Biochemical and Biophysical Research Communications 451 (2014) 148–151

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

# *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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ARTICLE INFO

*Article history:*
Received 12 July 2014
Available online 26 July 2014

*Keywords:*
Muscle regeneration
Fibro/adipogenic progenitors
Neural crest

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.


## 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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http://dx.doi.org/10.1016/j.bbrc.2014.07.089
0006-291X/

(>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% isofluorane, 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 450*g* 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

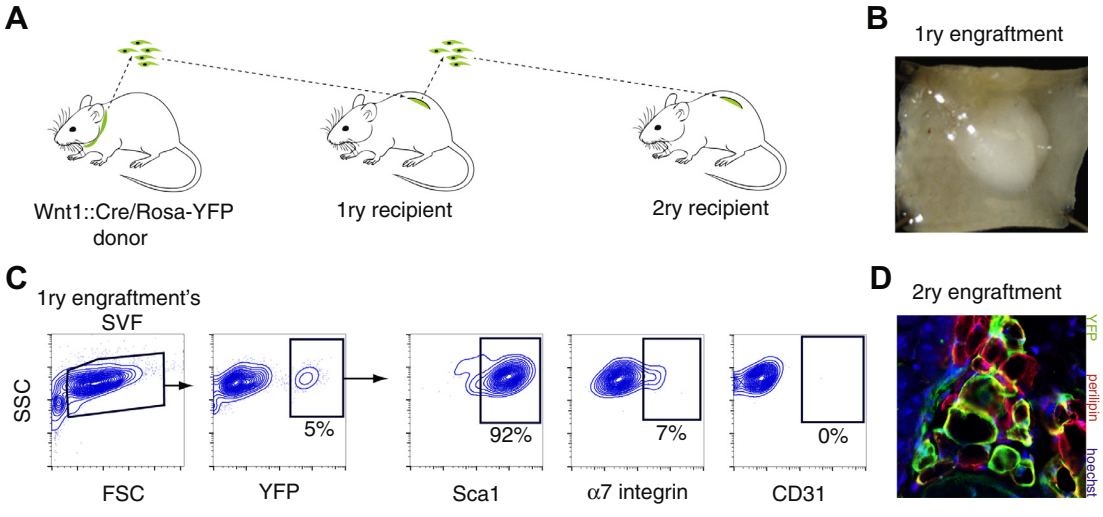


**Fig. 1.** (A) Serial transplantation of NCFAPs. A total of 200,000 CD31-:CD45-:a7-:Sca1+:PDGFRa+ YFP+ cells were purified from the craniofacial fat depot of Wnt1-Cre::Rosa26-YFP mice and injected subcutaneously in the subscapular region of WT recipient mice. (B) Two weeks after transplantation, mature 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.