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Isolation of progenitor cells from the blubber of northern elephant seals (*Mirounga angustirostris*) in order to obtain an *in vitro* adipocyte model—preliminary results

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Marine top predators are highly informative in understanding the quality and health of ocean habitats. Through bioamplification, they face a very large risk of exposure to toxic, persistent, and fat-soluble molecules such as polychlorinated biphenyls (PCBs), polybrominated diphenyl ethers (PBDEs), dichlorodimethyltrichloroethane (DDT), and methylmercury, which are preferentially stored in the adipose tissue. The life history of pinnipeds often includes extended periods of fasting on land. This is particularly true for phocid seals such as northern elephant seal (NES) (*Mirounga angustirostris*). This species indeed exhibits one of the most extreme terrestrial fasting durations (up to 3 mo) corresponding to breeding, lactation, and molting for females, postweaning development for pups, and maintaining a territory or competing for dominance rank on the breeding rookery for males (Le Boeuf *et al*. 1972). During such periods, individuals mobilize primarily lipids from their large adipose tissue stores (Costa *et al*. 1986, Noren *et al*. 2003). This mobilization of blubber lipids presents a risk through the release of environmental pollutants into the circulation. Previous studies in fasting NES pups and females elucidated the mobilization dynamics

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of PCBs, PBDEs, and DDT from blubber to blood and revealed that the mobilization patterns of these pollutants were not directly related to those of lipids (Debier *et al*. 2006, 2012; Louis *et al*. 2014*a*). Indeed, during the beginning of the fast, the pollutants appeared to be less efficiently mobilized from blubber than lipids. Their concentrations thus increased in the remaining immobilized blubber. The concentrations of pollutants into the blood circulation were more important at late fast. Similar dynamics were also observed in other phocid seal species such as the gray seal (*Halichoerus grypus*) (Debier *et al*. 2003, Vanden Berghe *et al*. 2012). The health impact of the release of these toxic molecules into the circulation is unclear, but is believed to be at the origin of physiological disruptions (Ross *et al*. 2000, Vanden Berghe *et al*. 2010).

So far, the mechanisms associated with the mobilization of fat-soluble pollutants from adipose tissue remain unknown. We investigated the development of primary cultures of adipocytes from NES, in order to study the biochemical aspects involved in the release of pollutants and lipids from the adipose tissue during periods of negative energy balance in large marine predators. Establishing such *in vitro* model for NES is a critical element to understanding the molecular mechanisms for mobilization and ultimately the toxicity of lipophilic pollutants in these and other marine mammals. There is indeed a lack of simple *in vitro* model of adipocytes, available for the characterization of accumulation kinetics, storage and release of fat-soluble pollutants. This model will also be useful to study the potential toxicological effects of pollutants on the endocrine function of adipose tissue. The present paper compiles the preliminary results concerning the *in vitro* differentiation of NES progenitor cells, collected on free-ranging animals, into adipocytes.

Sampling took place on NES weaned pups, at Año Nuevo State Reserve, California (37°06′30″N, 122°20′10″W) from January to April 2011. Two NES pups undergoing a postweaning fast were sampled for each primary culture in order to obtain 4–5 g of adipose tissue. This age class of animals was chosen due to the ease of catching. NES pups were initially immobilized with an intramuscular injection of Telazol at ~1 mg/kg of estimated body mass and sedation was maintained through subsequent intravenous injections of 50 mg of ketamine as needed (all drugs were from Fort Dodge Animal Health, Fort Dodge, IA). Blood was collected from the extradural vein into Vacutainer serum tubes (Becton-Dickinson, Franklin Lakes, NJ). Blubber biopsies were collected in the lateral pelvic area using 6 mm biopsy punches (Acupunch, Acuderm Help Medical, France) and stored in cold sterile phosphate buffered saline (PBS) (Sigma-Aldrich, Atlanta, GA). Once collected, samples were immediately kept at 4°C and transported to the laboratory. Blood samples were centrifuged at 1,300 × g for 15 min at 4°C in order to obtain serum, which was then heat-inactivated at 56°C for over 50 min, aliquoted and stored at −20°C until use. Progenitor cells from the adipose tissue were isolated and put into culture using a protocol adapted from Bourez *et al*. (2012) and Louis *et al*. (2014*b*) for rats. This protocol is illustrated in Figure 1. First, blubber was minced with scissors under sterile conditions and added to Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% (v:v) heat-inactivated fetal bovine serum (FBS) (PAA Laboratories Inc., Darthmouth, MA), 10 mM HEPES buffer solution (Invitrogen), antibiotic and antifungal mixture (0.5 K/mL penicillin, 0.5 K/mL streptomycin, 1.25 μg/mL amphotericin B mixture [Lonza, Walkersville, MD], 1 mg/mL gentamicin [Invitrogen], and 40 U/mL nystatin [Lonza]), and 1,250 U/mL collagenase (Type II, Sigma-Aldrich). The minced fat was then incubated in the medium at 37°C for over 40 min under gentle agitation to allow tissue digestion. The digested

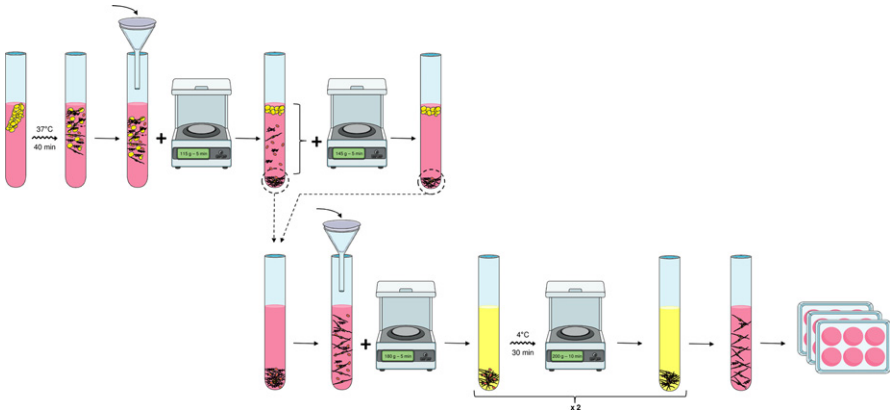

Figure 1. Schematic representation of isolation of progenitor cells from northern elephant seal adipose tissue. Blubber biopsies were sampled from weaned pups. They were then digested by collagenase. Filtrations and centrifugations allowed isolating progenitor cells. Cells were treated twice with ACK lysis buffer in order to eliminate red blood cells and then, seeded on 6 well culture plates. For more details refer to the text. Figure was produced using Servier Medical Art (available at http://www.servier.com/Powerpoint-image-bank).

tissue was then filtered through sterile 200 µm nylon mesh and the filtrate was centrifuged at 115 × g for 5 min at 4°C. The supernatant was collected and centrifuged at 145 × g for 5 min at 4°C. The pellets of the two centrifugations were pooled, filtered through sterile 25 µm nylon mesh and finally, centrifuged at 180 × g for 5 min at 4°C. The supernatant was discarded. ACK lysis buffer composed of 150 mM NH_4Cl, 1 mM $NaHCO_3$, and 1 µM EDTA (all from Sigma-Aldrich) was added to the final cell pellet at a ratio of 180:1 (v:v) and stored on ice for 30 min with gentle and regular stirring. The solution was then centrifuged at 200 × g for 10 min at 4°C and the supernatant was then removed. This step was repeated twice in order to eliminate red blood cells. After the second centrifugation, the lysis buffer was removed and the isolated progenitor cells were suspended in DMEM supplemented with 10% (v:v) heat-inactivated FBS and antibiotic and antifungal mixture as described above. Cells were seeded at a mean density of 21 × 10^3 cells/cm^2 on six well culture plates (Corning CellBind Surface, Corning Incorporated Life Sciences, Tewksbury, MA). Plates were stored in an incubator at 37°C and 5% CO_2 for 24 h to allow cell sedimentation and adhesion. Twenty-four hours after the precursor isolation (day 1), the culture medium was removed and replaced by a new medium composed of DMEM, 0.1 K/mL penicillin–0.1 K/ml streptomycin–0.25 µg/mL amphotericin B mixture, 10% (v:v) heat-inactivated NES serum, and a differentiation cocktail composed of 10 nM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 10 nM ciglitizone, and 8.6 µM insulin (Sigma-Aldrich) (adapted from Bourez *et al*. 2012 and Louis *et al*. 2014*b*). The culture medium was renewed every 2 d and a visual monitoring was regularly performed.

Differentiation Rates

The NES progenitor cells presented a typical fibroblastoid shape (Fig. 2A) and multiplied during the few days following the inoculation (Fig. 2B). After approximately 10 d, the first lipid droplets could be observed within cells. To document the

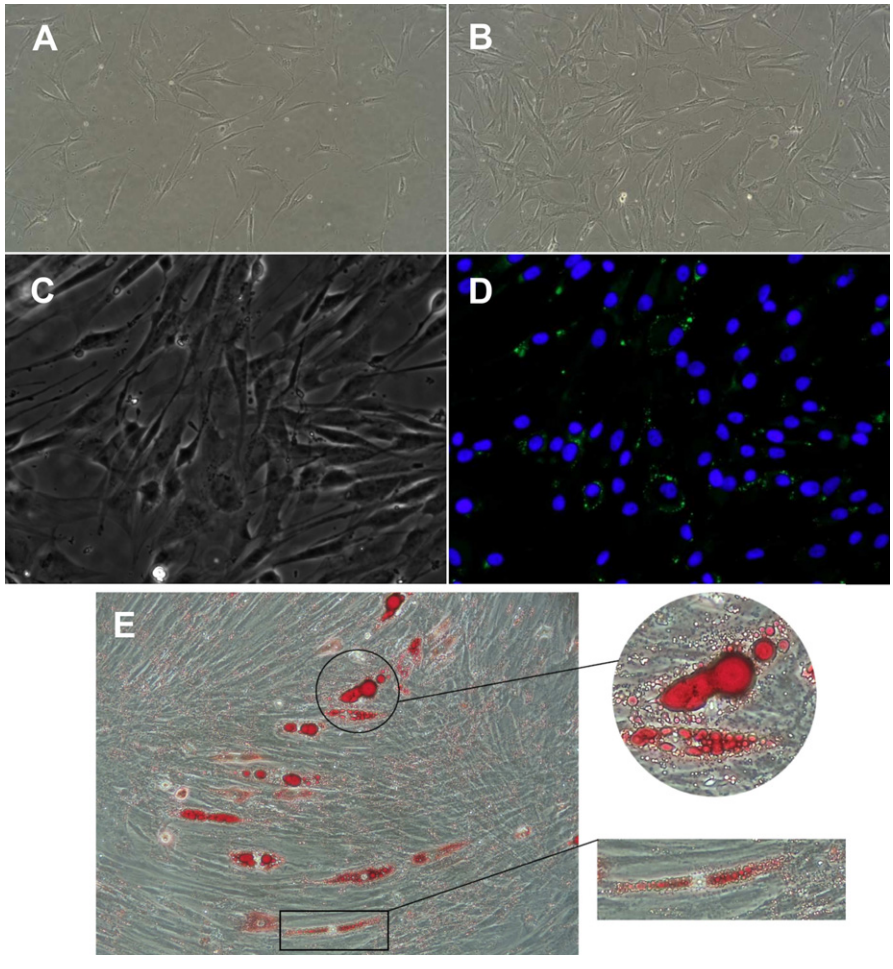

Figure 2. Progenitor cells from northern elephant seals (NES) were cultivated with 10% NES serum and the differentiation cocktail during 3 d (A) and 7 d (B) (light microscopy). They were then stained with diamino-2-phenylindole and AdipoRed after 11 d of culture (light microscopy) (C). The nuclei appear in blue and lipid droplets, in green with a fluorescence microscope (D). Similarly, after 20 d of culture, cells were fixed and stained with Oil Red O. The lipid droplets then appear in red (light microscopy) (E).

extent of adipocyte differentiation through microscopic observation, the rate of differentiation was evaluated by staining the lipids with AdipoRed (Cambrex Bio Science, Walkersville, MD), a specific fluorescent staining for intracellular lipid droplets. To do this, cells were seeded on 12 well culture plates, containing a glass cover slip on the bottom, at the step of inoculation of progenitor cells. They were cultivated in culture medium that was renewed every 2 d, as described above. After 11 d, cells were stained with AdipoRed according to the manufacturer's instructions for 15 min in darkness. They were then washed with warm PBS and fixed with 4% paraformaldehyde (v:v) in PBS for 40 min. The cover slip was then mounted on glass slide with

Vectashield (Vector Laboratories, Burlingame, CA). The mounting media contained diamino-2-phenylindole (DAPI) (Sigma-Aldrich), which marked the nuclei. Cells were observed with a fluorescent microscope (excitation λ = 385 nm and emission λ = 572 nm for AdipoRed; excitation λ = 372 nm and emission λ = 454 nm for DAPI). The lipid droplets thus appeared in green and the nuclei were stained in blue. Some cells were filled with small fat droplets (Fig. 2C, D). After 20 d of culture in 6 well culture plates, cells were stained with Oil Red O (ORO) (Li *et al*. 2008). To do this, the culture medium was removed at day 20 and cells were washed twice with warm PBS before being fixed with 4% paraformaldehyde (v:v) (Sigma-Aldrich) in PBS for 40 min. Cells were rinsed once with deionized water and incubated with ORO solution (Sigma-Aldrich) in 60% isopropanol for 15 min. Fixed cells were kept wet with deionized water. In this staining treatment, lipids within lipid droplets appeared red. Around 10% of the cells were considered as adipocytes after 3 wk of culture, while around 80% of the cells kept an elongated shape while accumulating very small fat droplets as evidenced by ORO staining on Figure 2E. Cells considered as adipocytes exhibited various forms (Fig. 2E).

Effect of Differentiation Cocktail

The differentiation cocktail used in the present study (*e.g.*, dexamethasone, IBMX, ciglitizone, and insulin) is an established cocktail for inducing adipocyte differentiation of rodent progenitor cells (Bourez *et al*. 2012, Louis *et al*. 2014*b*). Dexamethasone, a synthetic glucocorticoid, stimulates adipocyte differentiation in cell lines derived from mice (3T3-L1) (Yeh *et al*. 1995) and preadipocytes of rats, pigs and humans (Chen *et al*. 1995, Kras *et al*. 1999, Joyner *et al*. 2000, Shin *et al*. 2003). IBMX, a synthetic chemical, can induce adipocyte differentiation in 3T3-L1 cells (Birsoy *et al*. 2008). Both dexamethasone and IBMX induce two families of transcription factors, C/EBPs and PPAR-γ, which play major roles in the differentiation of preadipocytes (Cao *et al*. 1991, Shin *et al*. 2003). Similarly, ciglitizone, a member of thiazolidinedione family, has been shown to enhance the differentiation of murine C3H10T1/2 cell lines and human mesenchymal stem cells in adipocytes by acting as a selective ligand of PPAR-γ (Lehmann *et al*. 1995, Spiegelman 1998, Janderová *et al*. 2003, Rosen 2005). Insulin is known to promote differentiation of murine cell lines (3T3-L1 and 3T3-F442A) (Ailhaud 1982), and it can indeed upregulate the transcription of C/EBPs in 3T3-L1 cell line (MacDougald *et al*. 1995, Klemm *et al*. 2001) and stimulate the PPAR-γ mRNA and protein expression in human adipocytes (*in vivo* and in mature isolated cells) (Rieusset *et al*. 1999). In addition, insulin induces the translocation of glucose transporter type 4 from the cytosol to the plasma membrane of adipocytes. As a result, the extracellular glucose may be absorbed by the cells and converted to fatty acids through the lipogenesis pathway (Cushman and Wardzala 1980). The differentiation rate of NES progenitor cells into adipocytes obtained in the present study was lower than what is usually obtained for rodents and humans (Pittenger *et al*. 1999, Janderová *et al*. 2003, Bourez *et al*. 2012, Louis *et al*. 2014*b*). We believe this might be due to the fact that NES possess unique physiological specificities. For example, *in vivo* studies report different levels of insulin resistance in fasting individuals (Fowler *et al*. 2008, Viscarra *et al*. 2011). Therefore, it is possible that the differentiation cocktail used for rodents does not have the same impact on NES progenitor cells. To address this, we investigated the effect of the presence/absence of this cocktail on the differentiation of NES progenitor cells into adipocytes. After 20 d of culture, there was no accumulation of lipids inside the cells

when they were cultivated without the cocktail of differentiation (Fig. 3A). By microscopic observation, it appeared that without the cocktail of differentiation, cells do not have the ability to engage in the adipocyte differentiation pathway. This cocktail thus appears necessary to differentiate NES progenitor cells into adipocytes, even though its composition could be improved in order to be better adapted to NES physiology.

Effect of NES Serum

Our choice of using NES serum in the culture medium was made with the aim of getting as close as possible to the *in vivo* situation. In this section, we compared the impact of 10% (v:v) heat-inactivated NES serum on the rate of differentiation to the one of 10% (v:v) heat-inactivated FBS, which is usually used in the *in vitro* culture of rodent adipocytes (Bourez *et al*. 2012, Louis *et al*. 2014*b*). It appears from our visual observations that the presence of NES serum with the cocktail of differentiation (Fig. 3B) was more favorable to the adipocyte differentiation than FBS with the cocktail of differentiation (Fig. 3C), which might result from the specific composition of NES pup serum. This improved differentiation might be due to the high triglyceride (TG) content within the blood of NES pups, which is the result of the ingestion of

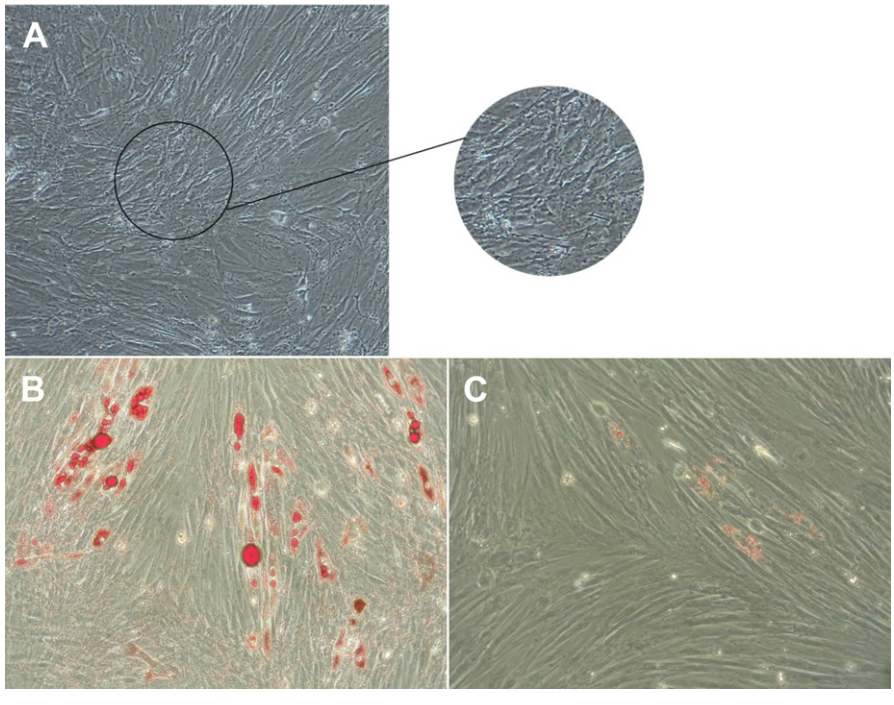

Figure 3. Northern elephant seal (NES) progenitor cells were cultivated for 20 d with 10% NES serum and without the cocktail of differentiation (A), with 10% NES serum and with the cocktail of differentiation (B) and with 10% fetal bovine serum and with the cocktail of differentiation (C). The potential accumulation of cytoplasmic triglycerides was investigated by Oil Red O staining (light microscopy).

highly lipid-rich milk during the nursing period (54% ± 5% lipid) (Le Boeuf and Ortiz 1977). The TG concentrations measured in the serum of suckled NES pups are elevated (2.1 ± 0.3 g TGs/L; CL, unpublished results, permit # 87-1743-00) compared to NES adults in different physiological states (*e.g.*, 0.67 ± 0.12 g TGs/L in fasting male serum at early breeding (Tift *et al*. 2011) and 0.58 ± 0.15 g TGs/L in fasting female serum at early molt (Fowler 2012)). NES pups gain 90 ± 27 kg during the 26 d nursing period and approximately 55% of this mass gain is composed of fat (Crocker *et al*. 2001). The circulating TGs packed in serum lipoproteins (chylomicrons and VLDL) can be hydrolyzed by lipoprotein lipase. Fatty acids are then picked up by adipose tissue and directly stored as TGs. It thus seems reasonable to think that, in the case of NES, cells accumulate lipids preferentially by direct uptake of fatty acids rather than by realizing fatty acid neosynthesis from glucose as for rodents. The levels of TGs in the serum of weaned NES pups used in the present study were approximately twice as high as the TG concentrations of FBS (1.2 ± 0.3 g TGs/L in NES serum vs. 0.46 ± 0.01 g TGs/L in FBS). We therefore suggest that the use of NES serum may thus promote the lipid accumulation within adipocytes by direct input of fatty acids. Additionally, because most fatty acids are also agonists of PPAR-γ (Tontonoz *et al*. 1994, Kliewer *et al*. 1997), the high TG levels in NES serum may further promote the adipogenesis. It is important to note that other molecules present in weaned NES serum (*e.g.*, glucagon and thyroid hormones) (Kirby and Ortiz 1994, Pucci *et al*. 2000, Ortiz *et al*. 2001) could inhibit an efficient accumulation of lipids within adipocytes. The use of serum from suckled pups might thus be preferable but requires further experiments in order to compare the lipid accretion within cells.

Conclusion

This study represents the first attempt to isolate and differentiate *in vitro* phocid progenitor cells into adipocytes. The differentiation of isolated NES cells towards adipocytes was induced by treating them with a cocktail of differentiation including dexamethasone, IBMX, ciglitizone, and insulin. The microscopic observations presented here are encouraging. The cocktail of differentiation was necessary to obtain *in vitro* adipocytes. Nevertheless, the composition of this cocktail should be further improved to adapt to the specific physiology of NES. The presence of high TG levels in the cell culture medium may enhance the accumulation of lipids in cultured NES adipocytes by direct intake of fatty acids or PPAR agonist activity. Identification of the NES serum constituents that promote adipocyte differentiation would facilitate more efficient and consistent *in vitro* differentiation of NES progenitor cells. Quantifying changes in gene expression correlating with NES adipocyte differentiation may aid in refining optimal differentiation induction conditions. The ability to differentiate NES progenitor cells would provide an *in vitro* system to characterize the molecular mechanisms controlling mobilization of lipophilic environmental pollutants in NES. Application of this innovative model to other marine mammals could then be used in physiological as well as toxicological studies to understand the behavior and the impact of lipophilic pollutants (*e.g.*, PCBs, PBDEs, DDT, and methylmercury) and thus, directly address the risk to marine mammals from these now ubiquitous pollutants. Adipose tissue is not only used for buoyancy, insulation, and energy, but it is also an endocrine organ in mammals. The availability of such tool can be indeed very useful to improve our knowledge regarding the blubber of marine mammals.

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