte lineage remain largely unknown. Similarly, although adipocytes are generally described to derive from mesoderm, the study of the developmental origin of MSCs and adipose tissues has been largely disregarded until now. Functional variations do exist between different adipose tissues, which suggest possible differences in their developmental origin and might explain why some depots are more associated than other to metabolic disorders. This review summarizes the surprising findings that have recently emerged from both embryonic stem cells and lineage-tracing studies *in vivo*, unravelling an unsuspected developmental origin for MSCs and adipocytes in the neural crest.

Introduction

The worldwide epidemic of obesity over the past fifty years has caused a surge of interest in the study of adipose tissue biology. Obesity is the result of an imbalance between energy intake and expenditure, and is often characterized by an increase in both adipocyte size and numbers. Two functionally different types of adipose tissues are classically described in mammals, which differ in several important properties: BAT (brown adipose tissue) and WAT (white adipose tissue). BAT and WAT are both involved in energy balance, but assume opposite functions. BAT is specialized in energy dissipation as heat during cold- and diet-induced thermogenesis, whereas WAT is mainly involved in energy storage and mobilization in the form of triacylglycerols. In addition, white adipocytes have intracrine, autocrine/paracrine and endocrine properties, such as the secretion of leptin and adiponectin (Ailhaud and Hauner, 2003). WAT is found in several depots throughout the body, and location varies between species. For mammals and birds, the largest fat tissues are intra-abdominal and subcutaneous, but WAT can also be found in other areas, such as the face and extremities, and within bone marrow. Different depots are not metabolically equivalent (reviewed in Rosen and MacDougald, 2006; Gesta et al., 2007). For example, adipose tissue in the breasts and thighs respond to sex hormones, whereas depots of the neck and upper back are more sensitive to glucocorticoids. Similarly, different adipose tissues show significantly different patterns of gene expression (Vidal, 2001; Vohl et al., 2004; Gesta et al., 2006; Tchkonia et al., 2007). These distinct molecular and physiological properties might partly explain why variations in WAT distribution are associated with metabolic disorders (Bjorntorp, 1990; Lafontan and Berlan, 2003). For instance, they might account for the higher risk of cardiovascular diseases observed in patients with an increased ratio of visceral to subcutaneous WAT. The distinct properties of different depots also suggest possible differences in their developmental origin. In accordance with this

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Key words: adipocyte, development, embryonic stem cell (ESC), mesenchymal stem cell (MSC), neural crest.

Abbreviations used: BAT, brown adipose tissue; *cre*, cyclization recombinase gene; E9.5 etc., embryonic day 9.5 etc.; EB, embryoid body; (h/m)ESC, (human/mouse) embryonic stem cell; β*geo*, β-galactosidase neomycin phosphotransferase; *gfp*, green fluorescent protein; HNK1, human natural killer 1 protein; LIF, leukaemia inhibitory factor; (h/m)MSC, (human/mouse) mesenchymal stem cell; NC, neural crest; NCC, NC cell; CNCC, cranial NCC; NCSC, NC stem cell; P28 , post-natal day 28; PDGFRα, platelet-derived growth factor receptor α; PSP, PDGFRα single-positive; RA, retinoic acid; SDIA, stromal cell-derived inducing activity; *tk*, herpes simplex thymidine kinase; TNC, trunk NC; TNCC, TNC cell; VEGFR-2, vascular endothelial growth factor receptor 2; VSP, VEGFR-2 single-positive, WAT, white adipose tissue; *yfp*, yellow fluorescent protein.

hypothesis, WAT depots exhibit a different timing of appearance during development (Gesta et al., 2007). In rodents, WAT develops mainly after birth, first in the peri-gonadal and subcutaneous depots, and only later in the omental depot. In humans, WAT formation begins during the second trimester of gestation and by birth both the visceral and subcutaneous depots are apparent.

Strikingly, the study of the developmental origin of fat tissues has received very little attention until now. Adipocytes, similar to muscle and bone cells, are generally described to be derived from MSCs (mesenchymal stem cells), which themselves are thought to arise from mesoderm. However, precise lineage-tracing studies to define MSCs and adipocytes origin have not been performed. It is worth noting that during development of higher vertebrates, the mesoderm is not the only germ-layer source of mesenchymal cells. In the head, for example, the facial bones, jaws and associated connective tissues have been shown to derive from the NC (neural crest). The NC is a vertebrate cell population that arises from the neuroectoderm. After neural tube closure, NCCs (NC cells) undergo an epithelial-to-mesenchyme transition and migrate to diverse regions in the developing embryo, where they differentiate into various cell types. NC derivatives include pigment cells, neurons and glial cells of the PNS (peripheral nervous system), as well as some endocrine cells. In the head and neck, the NC also yields mesenchymal precursors which differentiate into connective tissue cells, vascular smooth muscle cells, tendons, dermis, odontoblasts, cartilages and bones (reviewed in Le Douarin and Kalcheim, 1999; Le Douarin et al., 2004; Dupin et al., 2006).

Adipogenesis is generally described as a two-step process. The first step comprises the generation of committed adipocyte precursors (or preadipocytes) from MSCs. The second step involves the terminal differentiation of these preadipocytes into mature functional adipocytes. By definition, MSCs are endowed with self-renewal properties and a differentiation potential towards all mesenchymal cell types, whereas preadipocytes have lost the ability to differentiate into mesenchymal derivatives other than adipocytes. The differentiation of preadipocytes into adipocytes has been extensively studied *in vitro* (Farmer, 2006; Rosen and MacDougald, 2006) and will not be reviewed in this review. This was made possible by establishment of immortal preadipocyte cell lines that were selected from disaggregated mouse embryos or from adult adipose tissue for their ability to accumulate cytoplasmic triacylglycerols (Green and Kehinde, 1975, 1976; Negrel et al., 1978). These cell lines are believed to be faithful models of preadipocyte differentiation and they have provided important insights into the control of the late steps of adipogenesis. In contrast, the early steps leading to the generation and the commitment of MSCs to the adipocyte lineage remain largely unknown. Although there have been attempts to characterize the distinct cellular intermediates between MSCs and mature adipocytes, such studies have been hampered by the lack of specific cell-surface markers to identify and prospectively isolate these cells *in vivo*. Some knowledge about mesenchymal cell-fate decisions has been derived from studies on the immortalized mouse stromal cell line, C3H10T1/2, or mesenchymal precursor populations isolated from adult tissues. However, these cellular systems are not informative of the developmental origin of MSCs and adipocytes. Instead, the embryo might constitute a more suitable source of cells to address this issue. In particular, ESCs (embryonic stem cells) have provided an invaluable tool to model the earliest steps of mammalian development *in vitro*.

In this review, we provide a detailed overview of the advances that have been made in the generation and purification of MSCs and adipocytes from ESCs, and the surprising conclusions that they have pointed out regarding the ontogeny of such cells in the neural crest. We then assemble recent data from lineage-tracing studies that confirm these unexpected findings *in vivo*. We also outline new cellular systems that could help to unravel the molecular aspects of adipocyte generation from the neural crest. Finally, we discuss some of the exciting questions raised by these recent findings.

Dissection of adipocyte development in ESCs

mESC (mouse ESCs) are proliferating, pluripotent stem cells which have been isolated from the epiblast of blastocyst-stage mouse embryos (Evans and Kaufman, 1981; Martin, 1981; Brook and Gardner, 1997). They can be propagated indefinitely at the undifferentiated state *in vitro* in the presence of LIF

(leukaemia inhibitory factor) (Williams et al., 1988; Smith, 2001). Furthermore, when transplanted into a mouse blastocyst, mESCs integrate into the embryo and contribute to all cell lineages, including germ cells (Bradley et al., 1984). When mESCs are cultured without LIF on a non-adherent surface, they aggregate to form EBs (embryoid bodies), in which the cells form ectodermal, mesodermal and endodermal derivatives (Keller, 1995). The successful isolation of hESCs (human ESCs) has raised the hope to use these cells as a universal tissue source to treat human diseases (Lerou and Daley, 2005). In addition, ESCs are easily genetically modifiable and can be produced in large numbers, thus offering a unique cell culture model to study the earliest steps of mammalian development (Keller, 1995).

Generation of specific mesenchymal derivatives from ESCs

Directed differentiation of mESC towards the adipocyte lineage was first accomplished by Dani et al. (1997), who showed that functional adipocytes could be obtained when mESCs were exposed to appropriate extracellular cues. In this system, the generation of adipocytes is dependent on an early and transient exposure of differentiating mESCs to RA (retinoic acid) and a subsequent treatment with conventional adipogenic factors (e.g. insulin, tri-iodothyronine and rosiglitazone). Both lipogenic and lipolytic activities, as well as high levels of expression of adipocyte-specific genes, could be detected in mESC-derived adipocytes. Remarkably, the sequence of expression of key transcription factors that are known to govern preadipocyte differentiation, such as members of the C/EBP (CCAAT/enhancer-binding protein) and the PPAR (peroxisome-proliferator-activated receptor) families (Tontonoz et al., 1995; Mandrup and Lane, 1997), was closely conserved during mESC adipogenesis. Thus this model has provided a powerful system to address the different steps of adipocyte development (Dani et al., 1997). More recently, adipocytes have been obtained from hESCs using a protocol based on mESC studies (Xiong et al., 2005).

In addition to adipocytes, the derivation of other mesenchymal cell types from both mESCs and hESCs has been reported in several studies. These derivatives include osteoblasts (Dani et al., 1997; Buttery et al., 2001; Phillips et al., 2003; Sottile et al., 2003; Bielby et al., 2004; Kawaguchi et al., 2005; Duplomb et al., 2007a, 2007b), chondrocytes (Kramer et al., 2000; Hegert et al., 2002; Nakayama et al., 2003; Phillips et al., 2003; Kawaguchi et al., 2005) and skeletal myocytes (Barberi et al., 2005, 2007). Interestingly, in some reports, early treatment with RA turned out to be critical for quantitative induction of mesenchymal derivatives from mESCs, namely osteoblasts and chondrocytes (Dani et al., 1997; Phillips et al., 2003; Kawaguchi et al., 2005). Together with adipocytes, these lineages were generated in a mutually exclusive fashion by exposure of RA-treated mESCs to specific growth factors (Kawaguchi et al., 2005).

First attempts to generate purified populations of mesenchymal precursors from ESCs

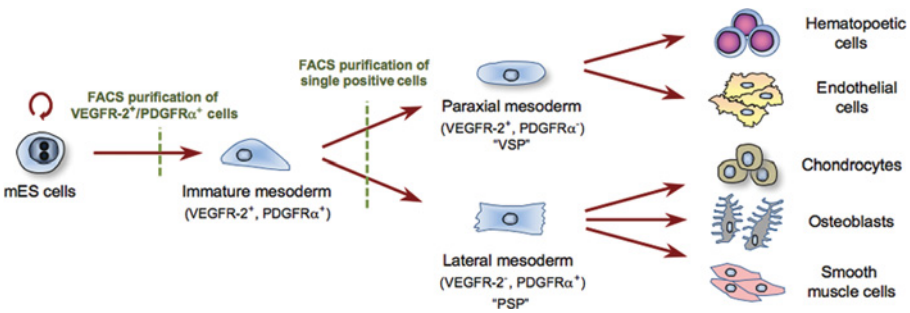
Very few attempts have been made to obtain purified populations of mesenchymal precursors from ESCs due to the lack of specific cell-surface markers for MSCs and mesenchymal precursors, as mentioned above. They are presented below.

In order to dissect the early processes of divergence of mesoderm into endothelial cell lineages compared with other mesodermal lineages in mESCs, the group of Nishikawa and colleagues used surface expression of VEGFR-2 (vascular endothelial growth factor receptor 2) and the PDGFRα (platelet-derived growth factor receptor α) to define lateral and paraxial mesoderm derivatives respectively (Nishikawa et al., 1998; Sakurai et al., 2006). They first generated VEGFR-2^+/PDGFRα^+ immature mesodermal precursors by plating mESCs on to collagen-IV-coated dishes in serum-containing medium. These precursors were then purified and shown to give rise to both VSP (VEGFR-2 single-positive; VEGFR-2^+/PDGFRα^-) and PSP (PDGFRα single-positive; VEGFR-2^-/PDGFRα^+) populations, exhibiting specific properties of paraxial and lateral mesoderm respectively. Indeed, the VSP population was able to produce endothelial and haematopoietic cells, whereas the PSP population had myogenic, osteogenic and chondrogenic potential *in vitro*. The PSP population might therefore constitute an interesting subset of mesoderm-derived mesenchymal precursors. This work is summarized on Figure 1.

In addition, a few groups reported the isolation of mesenchymal precursors from hESCs. Barberi et al. (2005) induced differentiation of hESCs by

Figure 1 | Generation and purification of mesodermal subpopulations endowed with endothelial and mesenchymal potentials from mESCs

Serum-exposure of mESCs seeded on to collagen-IV-coated culture dishes is used to obtain VEGFR-2$^+$/PDGFRα^+ immature mesodermal precursors, which can then be purified by FACS and give rise to VSP (i.e. VEGFR-2$^+$/PDGFRα^-) and PSP (i.e. VEGFR-2$^-$/PDGFRα^+) populations, exhibiting paraxial and lateral mesoderm properties respectively. The VSP population can produce endothelial and haematopoietic cells, whereas the PSP population has myogenic, osteogenic and chondrogenic potential, and thus constitutes a pool of mesoderm-derived mesenchymal precursors. Adapted with permission of AlphaMed Press, Inc., from H. Sakurai, T. Era, L.M. Jakt, M.Okada, S. Nakai, S. Nishikawa and S. Nishikawa, *In vitro* modeling of paraxial and lateral mesoderm differentiation reveals early reversibility, Stem Cells, vol. 24, pp. 575–586, 2006; permission conveyed through Copyright Clearance Center, Inc.



plating them on to a monolayer of murine OP9 stromal cells in the presence of serum. Using this method, 5 % of CD73$^+$ cells could be recovered and shown to be able to differentiate into fat, cartilage, bone and skeletal muscle cells. Strikingly, genome-wide expression analysis showed a remarkable overlap of global gene expression profiles between hESC-derived mesenchymal precursors and human bone marrow MSCs, suggesting that these two kinds of cells are highly related (Barberi et al., 2005). Using a similar strategy, Olivier et al. (2006) derived bipotential mesenchymal precursors from subcultures of hESCs under feeder-free conditions. These cells expressed specific markers of adult MSCs and were capable of adipogenic and osteogenic differentiation *in vitro* (Olivier et al., 2006).

It is worth mentioning, however, that none of these studies have assessed the differentiation potential of ESC-derived mesenchymal precursors in clonal assays. It is unknown, therefore, whether the aforementioned populations contain multipotent MSCs and/or a mixture of more committed precursors. Furthermore, the mechanisms involved in the formation of these ESC-derived MSCs and/or committed precursors are still unclear, although the PSP population obtained by Sakurai et al. (2006) is likely to have a mesodermal origin.

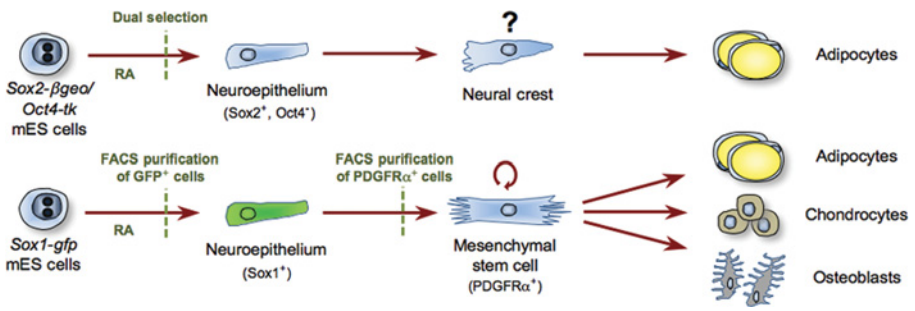
MSCs and adipocytes developing from RA-treated mESCs derive from the neuroectoderm, rather than from the mesoderm

In a first attempt to unravel the events underlying the formation of mesenchymal derivatives in RA-treated mESCs, Kawaguchi et al. (2005) examined the expression of various mesodermal and mesenchymal markers in early EBs. Surprisingly, they noticed that treatment with RA resulted in a sharp reduction in several mesodermal markers, as well as in the suppression of cardiomyocyte formation, suggesting that RA reduces overall mesoderm formation in mESCs. Since at high concentrations, RA was shown to promote neural differentiation of mESCs (Bain et al., 1995; Okabe et al., 1996), and as some mesenchymal tissues are known to be generated by the NC, which itself derives from neuroectoderm, Bain et al. (1995) and Okabe et al. (1996) then analysed the expression of various NC markers in mESCs. They showed that *sox9*, *sox10*, *foxD3* and *runx2*, which all play an important role in NC formation and/or mesenchymal condensation, were up-regulated on RA treatment. Together, these data suggest that neuroectoderm/NC is the major source of mesenchymal cells in RA-treated mESCs.

To test this hypothesis with respect to adipocytes, we have recently developed a genetic

Figure 2 | Genetic selection strategies used for the generation of MSCs and adipocytes of neuroepithelial origin in mESCs

Transient exposure of *sox2-βgeo/oct4-tk* and *sox1-gfp* mESCs to RA is used to drive neural differentiation. $Sox2^+/Oct4^-$ and $Sox1^+/GFP^+$ neuroepithelial cells are then purified using a dual selection strategy or FACS respectively. On adipogenic treatment, $Sox2^+/Oct4^-$ neuroepithelial cells can differentiate into adipocytes, possibly through a NC pathway. $Sox1^+/GFP^+$ neuroectoderm cells produce MSCs endowed with self-renewal properties and mesenchymal differentiation potentials towards adipocyte, chondrocyte and osteoblast lineages. Adapted from data presented in Billon et al. (2007) and Takashima et al. (2007).



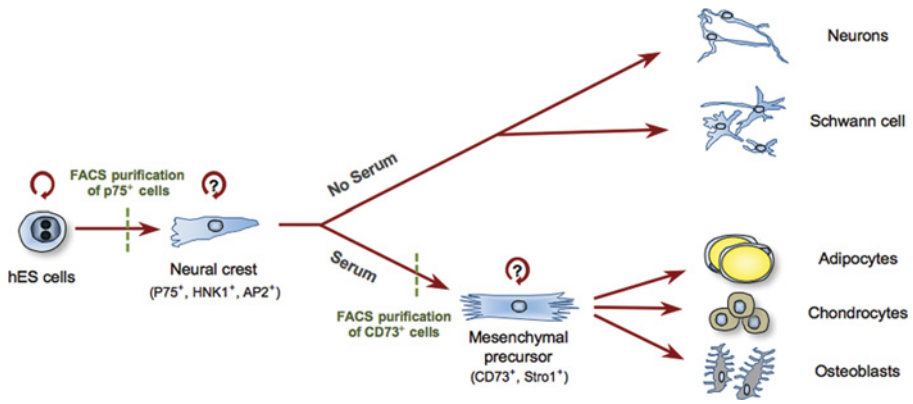
lineage-selection approach in mESCs, which is outlined on Figure 2. We used genetically engineered, selectable *sox2-βgeo/oct4-tk* (where *sox2* is the *sox2* promoter, *βgeo* is a neomycin-resistance gene, *oct4* is the *oct4* promoter and *tk* is a gene conferring sensitivity to the drug gancyclovir) mESCs that allow to select for neuroepithelial precursors ($Sox2^+$) and eliminate residual undifferentiated mESCs ($Oct4^+$) (Li et al., 1998; Billon et al., 2006). After induction of neural differentiation via RA treatment, highly enriched populations of neuroepithelial cells were selected in the presence of G418 and gancyclovir. We then exposed the cells to adipogenic signals and showed that, indeed, they could give rise to mature adipocytes within 14 days. Interestingly, a significant increase in *sox9*, *sox10* and *foxD3* mRNAs was observed prior to adipocyte formation, suggesting that NC-like cells present in the selected population could undergo adipocyte differentiation. Together, these data suggest that neuroectoderm/NC is the major source of adipocytes, at least in mESCs exposed to RA (Figure 2).

These findings were recently corroborated by Takashima et al. (2007), who used an elegant approach to unravel the origin of MSCs in both mESC cultures and during mouse development. They first developed two protocols to differentiate mESCs to mesodermal or neural/NC lineages. The 'mesoderm' method has been mentioned above and supported the generation of a mesodermal population that could then give rise to mesenchymal precursors (VSP population) (Nishikawa et al., 1998; Sakurai et al., 2006). The 'neural' method was an adaptation from the original technique, as described by Dani et al. (1997), for the generation of adipocytes, and comprised the early treatment of mESCs with RA. Takashima et al. (2007) showed that, although both methods could generate $PDGFR\alpha^+$ mesenchymal precursors, MSCs could only be generated from the RA method. MSCs were defined as being able to undergo sustained clonogenic proliferation while maintaining their potential to give rise to adipocytes, chondrocytes and osteocytes (Takashima et al., 2007). To confirm the neural origin of these MSCs, Takashima et al. (2007) then used a genetic labelling strategy similar to the *sox2-βgeo/oct4-tk* strategy described previously (Li et al., 1998; Billon et al., 2006, 2007), in which they could visualize and purify $Sox1^+$ neuroepithelial cells produced from a *sox1-gfp* (where *gfp* is green fluorescent protein) mESC line (Figure 2) (Aubert et al., 2002). They showed that MSCs could only be produced from the $Sox1^+/GFP^+$ cell population.

All together, these studies suggest that MSCs, as well as adipocytes generated from RA-exposed mESCs, arise from the neuroectoderm/NC, rather than from the mesoderm. Optimization of the methods allowing directed differentiation of mESCs toward a NC fate, and subsequent purification of NCCs, should therefore provide new opportunities to study NC specification towards MSCs and their derivatives *in vitro*. Recent progresses in that direction is summarized below.

Figure 3 | Generation and differentiation of NCSCs from hESCs

Stepwise isolation of NCSCs involves a combination of cell sorting, *in vitro* expansion and directed differentiation via extrinsic signals. Serum-free conditions allow differentiation of NCSCs into both sensory and autonomic neurons, as well as into Schwann cells. Serum exposure leads to rapid conversion of NCSCs into $CD73^+$ mesenchymal precursors, suggesting that $CD73^+$ cells may derive directly from NCSCs. This Figure is adapted from Lee et al. (2007) with permission from *Nature Biotechnology*



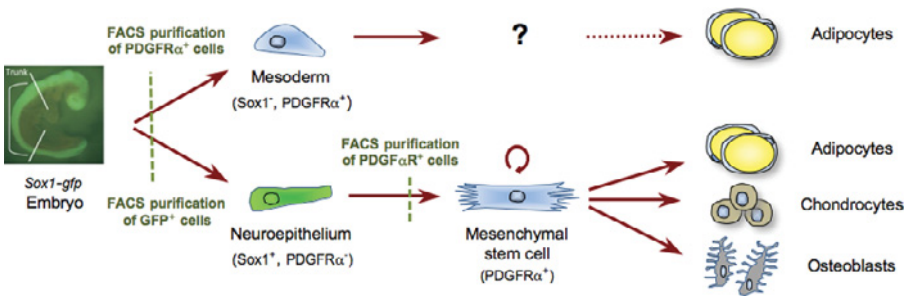
Dissecting NCC specification in ESCs

Very few studies have succeeded in generating NCCs from ESCs. In a recent report, c-kit was used as a cell-surface marker to enrich for NCCs in RA-exposed mESC cultures (Motohashi et al., 2006). $c\text{-}kit^+$ cells could be directed towards autonomic neuronal, glial and melanocyte fate in clonal assays, suggesting that this population included multipotent NC progenitors. Interestingly, RA-independent formation of NCCs has been reported in mouse and primate ESCs *in vitro* (Rathjen et al., 2002; Mizuseki et al., 2003; Pomp et al., 2005). In two approaches, NC induction involved exposure to SDIA (stromal cell-derived-inducing activity), and allowed the generation of PNS neurons and smooth muscle cells (Mizuseki et al., 2003; Pomp et al., 2005). However, the formation of other mesenchymal derivatives, in particular adipocytes, was not assessed in any of these studies. Similarly, the generation of MSCs with self-renewing capacities and extensive mesenchymal differentiation potential was not tested. More recently, Lee et al. (2007) reported the derivation of NC stem cells (NCSCs) from hESCs (Figure 3). They first induced neural differentiation through neural rosette formation and then purified NCCs by cell sorting on the basis of their expression of p75 and HNK1 (human natural killer 1 protein). Clonal analysis of $p75^+/HNK1^+$ cells revealed that they were multipotent and could differentiate into peripheral neurons, Schwann cells and myofibroblasts. Furthermore, after propagation *in vitro* in the presence of serum, these cells could be directed towards other mesenchymal lineages, including adipogenic, osteogenic and chondrogenic cells. After transplantation in the developing chick embryo or in adult mice, these hESC-derived NCSCs could differentiate into NC derivatives (Lee et al., 2007). Together, these data represent a significant breakthrough, since they open new avenues to study human NC development and further commitment to various lineages, including mesenchymal derivatives, such as adipocytes, at a single-cell level.

Conclusions regarding ESCs studies

Although significant progress has been made to derive specific mesenchymal cell types from ESCs, this system is still lacking defined culture conditions and good cell-surface markers for the isolation of pure MSCs and mesenchymal precursor populations. Furthermore, the molecular events leading to the formation of such populations remain unclear at present. Nevertheless, major themes emerge from the reports discussed here. First, it seems that several routes can be used to produce mesenchymal precursors and their derivatives from ESCs, which are likely to

Figure 4 | Generation of neuroepithelium-derived MSCs from *Sox1-gfp* E9.5 mouse embryos
Truncal neural tubes are isolated from *sox1-gfp embryos* at E9.5. After dissociation, GFP$^+$ neuroepithelial cells and PDGFRα^+ mesodermal cells can be separated by FACS. Although both populations can give rise to adipocytes, only the Sox1/GFP$^+$ neuroepithelial cells have the ability to generate PDGFRα^+ MSCs. Note that the adipogenic potential of mesodermal cells is reduced. Adapted from data presented in Takashima et al. (2007).



involve either mesodermal or neural/NC intermediates. While the strategies that include RA treatment, or neural rosettes formation, almost certainly involve a neural, rather than a mesodermal intermediate, the pathways involving exposure to SDIA via co-culture of ESCs on stromal cell lines are much less clear. Indeed, such approaches have been used to derive both mesodermal cells (Barberi et al., 2005), and neural/NC precursors (Kawasaki et al., 2000; Mizuseki et al., 2003; Pomp et al., 2005). Nevertheless, it is comforting to think that the different routes observed in the ESC system might mimic normal development, since mesenchymal precursors can be produced both from the mesoderm and the NC. The potential differences that distinguish mesenchymal precursors of mesodermal or neural crest origin remain to be elucidated. Interestingly, two studies recently addressed this matter by comparing gene expression profiles of these populations in both the ESC system (Takebe et al., 2006) and during craniofacial development (Bhattacherjee et al., 2007). Second, results obtained using mESCs suggest that, in contrast with mesenchymal precursors, true MSCs might only originate from a unique source, the neuroepithelium, rather than from the mesoderm (Takashima et al., 2007). Third, they indicate that adipocytes might also, in part, develop from the neuroepithelium through an MSC intermediate (Billon et al., 2007; Takashima et al., 2007). These data are surprising, since MSCs, as well as adipocytes, have been widely believed to derive from mesoderm (Dennis and Charbord, 2002).

Study of the developmental origin of MSCs and adipocytes by lineage-tracing approaches in mouse

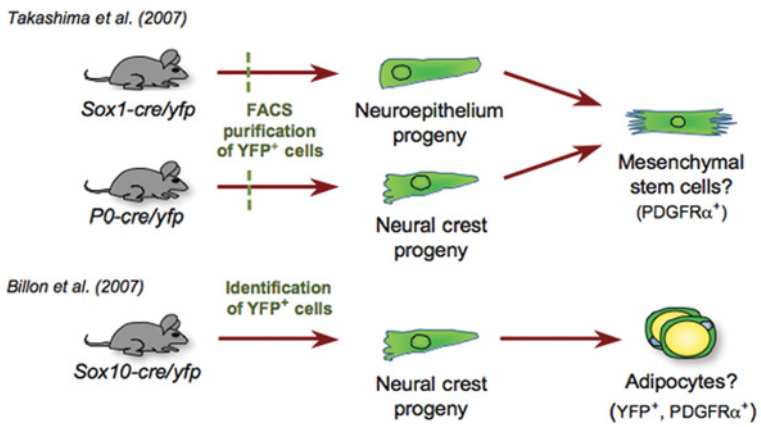
MSCs are generated in multiple waves of distinct origins

To confirm the relevance of these unexpected findings *in vivo*, Takashima et al. (2007) assessed the origin of MSCs in *sox1-gfp* embryos (Figure 4). They isolated the trunk of these embryos at E (embryonic day) 9.5 and purified neuroepithelial cells (Sox1$^+$, GFP$^+$), as well as mesodermal cells (Sox1$^-$, GFP$^-$, PDGFRα^+). They then tested the ability of these cells to give rise to proliferating MSCs and adipocytes *in vitro*. They demonstrated that, although both populations could give rise to adipocytes, only neuroepithelial cells could generate PDGFRα^+ MSCs. These data suggest that in mid-gestation embryos, as seen with mESCs, trunk MSCs originate entirely from neuroepithelium, and not from mesoderm. They also indicate that some adipocytes may arise from a non-neural pathway without transiting through an MSC intermediate.

To determine the correlation between Sox1$^+$ neuroepithelial progenitors in E9.5 embryos and MSCs in later life, Takashima et al. (2007) then carried out a persistent labelling of Sox1$^+$ neuroepithelial cells using *sox1-cre/rosa26-yfp* (where *sox1* is the *sox1* promoter, *cre* is cyclization recombinase gene, *rosa26* is the *rosa26* promoter and *yfp* is yellow fluorescent protein preceded by a floxed stop codon) mice. Considering the possibility that Sox1$^+$ cells give rise to MSCs via the neural crest, they also permanently labelled

Figure 5 | Permanent genetic lineage-labelling approaches used in mouse to follow neuroepithelium and NCC derivatives

Permanent genetic lineage labelling of neuroepithelium derivatives was achieved by crossing transgenic mice carrying a *Sox1-cre* construct with reporter mice (*R26-yfp*) carrying a floxed stop codon-*yfp* gene cassette inserted after the *rosa26* promoter. Cells in which *Sox1* has been activated are permanently marked and can be traced by the expression of the *yfp* transgene. Similarly, permanent genetic lineage labelling of the NC derivatives was achieved by crossing transgenic mice carrying a *P0-cre* or a *sox10-cre* construct with the *R26-yfp* reporter mice. Neuroepithelium and NC-derived YFP$^+$/PDGFRα^+ MSCs were purified by FACS at different stages of development in the trunk, and in adult bone marrow (Takashima et al., 2007). NC-derived YFP$^+$ adipocytes were identified in the cranial region of adult mice (Billon et al., 2007). Adapted from data presented in Billon et al. (2007) and Takashima et al. (2007).



NC progeny using *P0-cre/yfp* mice (P0 being a NC marker). The genetic strategies to permanently label neuroepithelium and NC progeny are shown in Figure 5. The presence of neuroepithelium/NC-derived YFP$^+$/PDGFRα^+ cells was assessed in the trunk of E14.5 embryos, as well as the presence of MSC within this population. Takashima et al. (2007) discovered that part of PDGFRα^+ cells in the E14.5 embryo trunks of both genotypes were also YFP$^+$, and thus derived from neuroepithelium/NC. In addition, some of these PDGFRα^+/YFP$^+$ cells were MSCs. MSCs could also be established from YFP$^-$/PDGFRα^+ cells, however, suggesting that, in contrast with E9.5 embryos, MSCs in E14.5 embryos derive both from a neural/NC pathway and a non-neural pathway.

Takashima et al. (2007) then analysed the contribution of these neural/NC-derived MSCs to the post-natal bone marrow, which is a well-studied source of adult MSCs. They found that the proportion of YFP$^+$/PDGFRα^+ cells in the bone marrow of neonates (P0) was far lower than in the embryonic trunk in both *sox1-cre/yfp* and *P0-cre/yfp* mice (0.032% and 2.39% respectively). Furthermore, although the YFP$^+$/PDGFRα^+ population was able to generate genuine MSCs, this population progressively decreased to negligible levels with aging [0.0021% and 0.35% for *sox1-cre/yfp* and *P0-cre/yfp* mice at P28 (post-natal day 28) respectively]. Finally, the frequency of PDGFRα^+ MSCs derived from YFP$^+$ compared with YFP$^-$ populations was nearly the same. Thus, in neonatal and adult bone preparations, MSCs were found within PDGFRα^+ population, but most of them derived from a non-neural/NC pathway.

Together, these results suggest that MSCs arise in multiple waves of distinct origins: the first MSCs in the embryo, which are identifiable by the method herein, are derived from neuroepithelium/NC. Later, this early, but transient, population is eventually replaced by MSCs derived from an as-yet-unidentified pathway (Takashima et al., 2007). An important question raised by this study (Takashima et al., 2007) is what is the other source of MSCs?Until recently, MSCs have been described to derive from mesoderm, but the results presented by Takashima et al. (2007) strongly argue against this hypothesis, since YFP$^-$/PDGFRα^+ somitic cells in *sox1-cre/yfp* E9.5 embryos could not generate MSCs. The authors speculate, on the basis of recent reports, that haematopoietic

cells might constitute the progenitors of MSCs in adult bone marrow (Ogawa et al., 2006). However, one cannot rule out the possibility that *in vitro* conditions used in this study (Takashima et al., 2007) to measure the clonogenicity and the differentiation potential of PDGFRα^+ cells were not appropriate to reveal the MSC potential of mesoderm-derived cells. Conversely, this study (Takashima et al., 2007) did not test whether neuroepithelium/NC-derived MSCs could form mesenchymal derivatives *in vivo*.

A subset of adipocytes is generated from the NC *in vivo*

Recently, we have used several approaches to address the origin of the adipocyte lineage *in vivo* and provided direct evidence for the contribution of the neural crest (Billon et al., 2007). We have investigated whether subsets of adipocytes originate from the NC using *sox10-cre/yfp* transgenic mice (where *sox10* is the *sox10* promoter and *yfp* is a floxed stop codon-*yfp* gene cassette inserted after the *rosa26* promoter) to map NC derivatives *in vivo*, because, to date, Sox10 is considered as the best NC marker (Figure 5) (Matsuoka et al., 2005). Indeed, *sox10* is strongly and specifically expressed in the NC from early embryonic development, and is not expressed in mesoderm (Kuhlbrodt et al., 1998; Ferguson and Graham, 2004). We examined *sox10-cre/yfp* offspring for the presence of YFP$^+$ adipocytes at P28, since, at this stage, adipose tissues can be detected at both cephalic and trunk levels. This analysis revealed YFP$^+$ lipid-filled adipocytes in cephalic adipose depots, between the salivary gland and the ear area. In contrast, no YFP$^+$ adipocytes could be detected in truncal adipose depots, including subcutaneous, perirenal, periepididymal and interscapular tissues. These results therefore provide new information about the ontogeny of the adipocyte lineage, and demonstrate that during normal development a subset of adipocytes in the face originates from NC, and not from mesoderm.

The contribution of NC to mesenchymal cell types is not restricted to the adipocyte lineage and has been established in various classes of vertebrates. The replacement of the cephalic NC by its quail counterpart in chick embryos showed that most of the facial skeleton is NC-derived. Moreover, much of the dermis, the connective components of facial musculature, and the muscular walls of blood vessels that vascularize the face and forebrain, have a NC origin (Le Douarin et al., 2004). The contribution of cephalic NC cells to the skull and to the cardiac outflow tract has been confirmed in the mouse, using genetic *wnt1-cre* fate mapping (Chai et al., 2000; Jiang et al., 2000; Santagati and Rijli, 2003). Our findings that mature adipocytes in the ear region arise from the NC during mouse development provide further evidence of the crucial role of the cephalic NC in the generation of the various mesenchymal derivatives, forming head structures and tissues, including adipocytes.

Study of adipocyte differentiation from NCCs *in vitro*

To better understand adipocyte lineage specification from the NC, we verified whether adipocytes could be obtained from NCCs isolated from a normal developing embryo. We used *in vitro* cultures of quail NCCs, since they have been instrumental in establishing the developmental potentialities of the NC (Baroffio et al., 1988; Dupin et al., 1990; Baroffio et al., 1991; Lahav et al., 1998; Trentin et al., 2004; Calloni et al., 2007). We isolated NCCs from both the cephalic and thoracic level and grew them in culture medium that is permissive for adipocyte differentiation (Student et al., 1980; Rodriguez et al., 2004). This analysis revealed that typical mature adipocytes could readily be produced from cephalic NCCs, and, to a lesser extent, from truncal NCCs. Therefore, at least *in vitro*, quail NCCs from both the cephalic and the thoracic level exhibit an adipogenic developmental potential. These findings open exciting new opportunities to study the events regulating the earliest stages of adipocyte lineage induction and differentiation from the NC. A major challenge now is to discover how and when the adipocyte lineage segregates in NC-derived cells. Analysis of the progeny of single NC cells should allow the determination of whether adipocytes arise from multipotent NCSCs or from early committed cells.

General discussion

Hidden mesenchymal potentialities for the TNC (trunk NC)?

NCC fate is not the same along the neural axis, as established by mapping the antero–posterior origin of NC derivatives in quail–chick chimaeras (Le Douarin et al., 2004). The generation of mesenchymal derivatives was found to be restricted to the cephalic

NC region in higher vertebrates. Indeed, when the quail TNC is orthotopically implanted in the chick, no quail mesenchymal cells are ever present in the host (Nakamura and Ayer-le Lievre, 1982; Le Douarin et al., 2004), although one study suggested that TNCCs (TNC cells) might generate a small subset of fibroblasts in the mouse sciatic nerve (Joseph et al., 2004). TNCCs may also acquire very cryptic mesenchymal differentiation potentials when exposed to cephalic signals in grafting experiments (Nakamura and Ayer-le Lievre, 1982; Lumsden, 1988). However, taken together, these data support the widely accepted view that TNCCs do not contribute to mesenchymal cell lineages in normal development, in contrast with CNCCs (cranial NCCs). This idea is challenged by the recent report of Takashima et al. (2007), which shows that neuroepithelium ($Sox1^+$) and NC ($P0^+$)-derived cells in the trunk region of E9.5 mouse embryos can produce a good proportion, if not all, MSCs present at this stage. As the contamination of $Sox1^+$ trunk neuroepithelium by cranial NCCs is unlikely, these data suggest that TNCCs could be endowed with more mesenchymal potentialities than previously suspected. It is worth mentioning, however, that this study (Takashima et al., 2007) did not test whether truncal neuroepithelium/NC-derived MSCs could indeed differentiate and form mesenchymal derivatives *in vivo*. In contrast, we directly assessed for the formation of adipocytes from all levels of the NC in *sox10-cre/yfp* mice (Billon et al., 2007). Although a subset of adipocytes was found to derive from the NC in the cephalic region, we could not detect any NC-derived adipocytes at the truncal level, at least at P28 and during later life. These data suggest that, during normal development, the property of NC to form adipocytes is restricted to the cephalic part of the neural axis, just as it is the case for the other mesenchymal derivatives produced from the NC. The possibility that the TNC might form adipocyte precursors during embryogenesis remains to be assessed, since no markers are presently available to prospectively identify these cells, and the adipose tissue cannot be detected macroscopically before birth. This possibility would imply, however, that these hypothetical TNC-derived adipocyte precursors disappear in latter life, or are maintained in the undifferentiated state, at least until P28, since we did not detect NC-derived adipocytes in adipose tissues of the trunk of *sox10-cre/yfp* mice. Of note, NC-derived adipocyte precursors could not be detected in several truncal adipose depots of adult *wnt1-cre/yfp* mice, suggesting again that truncal adult adipocytes are not derived from the NC (Wrage et al., 2008).

It is worth mentioning that in cultures of quail NCCs, the ability to generate adipocytes *in vitro* was not restricted to CNCCs, but was also shared by TNCCs (Billon et al., 2007). These data suggest that, although the property of the NC to form adipocytes seems to be restricted to the cephalic part of the neural axis *in vivo*, a hidden capacity of the TNC to yield mesenchymal cells can be revealed *in vitro* by appropriate environmental cues. In support of this hypothesis, TNCCs from avian and mammalian embryos were reported to generate myofibroblasts, as well as chondrocytes *in vitro* (Shah et al., 1996; McGonnell and Graham, 2002; Abzhanov et al., 2003; Trentin et al., 2004; Ido and Ito, 2006; Calloni et al., 2007). The *in vitro* adipogenic and chondrogenic differentiation potentials in the trunk was far lower than in the cranial region, where a significant subset of NCCs are multipotent progenitors endowed with both neural and mesenchymal potentials (Billon et al., 2007; Calloni et al., 2007). In view of these considerations, the finding by Takashima et al. (2007) that MSCs are generated by the TNC during embryogenesis does not necessarily mean that these MSCs, indeed, generate mesenchymal derivatives *in vivo*, since these results were based on *in vitro* studies.

Do MSCs and adipocytes produced from different sources differ in their biological properties?

The recent findings that subsets of MSCs, as well as adipocytes, have an alternative origin in the NC, raise an important question: do MSCs and adipocytes of different origin also differ in their biological functions?Takashima et al. (2007) demonstrated that from late embryogenesis, neuroepithelium/NC-derived MSCs are progressively replaced by MSCs from another, at present unknown, source(s). Therefore, one can argue that embryonic MSCs might only be important for the genesis of embryonic tissues, whereas later MSCs might be involved in organ repair and maintenance in postnatal life (S.I. Nishikawa, personal communication). At early developmental stages, TNCCs might be the origin of MSCs/precursors that may possibly provide a scaffold facilitating, at later stages, further colonization

of embryonic tissues by new MSCs/precursors of another yet undefined source. More experiments are needed to support these hypotheses. Similarly, our findings indicate that, similar to other mesenchymal cells, such as chondrocytes and osteocytes, white adipocytes have a different origin along the antero–posterior axis: in the cephalic region, they arise from the NC, whereas in the trunk, they are thought to derive from the mesoderm, although further mesodermal lineage-mapping studies are still required to strengthen this point (Billon et al., 2007). Of note, morphological and functional differences have been reported for different fat depots in rodents and humans: VAT (visceral adipose tissue) and SAT (subcutaneous adipose tissue), for example, differ in various biochemical properties, such as insulin and adrenergic response (Montague and O'Rahilly, 2000; Lafontan and Berlan, 2003). Interestingly, these variations might explain why some depots are linked to the metabolic disorders associated with obesity, whereas other are not (Rosen and MacDougald, 2006; Gesta et al., 2007). Do cephalic versus truncal adipose depots also present site-specific regulation?

The recent advances presented in this review in the generation and isolation of MSCs and adipocytes from different cellular models, such as murine and human ESCs, and avian NCCs, open exciting new opportunities to study the earliest stages of MSC and adipocyte specification from the NC. Together, these new systems promise to keep bringing unexpected and fundamental insights into the biology of MSCs and the adipocyte lineage.

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