In vivo characterization of neural crest-derived fibro/adipogenic progenitor cells as a likely cellular substrate for craniofacial fibrofatty infiltrating disorders

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ABSTRACT

The cellular substrate underlying aberrant craniofacial connective tissue accumulation that occurs in disorders such as congenital infiltration of the face (CILF) remain elusive. Here we analyze the *in vivo* properties of a recently identified population of neural crest-derived CD31-:CD45-:alpha7-:Sca1+:PDGFRa+ fibro/adipogenic progenitors (NCFAPs). In serial transplantation experiments in which NCFAPs were prospectively purified and transplanted into wild type mice, NCFAPs were found to be capable of self-renewal while keeping their adipogenic potential. NCFAPs constitute the main responsive FAP fraction following acute masseter muscle damage, surpassing the number of mesoderm-derived FAPs (MFAPs) during the regenerative response. Lastly, NCFAPs differentiate into adipocytes during muscle regeneration in response to pro-adipogenic systemic cues. Altogether our data indicate that NCFAPs are a population of stem/primitive progenitor cells primarily involved in craniofacial muscle regeneration that can cause tissue degeneration when the damage co-occurs with an obesity inducing diet.

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1. Introduction

A number of craniofacial pathologies such as congenital infiltrating lipomatosis of the face (CILF) [1,2] and Graves' ophthalmopathy (also known as Thyroid Eye Disease, TED) [3] are characterized by excessive local accumulation of fat and connective tissue. While the signals involved may vary, both CILF and TED share a common feature: the aberrant accumulation of adipocytes and connective tissue is restricted to the head, with little or no signs of abnormal fat expansion or fibrosis in other parts of the body. This anatomical attribute strongly suggests that cells from the neuroepithelium lineage may be specifically affected in both disorders.

We have recently identified a population of neural crest-derived CD31-:CD45-:a7-:Sca1+:PDGFRa+ fibro/adipogenic progenitor (NCFAP) cells by means of lineage tracing, using a transgenic mouse in which expression of recombinant yellow fluorescent protein (YFP) is driven by the neuroectoderm marker WNT1 [4,5]. Neural crest-derived FAPs (NCFAPs) specifically reside in the stromal fraction of head and craniofacial tissues including fat and muscle, are phenotypically similar to mesoderm-derived FAPs (MFAPs) [5], and become activated early after the occurrence of acute craniofacial muscle damage [5]. During muscle regeneration FAPs exert a pro-regenerative effect, increasing myoblast maturation/differentiation *in vitro* [6] and *in vivo* [7], possibly through the secretion of pro-myogenic factors [5,6]. NCFAPs proliferate following craniofacial muscle damage and infiltrate the damaged area, a process during which recapitulation of the neural crest gene program occurs [5]. When exposed to a local degenerative environment, however, FAPs adopt the adipogenic lineage and contribute to intramuscular fat accumulation and tissue degeneration [5,6,8]. In order to further characterize the properties of NCFAPs, we tested their ability to self-renew, undergo preferential expansion following acute damage and to produce ectopic fat in response to metabolic systemic cues, *in vivo*.

2. Materials and methods

2.1. Animals and muscle damage

Wnt1-Cre::Rosa26-YFP mice were generated by crossing *WNT1-Cre$^{+/-}$* (The Jackson Laboratory) with *R26-YFP$^{+/-}$* mice (The Jackson Laboratory). *R26-YFP* littermates were used as controls. Adult

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(>8 weeks) C57BL/6J-CMV-β actin-EGFP transgenic mice were used as donors in transplantation experiments. PDGFRα-H2B::EGFP mice (The Jackson Laboratory) were used for flow cytometry analysis. For muscle damage experiments, *Wnt1-Cre::Rosa26-YFP* and control mice were anesthetized with 0.5–2% isoflurane, and the damage was induced by intramuscular injection of 0.15 μg notexin (Latoxan) into the masseter muscle. All mice were maintained in a pathogen-free facility and all experiments were performed in accordance to the University of British Columbia Animal Care Committee regulations.

2.2. Flow cytometry

Sample preparation and flow cytometry were performed as previously described [6]. The following monoclonal primary antibodies were used: anti-CD31 (clones MEC13.3, Becton Dickenson, and 390, Cedarlane Laboratories), anti-CD34 (clone RAM34, eBioscience), anti-CD45 (clone 30-F11, Becton Dickenson), anti-Sca-1 (clone D7, eBiosciences) and anti-α7 integrin (produced in-house). The antibody dilutions were as previously reported [6]. Analysis was performed on LSRII (Becton Dickenson) equipped with three lasers. Data were collected using FacsDIVA software. Sorts were performed on a FACS Vantage SE (Becton Dickenson) or FACS Aria (Becton Dickenson), both equipped with three lasers. Sorting gates were strictly defined based on isotype control (fluorescence minus one) stains. Data analysis was performed using FlowJo 8.7 (Treestar) software.

2.3. Transplantation

CD31-:CD45-:a7-:Sca1+:PDGFRa+:YFP+ cells were prospectively purified from intact masseter muscles of *Wnt1-Cre::Rosa26-YFP* mice and collected in cold DMEM and collected by centrifugation at 450g for 5 min. Cells were resuspended in 25 μl Matrigel and loaded into an ice-cold needle and syringe immediately before injection. Cells were injected either into the subscapular region or intramuscularly into the TAs of WT recipient mice.

3. Results

One of the main questions regarding the "stemmness" of progenitor cells is their ability to reconstitute/maintain the niche. To test the ability of NCFAPs to renew the progenitor cell (e.g. Sca1+) niche we performed a serial transplantation experiment (Fig. 1A). We prospectively purified NCFAPs from the subcutaneous cephalic fat depot of 6 week-old Wnt1::ROSA-YFP mice and transplanted them into WT mice by means of subcutaneous injection. This method allows the easy tracing of neuroepithelium-derived cells by detection of YFP reporter expression. Three weeks after transplantation, fat fads of approximately 2 mm of diameter were observed at the site of injection (Fig. 1B). As previously reported, those pads were enriched in YFP+ adipocytes [5]. Importantly too, YFP+ cells that did not show signs of adipogenic differentiation were also observed in the stromal vascular fraction (SVF) of the newly formed fat pads. Analysis of the NC-derived fraction of the SVF revealed that 92% of the cells were Sca1+, while 7% expressed α7 integrin (Fig. 1B). On the other hand, CD31+ cells were not detected (Fig. 1B), indicating that NCFAPs do not give raise to endothelial cells *in vivo*. Altogether this analysis revealed that while a small percentage of NCFAPs present in the SVF of the engraftments adopted the myofibroblast (α7+) lineage, the vast majority of NCFAPs remained undifferentiated. In order to test whether those undifferentiated cells retained the potential to differentiate *in vivo*, we purified Sca1+ YFP+ cells from the SVF of the primary engraftments and injected them subcutaneously into secondary recipients. After three weeks the engraftments were dissected and the presence of YFP+ adipocytes was detected by immunofluorescence (Fig. 1D). This result indicated that a subset of NCFAPs remains undifferentiated within the niche, yet the cells retain their adipogenic potential.

Our previous data indicated that NCFAPs adopt the adipogenic lineage in response to local signals during craniofacial muscle regeneration [5]. We next asked whether NCFAPs can respond to metabolic systemic cues. To this end we induced acute damage in the TAs of mice that had been kept under either high fat diet (HFD) or normal diet for two weeks (Fig. 2). NCFAPs were purified from the cephalic fat depot in the neck area and injected into the
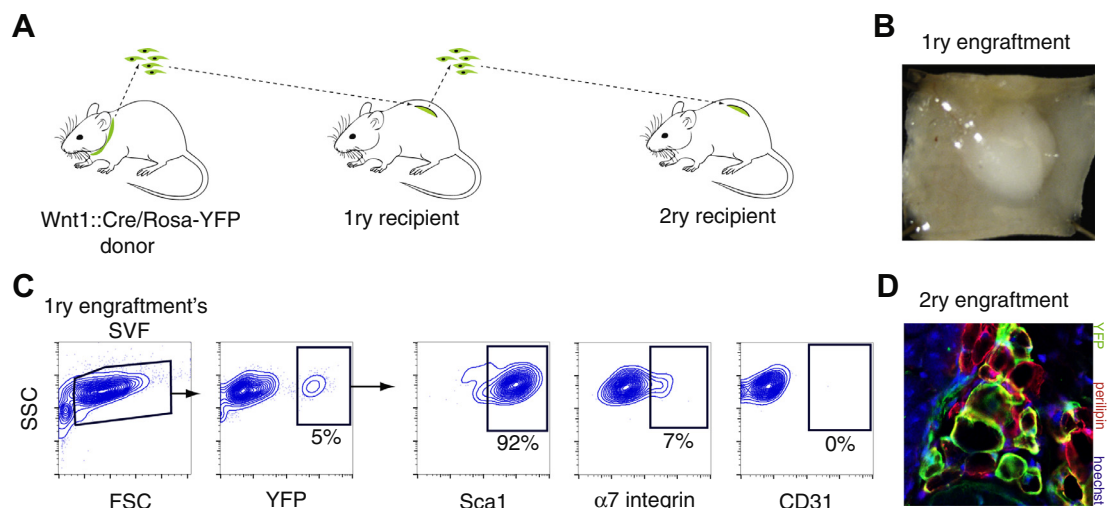

Fig. 1. (A) Serial transplantation of NCFAPs. A total of 200,000 CD31-:CD45-:a7-:Sca1+:PDGFRa+ YFP+ cells were purified from the craniofacial fat depot of Wnt1-Cre::Rosa26-YFP mice and injected subcutaneously in the subscapular region of WT recipient mice. (B) Two weeks after transplantation, mature subcutaneous fat pads were observed. (C) The fat pads were dissected and digested and the stromal vacular fraction was analyzed for the presence of stem/progenitor cells (Sca1), muscle progenitors (α7) and endothelial progenitors (CD31) by FACS. (D) Sca1+ YFP+ cells were prospectively purified by cell sorting and transplanted into the subscapular region of secondary WT recipient mice. Two weeks after transplantation, the newly formed fat blobs were dissected and the presence of neural crest-derived (YFP+) adipocytes was analyzed using an antibody anti-perilipin.
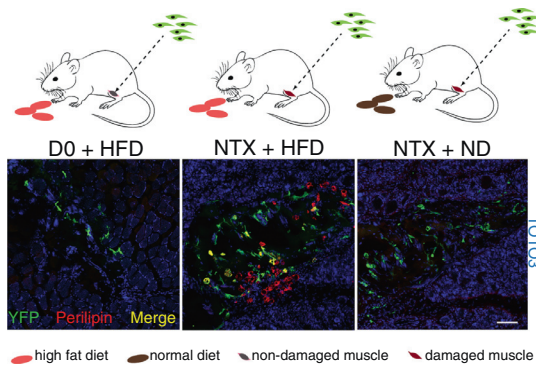
Fig. 2. NCFAPs differentiate to adipocytes in response to metabolic systemic signals. NCFAPs were prospectively purified from the cephalic fat depot and injected into the TAs of recipient mice that had been previously kept under either high fat diet (HFD) or normal diet (ND) for two weeks. 100,000 cells were injected into the TAs along with the injury-causing agent notexin (NTX). The NCFAPs were also injected into control non-damaged TAs (D0) from animals on HFD. The TAs were collected seven days after damage/transplantation and processed for immunofluorescence histology. An antibody against the adipocyte marker perilipin was used to detect adipogenic differentiation.

TAs along with notexin (i.e. at the time of damage). The TAs were dissected one week after the induction of damage and injection of cells. NCFAP-derived adipocytes were observed in the regenerating muscles of HFD-fed mice, but not in the muscles of normal diet (ND) fed mice (Fig. 2). Importantly, only undifferentiated NCFAPs were observed in non-damaged TAs of mice under HFD (Fig. 2). This result indicated that systemic metabolic cues associated with an obesity-inducing diet trigger NCFAP differentiation during the regenerative response.

A feature unique to NCFAPs is that even though their number declines throughout life, this subpopulation remains the most responsive in the event of muscle damage. NCFAPs constitute 100% of the craniofacial FAP population one week after birth, but in 8-week old and 20 week-old mice those cells comprise 30% and 20% of the craniofacial FAP population, respectively [5]. We next tested whether NCFAPs are still responsive to craniofacial muscle damage in adult mice. To this end, we measured the expansion of both FAP populations *in vivo* following injection of the snake venom notexin into the masseter muscle of 8 week-old mice. We found that the damage triggers an asymmetric expansion of NCFAPs, which initially accounted for 30% of the FAPs and grew to 70% of the total FAP population by day 3 post-damage (Fig. 3). This result indicated that despite constituting the smaller

subpopulation, NCFAPs remain the FAP fraction most responsive to acute injury.

4. Discussion

In the present study we have gained further insight into the dynamics of NCFAP activation and differentiation *in vivo*. The asymmetric response to damage in which NCFAP expansion prevails over MFAP expansion suggests that the signals involved in craniofacial FAP activation are different from the ones involved in limb muscle regeneration. Alternatively, the same signals might be present in regenerating craniofacial and limb structures, yet NCFAPs are more responsive to them than MFAPs. In support of the first hypothesis, the expression of endothelin receptor type A (ETA) increases in NC-FAPs following facial muscle damage [5], suggesting a role for the endothelin axis in NCFAP activation and expansion. Activation of ETA by endothelin 1 (ET1-1) stabilizes Snail and contributes to the repression of E-Cadherin (ECad) during endothelial-to-mesenchymal transition (EMT) [9,10]. The endothelin system plays an important role in craniofacial development with mesoderm-derived ET-1 contributing to NC migration and cell survival [11,12]. Activation of the pathway has also been linked to the development of tumor invasiveness and metastasis in certain types of cancer, including nasopharyngeal and oral cancers [13,14]. In this regard, therapies aimed at inhibiting the ET-ETA axis have been used with encouraging results in the treatment of various cancers [10]. In particular, the orally bioavailable ETA antagonist ZD4054, has been shown to inhibit the growth breast cancer cells *in vitro* and *in vivo* [10]. Taking into account the activation of the ET pathway during NCFAP proliferation, a therapeutic strategy including the use of ETA antagonists may prove effective in the treatment of orbitofacial infiltration caused by fibroblasts and adipocytes.

A key characteristic of NCFAPs is their ability to infiltrate craniofacial tissue and to differentiate. As observed during craniofacial muscle regeneration under healthy conditions, the NCFAP population contracts after a few days and returns to pre-damage levels [5]. In the presence of systemic or local pro-adipogenic signals, however, NCFAPs undergo *in situ* adipogenesis, giving rise to ectopic fat deposition and extensive adipogenic infiltration of craniofacial muscles (Fig. 3) [5]. This functional characteristic, in addition to the fact that NCFAPs proliferate more aggressively than their mesoderm-derived counterpart, leads to the fact that neural crest-derived adipocytes are predominantly found within the infiltrated craniofacial structures (Fig. 4) [5].

In sum, NCFAPs represent the predominant adipogenic population in the craniofacial region. On the basis of its reactivity to
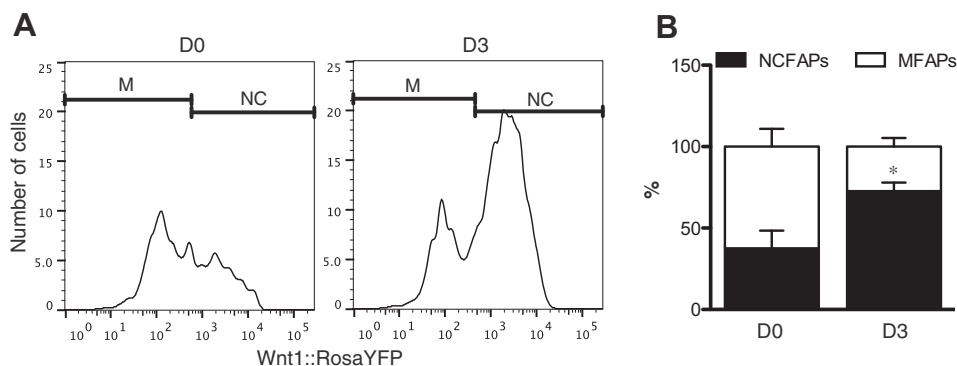

Fig. 3. Flow cytometry analysis of NCFAPs and MFAPs in the masseter muscle of adult Wnt1-Cre::Rosa26-YFP mice. (A) Number of cells was analyzed in non-damaged (healthy) masseteres *vs.* masseters on day 3 post notexin injection (regeneration). M = mesoderm-derived, NC = neural crest-derived. (B) Percentage of the whole FAP population was quantified for each subpopulation. *$p < 0.05$ *vs.* healthy NCFAPs. $n = 4$.
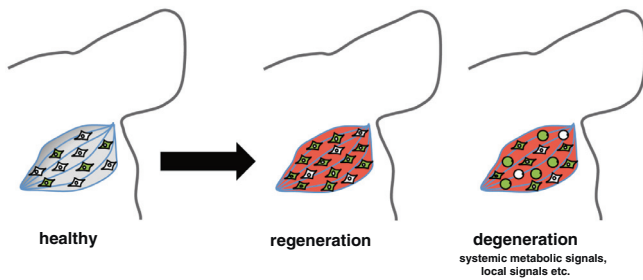
Fig. 4. Model of NCFAP behavior in healthy and damaged craniofacial muscle. In the healthy adult masseter, NCFAPs (gray cells) constitute the smaller fraction of the resident FAP population. Following damage, however, NCFAPs expand to become the predominant FAP subpopulation, and participate in masseter regeneration via secretion of pro-regenerative cytokines. When exposed to systemic and/or local pro-adipogenic signals, NCFAPs differentiate into adipocytes during the regenerative process, causing fatty muscle infiltration.

injury, robust fibrogenic capacity, and its ability to infiltrate the muscles, this progenitor population represents an ideal target for the study of the cellular mechanisms of CILF and TED.

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