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A Method to Induce Brown/Beige Adipocyte Differentiation from Murine Preadipocytes

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[Abstract] Adipocytes exhibit different morphological and functional characteristics, depending on their anatomical location, developmental origin, and stimulus. While white adipocytes tend to accumulate energy as triglycerides, brown and beige adipocytes tend to direct carbon sources to fuel thermogenesis. White and beige adipocytes originate from common progenitor cells, which are distinct from brown adipocyte precursors. Having a method to study white vs. beige vs. brown adipocyte differentiation may help to unveil the mechanisms driving distinct adipogenic programs. Preadipocytes can be cultured and differentiated *in vitro* using a combination of compounds to stimulate adipogenesis. Here, we describe and compare protocols designed to stimulate adipocyte differentiation and induce brown/beige-like or white-like characteristics in differentiating adipocytes. The protocols consist in exposing murine preadipocytes to pharmacological stimuli aimed at triggering adipogenesis and inducing (or not) a thermogenic gene expression program. After 8 days of differentiation with a pro-browning cocktail, immortalized preadipocytes isolated from interscapular brown fat (9B cells) or inguinal white fat (9W cells) from the same mouse expressed higher levels of brown/beige adipocyte markers (*e.g.*, *Ucp1*) and pan-adipocyte differentiation markers (*e.g.*, *Pparg*, *Cebpa* and *aP2*) when compared to the same cells differentiated with a cocktail that lacked brown/beige adipogenic inducers (*i.e.*, rosiglitazone, T3, and indomethacin). Consistent with a higher thermogenic potential of brown vs. beige adipocytes, differentiated 9B cells expressed higher *Ucp1* levels than differentiated 9W cells. This simple protocol may help researchers to understand mechanisms of adipogenesis and how adipocytes become thermogenic.

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Keywords: Beige adipocytes, Adipocyte differentiation, Thermogenesis, Preadipocytes, Brown adipocytes

[Background] Obesity is characterized by excess body fat and represents a risk factor for several chronic non-transmissible diseases (World Health Organization, 2015). The activation of brown adipose tissue (BAT) and the recruitment of newly formed beige adipocytes in white adipose tissue (WAT) leads to increased thermogenesis and higher energy expenditure, thus representing a promising intervention to treat obesity and its complications. Brown adipocytes contain multiple lipid droplets in their cytoplasm. They are also filled with mitochondria that promote energy dissipation in the form of heat, by leaking protons across the inner mitochondrial membrane, thus uncoupling oxidative phosphorylation from ATP synthesis. This mechanism is mediated by uncoupling protein 1 (UCP1), in a process called non-shivering thermogenesis (Nedergaard *et al*., 2001; Cannon and Nedergaard, 2004). White adipocytes have a single and large lipid droplet, few mitochondria, and produce many types of adipokines (Rosen and Spiegelman, 2014). Adipocytes with increased thermogenic capacity also appear in WAT depots, under states such as cold exposure, exercise, and caloric restriction (Cinti *et al*., 2005; Wu *et al*., 2012; Fabbiano *et al*., 2016), and are called beige adipocytes. Although beige adipocytes are functionally similar to brown adipocytes, these two types of thermogenic cells differ in their developmental origin (Ikeda *et al*., 2018). In fact, beige adipocytes share common progenitor cells with white adipocytes (Ikeda *et al*., 2018). The process of beige adipogenesis is often called browning, and normally occurs in subcutaneous fat depots. Together, these observations suggest that cell extrinsic and intrinsic factors contribute to determine the fate of adipocyte differentiation. Understanding these factors may help elucidating adipogenesis and lead to the identification of new therapeutic targets (Vegiopoulos *et al*., 2017). Here, we used murine preadipocyte cell lines isolated from different fat depots to characterize and compare protocols to promote brown, beige, or white adipocyte differentiation. These protocols were based on previous studies (Morrison and Farmer, 1999; Fasshauer *et al*., 2001; Tseng *et al*., 2004), although they have been slightly modified in our laboratory, where they have been replicated and optimized to apply basic procedures, budget-friendly reagents, and minimal infrastructure.

Materials and Reagents

1. 12-well Cell Culture Plate (Corning, 12-565-321)
2. 0.22 µm sterile filter
3. 9B preadipocytes: these cells were isolated from the interscapular brown fat of a *Dicer*$^{lox/lox}$*; aP2-Cre-ERT2* mouse and immortalized using SV-40-large T antigen as described before (Mori *et al*., 2012 and 2014). They behave like wild type cells unless 4-hydroxytamoxifen is added to the medium
4. 9W preadipocytes: these cells were isolated from the subcutaneous white fat of the same mouse used to isolate 9B cells. These cells were immortalized using SV-40-large T antigen and also behave like wild type cells unless 4-hydroxytamoxifen is added to the medium

5. 20 nM insulin* (Sigma-Aldrich, catalog number: I6634, powder storage temperature -20°C. Store at 4°C after dilution)
6. 1 nM triiodothyronine (T3)* (Sigma-Aldrich, catalog number: T2877, storage temperature -20°C)
7. 0.5 mM 3-Isobutyl-1-methylxanthine (IBMX)* (Sigma-Aldrich, catalog number: I5879, storage temperature -20°C)
8. 1 µM dexamethasone* (Sigma-Aldrich, catalog number: D4902, storage temperature -20°C)
9. 0.125 mM indomethacin* (Sigma-Aldrich, catalog number: I7378, storage temperature -20°C)
10. 2.8 µM rosiglitazone* (Sigma-Aldrich, catalog number: R2408, storage temperature -20°C)
11. Fetal bovine serum (FBS) (Thermo Fisher Scientific, catalog number: 12657-029, storage temperature -20°C)
12. Penicillin-Streptomycin (Pen/Strep) (Thermo Fisher Scientific, catalog number: 15140122, storage temperature -20°C)
13. Dulbecco's modified Eagle's medium (DMEM), high glucose (Life Technologies, Invitrogen™, catalog number: 11965, storage temperature 4°C)
14. 1 mM insulin stock solution (see Recipes)
15. 10 µM T3 stock solution (see Recipes)
16. 0.25 M IBMX stock solution (see Recipes)
17. 1 mM dexamethasone stock solution (see Recipes)
18. 0.125 M indomethacin stock solution (see Recipes)
19. 2.8 mM rosiglitazone stock solution (see Recipes)
20. Maintenance medium (see Recipes)
21. Differentiation medium day 2 (see Recipes)
22. Differentiation medium day 4 (see Recipes)
23. Differentiation medium day 6 (see Recipes)

**Final concentration*

Equipment

1. Heating block
2. Tissue culture incubator (37°C, 5% CO_2, Sheldon Manufacturing, Inc.)
3. Water bath (Fisher Scientific, FS-205)

Software

1. Graphpad Prism 9® software

Procedure

Note: Pre-warm media (see Recipes) to 37°C for 10 min prior to the procedure.

A. Seed cells

1. Seed 1 × 10^5 preadipocytes per well in a 12-well plate and culture in 1 ml of “Maintenance medium” (see Recipes: B1 for pro-browning induction, or C1 for pro-whitening).
2. Change media every 2 days until 90% confluence.

B. Adipocyte differentiation

Day 0: Once cells reach 90% confluence, change media and culture for 2 days with “Maintenance medium”.

Day 2: Replace media with 1 ml of “Differentiation medium day 2” (see Recipes: B2 for pro-browning, or C2 for pro-whitening) and culture for 2 days.

Day 4: Remove media and replace with 1 ml of “Differentiation medium day 4” (see Recipes: B3 for pro-browning, or C3 for pro-whitening).

Day 6: After 2 days, replace the media with 1 ml of “Differentiation medium day 6” (see Recipes: B4 for pro-browning, or C4 for pro-whitening).

Day 8: Cells are fully differentiated and ready to use.

Data analysis

Validation

Here, we describe a method to induce adipocyte differentiation *in vitro* from murine preadipocytes. We describe two types of differentiation protocols: one that leads to the induction of a brown/beige adipogenic profile (“Pro-Browning Cocktail Recipe”) and another that leads to differentiation of white adipocytes (“Pro-Whitening Cocktail Recipe”). In addition to the cells described here, we have applied these protocols in a wide range of murine preadipocyte cell lines, including 3T3-F442A, C3H10T1/2, and others (data not shown and Rocha *et al.*, 2020). The protocols lead to 50-90% of cells exhibiting lipid droplets in their cytoplasm, although the proportion of differentiated cells and the size of the lipid droplets will largely depend on a number of factors, including the lot of FBS, and the type, passage, commitment, and confluency of the preadipocytes. To illustrate the protocol’s efficiency, we used immortalized preadipocytes isolated from the subcutaneous white adipose tissue (9W cells) and brown adipose tissue (9B cells) of the same mouse and differentiated them with the Pro-Browning Cocktail Recipe or the Pro-Whitening Cocktail Recipe. The Pro-Browning Cocktail Recipe induced the expression of *Ucp1* in both cell lines, although the level of induction was higher in 9B cells by several orders of magnitude (Figure 1A). Additionally, it further induced pan-adipocyte markers *Cebpa* (Figure 1B), *Pparg* (Figure 1C), and *aP2* (Figure 1D) when compared to the Pro-Whitening Cocktail Recipe. These results indicate that not only 9B preadipocytes are more committed to adipogenesis, but also that the Pro-Browning Cocktail is more potent than the Pro-Whitening Cocktail to induce adipocyte differentiation.

Adipocytes differentiated using the procedures described here can be used in several different applications. In our original research paper where the Pro-Browning Cocktail protocol was applied (Rocha *et al.*, 2020), we performed multiple experimental procedures using differentiated adipocytes,

including gene expression analysis, measurement of oxygen consumption, and confocal and fluorescence lifetime imaging microscopy. Brown/beige adipocyte-like features were observed in 9W cells differentiated with the Pro-Browning Cocktail protocol. These features included the expression of a thermogenic gene profile, increased mitochondrial content, multilocular lipid accumulation, elevated oxygen consumption rate with high uncoupled respiration, and reduced NADH and FAD lifetime. All data and information about data processing and analysis, including statistics and details of replicates, are included in the original research paper (Rocha *et al*., 2020).

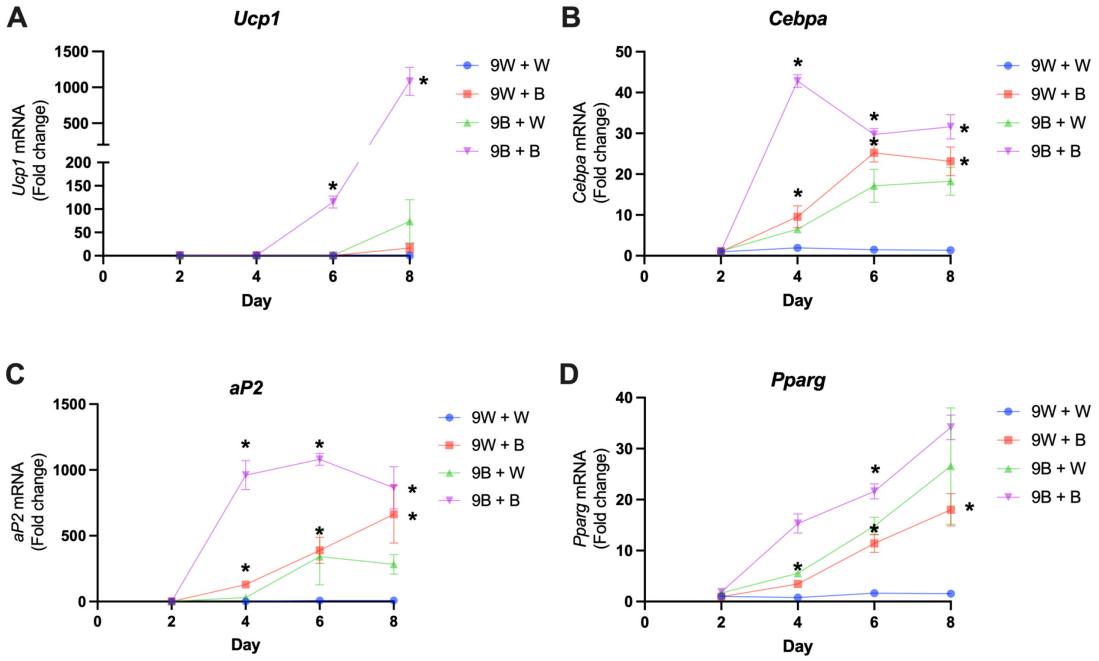

Figure 1. Comparison between the pro-browning (B) and pro-whitening (W) cocktails to induce adipocyte differentiation using murine immortalized white (9W) or brown (9B) preadipocytes.

White (9W) and brown (9B) preadipocytes were differentiated using the pro-whitening (9W + W and 9B + W), or the pro-browning (9W + B and 9B + B) cocktails. Expression of (A) *Ucp1*, (B) *Cebpa,* (C) *aP2,* and (D) *Pparg* mRNA was assessed by real-time qPCR (n = 3 independent pools of cells per group). *36B4* mRNA expression was used for normalization. Values are the mean ± SEM.*P < 0.05 versus the pro-whitening cocktail (W) (One-way ANOVA with Tukey's multiple comparison test). Primer sequences are available upon request.

Statistical analysis

The results were expressed as mean ± standard error of the mean (SEM) and analyzed statistically by the One-way ANOVA with Tukey's multiple comparison test, adopting a minimum significance limit of P < 0.05 in the Graphpad Prism 7® software.

Recipes

A. Preparation of stock solutions
   1. 1 mM insulin stock solution in 1 ml of distilled water (add 0.5 µl HCl 16 N for better solubilization). Filter the stock solution through a 0.22 µm sterile filter.
   2. 10 µM T3 stock solution in ethanol
   3. 0.25 M IBMX stock solution in 0.5 M KOH. Filter the stock solution through a 0.22 µm sterile filter
   4. 1 mM dexamethasone stock solution in ethanol
   5. 0.125 M indomethacin stock solution in ethanol
   6. 2.8 mM rosiglitazone stock solution in ethanol

   Notes:
   a. Stocks may be stored for several months at -20°C, except for insulin, which needs to be stored at 4°C.
   b. To help with solubilization, (3) and (5) may be heated to 75°C for 1 min using a heating block.

B. Pro-Browning Cocktail Recipe
   1. Maintenance medium
      DMEM high glucose supplemented with 10% FBS and 1% of Pen/Strep
   2. Differentiation medium day 2
      DMEM high glucose supplemented with 20 nM insulin, 1 µM dexamethasone, 0.5 mM IBMX, 1 nM T3, 0.125 mM indomethacin, and 2.8 µM rosiglitazone.
   3. Differentiation medium day 4
      DMEM high glucose supplemented with 20 nM insulin, 1 nM T3, and 2.8 µM rosiglitazone.
   4. Differentiation medium day 6
      DMEM high glucose supplemented with 20 nM insulin, and 2.8 µM rosiglitazone.

C. Pro-Whitening Cocktail Recipe
   1. Maintenance medium
      DMEM high glucose supplemented with 10% FBS and 1% of Pen/Strep.
   2. Differentiation medium day 2
      DMEM high glucose supplemented with 20 nM insulin, 1 µM dexamethasone, and 0.5 mM IBMX.
   3. Differentiation medium day 4
      DMEM high glucose supplemented with 20 nM insulin.
   4. Differentiation medium day 6
      DMEM high glucose supplemented with 20 nM insulin.

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Competing interests

The authors declare no competing interests.

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