ADIPOCYTE
2018, VOL. 7, NO. 3, 166–179
https://doi.org/10.1080/21623945.2018.1479578

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RESEARCH PAPER

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Promotion of lipid storage rather than of thermogenic competence by fetal versus newborn calf serum in primary cultures of brown adipocytes

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ABSTRACT

Much current understanding of brown adipocyte development comes from in-vitro cell models. Serum type may affect the behavior of cultured cells and thus conclusions drawn. Here, we investigate effects of serum type ("fetal bovine" versus "newborn calf") on responses to differentiation inducers (the PPARγ agonist rosiglitazone or the neurotransmitter norepinephrine) in cultured primary brown adipocytes. Lipid storage was enhanced by fetal versus newborn serum. However, molecular adipose conversion (*Pparg2* and *Fabp4* expression) was not affected by serum type. Rosiglitazone-induced (7-days) expression of thermogenic genes (i.e. *Ucp1, Pgc1a, Dio2* and *Elovl3*) was not systematically affected by serum type. However, importantly, acute (2 h) norepinephrine-induced thermogenic gene expression was overall markedly higher (and adipose genes somewhat lower) in cells cultured in newborn serum. Thus, newborn serum promotes thermogenic competence, and the use of fetal serum in brown adipocyte cultures (as is often routine) counteracts adequate differentiation. Agents that counteract this inhibition may therefore confoundingly be ascribed genuine thermogenic competence-inducing properties.

ARTICLE HISTORY
Received 28 March 2018
Accepted 10 May 2018

KEYWORDS
brown adipocytes; thermogenesis; norepinephrine; UCP1; adipose conversion; differentiation; fetal bovine serum; newborn calf serum

Introduction

Recruitment and activation of the energy-consuming brown adipocytes is currently considered a potential therapeutic approach to treat metabolic diseases (e.g. [1-3]). An enhanced understanding of brown adipocyte development and function is necessary to be able to utilize this therapeutic potential. A significant contribution to our understanding of brown adipocyte signaling and formation comes from in vitro cell culture systems. It is clearly important that brown adipocytes differentiating in such systems reflect physiologically relevant states and have the ability to acquire adequate thermogenic potential.

Brown adipocytes in culture may either be cell lines or they may be primary cultures, directly derived from progenitors isolated as stromal-vascular fractions from classical brown adipose tissue depots. The cell lines tend to demonstrate a decreased ability to express the essential uncoupling protein-1 (UCP1) with increasing number of passages.[4,5] In contrast, new preparations of primary brown adipocyte cultures will always demonstrate the ability to express *Ucp1* qualitatively and quantitatively similarly to what has been observed in earlier preparations.[6]

Primary brown adipocyte cultures are grown in media that generally are to 90% fully defined, as they are based on mixtures of chemically defined components; typically, variations of Eagle's Minimum Essential Medium (e.g. DMEM and α-MEM) or HAM's F12 medium are used. However, similarly to the majority of cultured cells, brown preadipocytes require serum(-derived factors) for proliferation (reviewed by[7]). Therefore, different kinds of serum are added, typically to 10% by volume. As primary cultures are primarily made from mice or rats, it would have been natural to utilize cognate serum (i.e. mouse or rat serum), but practical reasons have led to the use of serum from other sources, particularly fetal bovine serum (FBS) or newborn calf serum (NCS).

For adipocyte cultures in general, fetal bovine serum (FBS) has classically been the serum of choice. This choice is apparently due to early (incidental) reports with 3T3 adipocyte cell lines,[8,9] where it was found that FBS (rather than NCS) allowed for more efficient conversion of fibroblast-like precursors into lipid-filled adipocytes. More recent studies have similarly reported increased adipogenic conversion of 3T3-L1 cells cultured in FBS as compared to adult bovine serum.[10,11]

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Supplemental data for this article can be accessed here.

FBS is thus the most commonly used serum for most cell culture systems, and it is also the most common serum of choice for adipocyte cell line cultures. Probably therefore, it is often used even for primary brown adipocyte cultures (e.g. [12,13]). However, newborn calf serum (NCS) has also been used in several laboratories (including our own) for primary (brown and white) adipocyte cultures.[14-18]

Although fetal bovine serum and newborn calf serum are obtained from the same species, they may be considered different serum types. Serum is added primarily to promote cell proliferation, and serum obtained from the rapidly developing fetus may possess more proliferative capacity than bovine serum obtained after birth. However, promotion of proliferation is generally considered to suppress differentiation (and vice versa). Thus, optimizing proliferation may not be optimal to obtain the most advanced levels of differentiation.

In general, differentiation of adipocytes is followed morphologically. An adipocyte morphology ("adipose conversion"), with prominent lipid accumulation, is thus a measure of successful adipocyte differentiation. Classically, brown adipocytes and white adipocytes were considered closely related cell types, and adipose conversion was therefore considered a positive sign of differentiation even of brown adipocytes. However, in the early phases of differentiation, brown adipocytes express a series of myogenic genes, both transcription factors and structural proteins,[19] and the lineage of classical brown adipocytes is closely related to that of skeletal muscle.[20] Thus, acquisition of an adipocyte phenotype may not indicate progression to fully differentiated brown adipocyte characteristics. As the myogenic gene expression is transient during brown adipocyte development, myogenic genes cannot be chosen as differentiation markers. Rather, the ability to express thermogenesis-related genes may be considered signs of "brown adipose conversion"[21]; we here refer to this ability as "thermogenic competence". It should be noted that this ability is distinct from the actual expression of the genes, since thermogenic competence refers not to gene expression as such but to the ability to display a gene expression that is evoked acutely e.g. by stimulation with norepinephrine. It is thus also different from thermogenesis as such, as thermogenesis demands the prior expression of the thermogenic genes. Thermogenic competence thus basically reflects an "opening" of the thermogenic genes allowing them to respond to the relevant stimuli. Acquirement of optimal adipose conversion may accordingly not necessarily be paralleled by optimal thermogenic competence, and despite the general impression that FBS is more beneficial for adipose conversion than is NCS, it may be so that FBS is not optimal for thermogenic competence.

Therefore, to examine whether serum type (FBS versus NCS) can significantly affect the differentiation state of cultured primary brown adipocytes, we studied primary brown adipose cultures maintained in media containing 10% of either fetal or newborn bovine serum. To ensure that our conclusions were general for differences between FBS and NCS – and not simply batch differences – we studied different batches obtained from different providers. Our observations show that lipid accumulation and thermogenic competence are indeed consistently affected by serum type (with some batch-to-batch variation). Particularly, we find that the use of FBS versus NCS reduces thermogenic competence, i.e. the ability to express important thermogenesis-related genes.

Results

To examine whether consistent differentiation-promoting effects of fetal bovine versus newborn calf sera could be established, we acquired from different providers 5 batches of fetal bovine serum and 5 batches of newborn calf serum (NCS), identified with numbers (FBS &1-&5 and NCS &1-&5) as specified in Table 1. We found clear batch differences in the parameters followed (as shown in the figures) but we will concentrate here on general effects of the two serum types versus each other. To follow differentiation, we followed adipose conversion morphologically and measured the expression level of classical adipose-related genes. To identify the gain of thermogenic competence, we measured the expression level of accepted thermogenesis markers and we added known differentiation-promoting agents (rosiglitazone and norepinephrine) to the media and examined whether the ability of these inducers to promote thermogenic gene expression was differently affected by the two different serum types.

Differentiation in the absence of inducers

We first examined to what extent the differentiation process was affected by serum type when no differentiation inducer was added. Physiologically, this would resemble the differentiation state in brown adipose tissue in

Table 1. Serum information.

Indication in text	Vendor	Product no.	Lot no.
FBS &1	Sigma-Aldrich	F6178	14A173
FBS &2	BioWest	S1810	S11971S1810
FBS &3	Thermo Fisher Scientific	26140079	1495485
FBS &4	Sigma-Aldrich	F7524	011M3398
FBS &5	Sigma-Aldrich	F7524	014M3395
NCS &1	Thermo Fisher Scientific	16010159	6330496D
NCS &2	Thermo Fisher Scientific	16010159	1455367
NCS &3	GE Healthcare Hyclone	SH30118.03	APE21200
NCS &4	Sigma-Aldrich	N4637	15D230
NCS &5	BioWest	S0750	S10820S0750

animals where the tissue is not activated, i.e. in animals that are at thermoneutrality and are not obese or exposed to any diet that would activate the tissue.

Fetal bovine serum promotes lipid accumulation in brown adipocyte cultures

We obtained stromal-vascular cells from brown adipose tissue and followed their development in vitro. The cells were cultured in media each containing 10% of the different sera (Table 1) for 7 days; for technical reasons, morphology was only documented in 3 (out of the 5) sera of each type. We found that brown primary cells cultured in any batch of FBS tested contained larger lipid droplets than cells cultured in the NCS batches tested (Figure 1ABC versus DEF). To examine whether the appearance of large lipid droplets reflected totally higher lipid content or different propensities for lipid droplet coalescence, we measured total lipid content. Although the difference between the two serum types was not as marked as for the size of the lipid droplets, cells grown in FBS had increased levels of triglyceride storage as compared to NCS, as analyzed by quantification of Oil Red O staining (Figure 1G), a method that was developed to functionally quantify adipose conversion.[22] The total number of cells was largely unaffected by the choice of FBS or NCS, as indicated by quantification of DNA (Figure 1H), and the mean lipid content per cell thus tended to be higher in FBS than in NCS (Figure 1I).

Based on these data, particularly on the morphological appearance, it would traditionally be concluded that cells cultured in FBS were more differentiated than cells cultured in NCS.

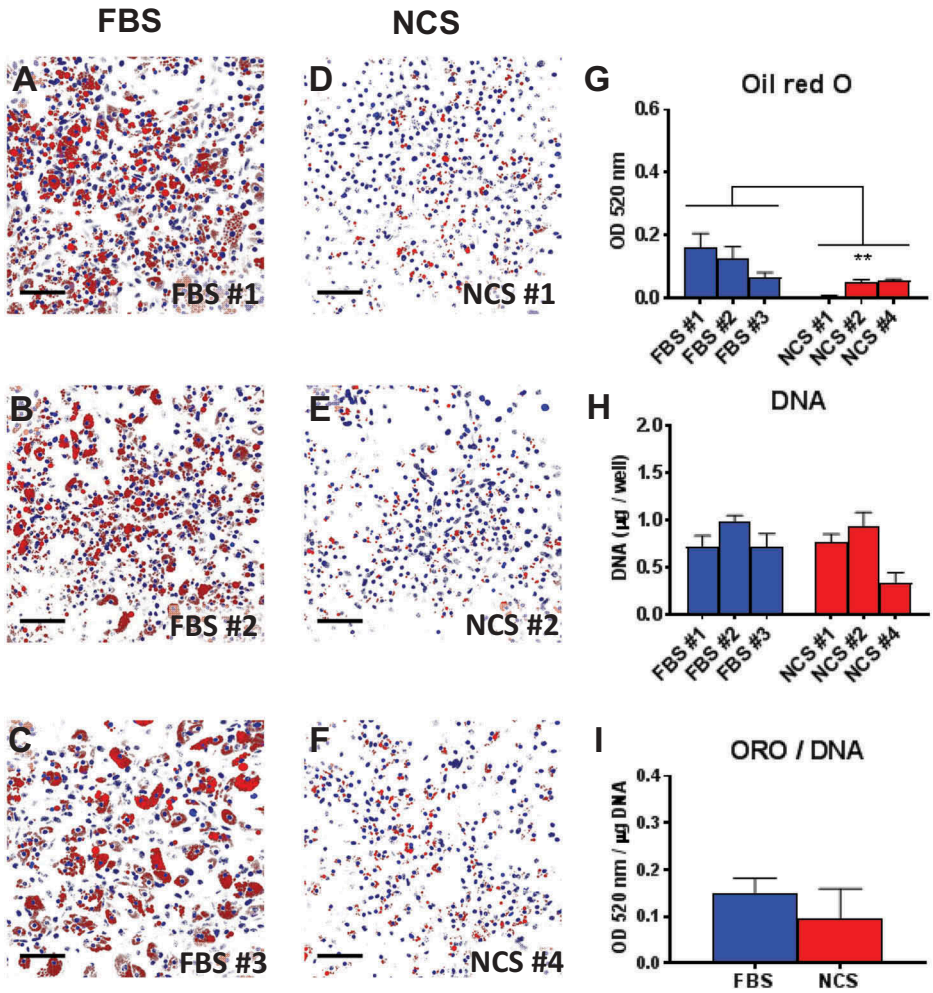

Figure 1. Fetal bovine serum (versus newborn calf serum) promotes lipid accumulation in brown adipose primary cultures. A-F: Confocal microscopy images of day-7 brown adipose primary cultures grown in the presence of the indicated batches of FBS (ABC) or NCS (DEF). Lipid droplets were stained with LipidTox (depicted as red), nuclei with Hoechst (blue). For clarity, background was rendered white. The scale bar is 75 µm. G: Quantification of Oil red O staining of cells grown in the presence of the indicated serum batches (n = 3 independent cultures for each serum). H: Quantification of DNA content in µg DNA/well. I: Mean FBS and NCS Oil red O quantification normalized per DNA content. For statistical comparison (by Student's *t*-test) in GHI, it was here considered that 9 cell cultures were grown with a batch of FBS or of NCS present. ** $P < 0.01$.

Despite promoting adipose conversion, fetal serum does not promote expression of general adipogenesis markers

To further characterize the effects of serum type on adipose conversion, we analyzed gene expression levels of two adipocyte markers in each of the 5 FBS and 5 NCS batches. One was *Pparg2* (Peroxisome Proliferator Activated Receptor gamma 2), a transcription factor closely related to adipocyte differentiation,[23] also discussed concerning brown adipocytes.[24] The other was *Fabp4* (fatty acid binding protein 4, also known as aP2).[25,26] Despite the clear difference in adipocyte appearance and lipid accumulation (Figure 1) and quite some variation in expression between serum batches (Figure 2A and C), *Pparg2* and *Fabp4* expression was not affected by serum type (Figure 2B and D). Thus molecularly, adipose conversion of primary brown adipose cultures – as indicated by *Pparg2* and *Fabp4* – was not affected by the type of serum (FBS or NCS).

Serum type does not consistently affect basal expression of genes related to thermogenesis in brown primary cultures

To examine brown adipose conversion, we analyzed the effect of each of the 5 FBS and 5 NCS batches on the expression levels of four genes generally related to brown adipocyte function.

PPARγ coactivator 1-alpha (*Pgc1a*)[27] expression was different in the different batches of the same type of serum (Figure 2E), but – despite this variation – there was a statistically significant indication that expression of *Pgc1a* was higher in the presence of NCS than in the presence of FBS (Figure 2F). The expression of Elongase of very long-chain fatty acids 3 (*Elovl3*)[28] was rather similar between cells cultured in FBS and NCS (Figure 2G), and the difference did not reach statistical significance (Figure 2H). Type 2 deiodinase (*Dio2*)[29] displayed less batch-to-batch variability and was overall expressed at higher levels in cells

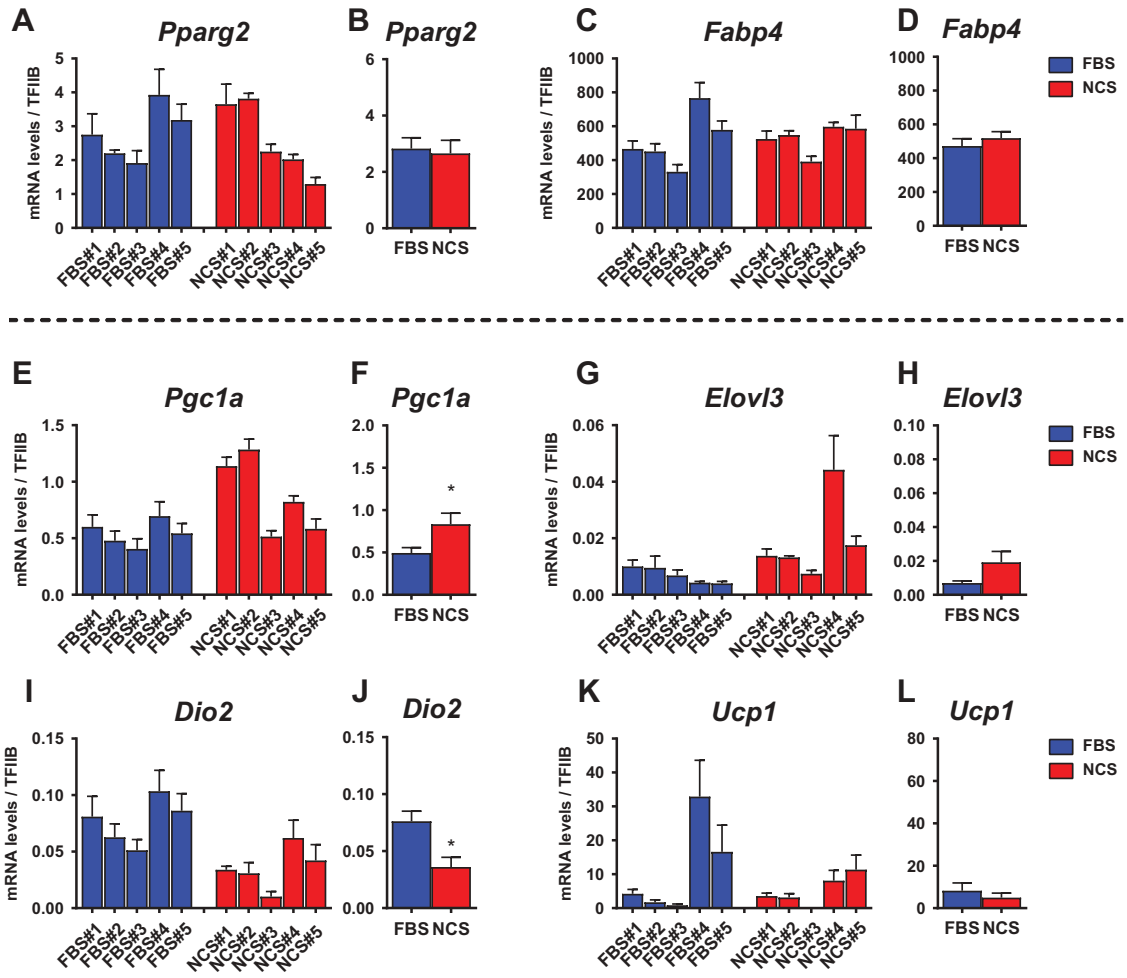

Figure 2. Basal gene expression in brown adipose primary cultures in various serum conditions. Brown primary adipose cultures were grown for 7 days in the indicated batches of FBS (blue bars) or NCS (red bars). Expression levels of the indicated genes are presented per individual serum batch (for each batch, n = 4–5 independent cultures) and as means of FBS versus NCS (B, D, F, H, J, L). Statistical significance was calculated on these mean values by Student's *t*-test, * $P < 0.05$, n = 5 (sera).

differentiated in the presence of FBS compared to NCS (Figure 2IJ). The main functional gene of brown adipocyte function, Uncoupling protein-1 (*Ucp1*),[30,31] displayed rather wide variation in expression between different batches of each serum type (Figure 2K). However, no consistent difference between FBS and NCS on basal *Ucp1* expression was observed (Figure 2L).

Thus, although morphological characteristics clearly pointed to FBS as the best inducer of adipose conversion, this observation was not paralleled with higher adipose gene expression; neither did the thermogenesis-associated genes systematically display expression patterns indicating that under these unstimulated conditions, FBS promoted cell differentiation in this important aspect.

Differentiation in the presence of the differentiation inducer rosiglitazone

A class of PPARγ-agonists, the thiazolidinediones, potently promote adipogenesis[32]; and also promote development of the brown phenotype in culture.[16,21,24,33,34] However, this type of inducer of differentiation does not activate thermogenesis as such. The physiological relevance of these inducers is not settled, but they may mimic processes taking place in brown adipose tissue during conditions such as prenatal differentiation of the tissue in precocial newborns and preparatory differentiation for hibernation in true hibernators.[16] We investigated whether the differentiation-promoting properties of the thiazolidinedione rosiglitazone was affected by serum type.

Effects of serum type on triglyceride storage remain apparent even in the presence of rosiglitazone

We treated the primary brown cell cultures in the different FBS and NCS batches with 1 μM rosiglitazone throughout the 7-day culture period. Similarly to the observations in cells grown in the absence of rosiglitazone (Figure 1A-F), lipid droplet formation was strongly enhanced in the presence of FBS as compared to NCS in the rosiglitazone-treated cells (Figure 3 ABC versus DEF).

From the quantification of Oil red O incorporation as a measure of triglyceride amount (Figure 3G) it was seen that rosiglitazone in any serum type promoted lipid accumulation (although not statistically significant in NCS). However, it was clear that the presence of FBS augmented rosiglitazone-induced lipid accumulation as compared to the presence of NCS, with an almost 3-fold higher total accumulation. Thus, cultured brown adipocytes treated with rosiglitazone stored more triglycerides in the presence of FBS than of NCS. Rosiglitazone also promoted cell proliferation (Figure 3H) but not to the same extent as it promoted lipid accumulation. This meant that the lipid amount per cell was increased due to rosiglitazone in the presence of FBS and that the cells became double as lipid filled in the presence of FBS than in the presence of NCS (Figure 3I).

Pparg2 expression remained generally unaffected by rosiglitazone treatment (Figure 4AB), and there was no effect of serum type. – We have earlier observed a reduction in *Pparg* expression in brown adipose cultures (cultured in NCS) upon chronic rosiglitazone treatment[16] but this effect is clearly batch-dependent (Figure 4A).

We observed a very consistent induction (2–3 fold) of *Fabp4* expression in response to rosiglitazone treatment (Figure 4CD). *Fabp4* expression has been reported to be induced upon rosiglitazone treatment in primary cultures from various adipose depots.[16,21,35] No differences between FBS and NCS were observed (Figure 4D).

Thus, again, the morphological difference in lipid accumulation consistently observed as a consequence of serum type was not consistently reflected in data for gene expression of adipose conversion-related genes.

Rosiglitazone-induced expression of thermogenesis-related genes is not consistently affected by serum type

We next analyzed whether serum type affected the rosiglitazone-induced expression of the genes indicative of brown adipocyte differentiation. Overall, rosiglitazone – as observed before[21] – increased *Pgc1a* mRNA levels, and the rosiglitazone-induced *Pgc1a* mRNA levels were significantly higher in cells cultured in NCS than in FBS (Figure 4F). *Elovl3*, which is highly induced by rosiglitazone treatment,[21] displayed substantial induction (at least 77-fold) in every serum batch analyzed (Figure 4G); no overall difference could be seen between FBS and NCS (Figure 4H). *Dio2*, highly induced in BAT in the cold,[36,37] displayed little change in response to rosiglitazone (Figure 4IJ).

Ucp1, significantly induced by rosiglitazone in all serum batches analyzed (Figure 4K), did not show an overall difference between FBS and NCS (Figure 4L). However, batch-to-batch variation was very apparent, resulting in as much as a 4-fold difference in the magnitude of the rosiglitazone-induced *Ucp1* expression between batches (Figure 4K).

Overall, no consistent effects of serum type on rosiglitazone-induced thermogenic gene expression were observed. Thus, although it could have been concluded from the lipid accumulation data that FBS augmented the

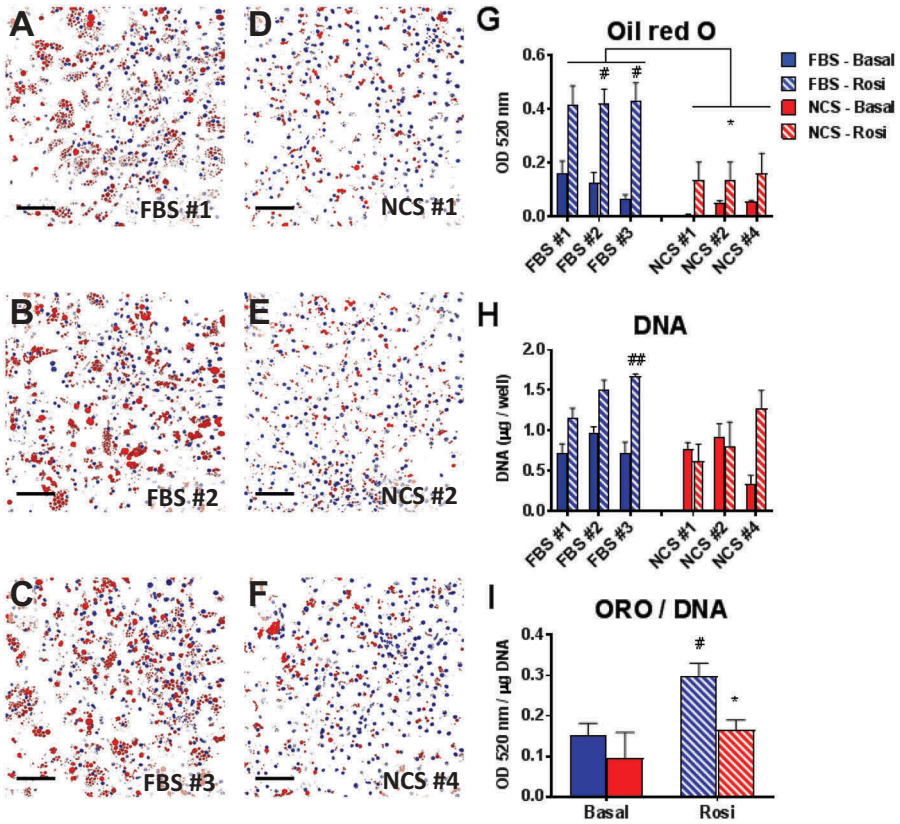

Figure 3. Lipid accumulation in brown adipose primary cultures with different serum types in the presence of rosiglitazone. A-F: Confocal microscopy images of brown adipose primary cultures differentiated for 7 days in 3 different batches of FBS (ABC) or NCS (DEF) in the presence of 1 µM rosiglitazone. As in Figure 1, lipid droplets were stained with LipidTox (red), nuclei with Hoechst (blue); background was rendered white. The scale bar is 75 µm. G: Quantification of Oil red O staining of cells grown in the presence of the indicated serum batches, basal values in GHI are those from Figure 1 (n = 3 independent cultures for each serum). H: Quantification of DNA content in µg DNA/well. I: Mean FBS and NCS Oil red O quantification per DNA content. Statistical significance of rosiglitazone-effect in individual batches (G-H) was calculated by Student's *t*-tests. In G, the overall effect of FBS vs NCS in the presence of Rosi was calculated by Student's t-test (3 vs 3). Statistical significance of differences between mean values (I) was calculated by Student's *t*-tests. * indicates serum-effect; & indicates rosiglitazone-effect. */& $P < 0.05$, && $P < 0.01$, &&& $P < 0.001$. In GH, statistical significance of rosiglitazone-effect in individual batches (GH) was calculated by multiple *t*-tests using the Holm-Sidak method for multiple comparisons; & $P < 0.05$, && $P < 0.01$. Statistical differences in I were determined by Student's *t*-test (n = 3 means for each serum type). * $P < 0.05$, *** $P < 0.001$ for effect of serum, & $P < 0.05$ for effect of rosiglitazone.

differentiation-inducing effect of rosiglitazone (Figure 3), the rosiglitazone-induced expression levels of thermogenic genes were actually not augmented in FBS (Figure 4).

Differentiation in the presence of the differentiation inducer norepinephrine

Physiologically, it is believed to be primarily norepinephrine that induces expression of the thermogenesis-related genes in brown adipose tissue.[38] Thus, culturing brown adipocytes in the absence of inducers presumably reflects the conditions in vivo in animals at thermoneutrality and in the absence of diet-induced thermogenesis, and the acute addition of norepinephrine to these cultures should reflect the activation of the tissue that is induced by acute cold[38] or by meals.[39]

Acute norepinephrine effects on adipogenesis-related genes

For investigation of the acute effects of norepinephrine, the cultures were treated for only 2 hours and therefore did not display marked differences in morphological appearance compared to untreated cells (as those shown in Figure 1) (therefore not shown). In cells grown in FBS-containing media, *Pparg2* expression was consistently unaffected by acute NE treatment (Figure 5A). However, in the presence of NCS, norepinephrine generally did induce a downregulation of *Pparg2* expression (Figure 5AB), in agreement with earlier reports.[16,40] Norepinephrine only slightly decreased *Fabp4* expression in cells cultured in either serum type (Figure 5D). Thus, in general, norepinephrine decreases the propensity of cells to display adipocyte characteristics.

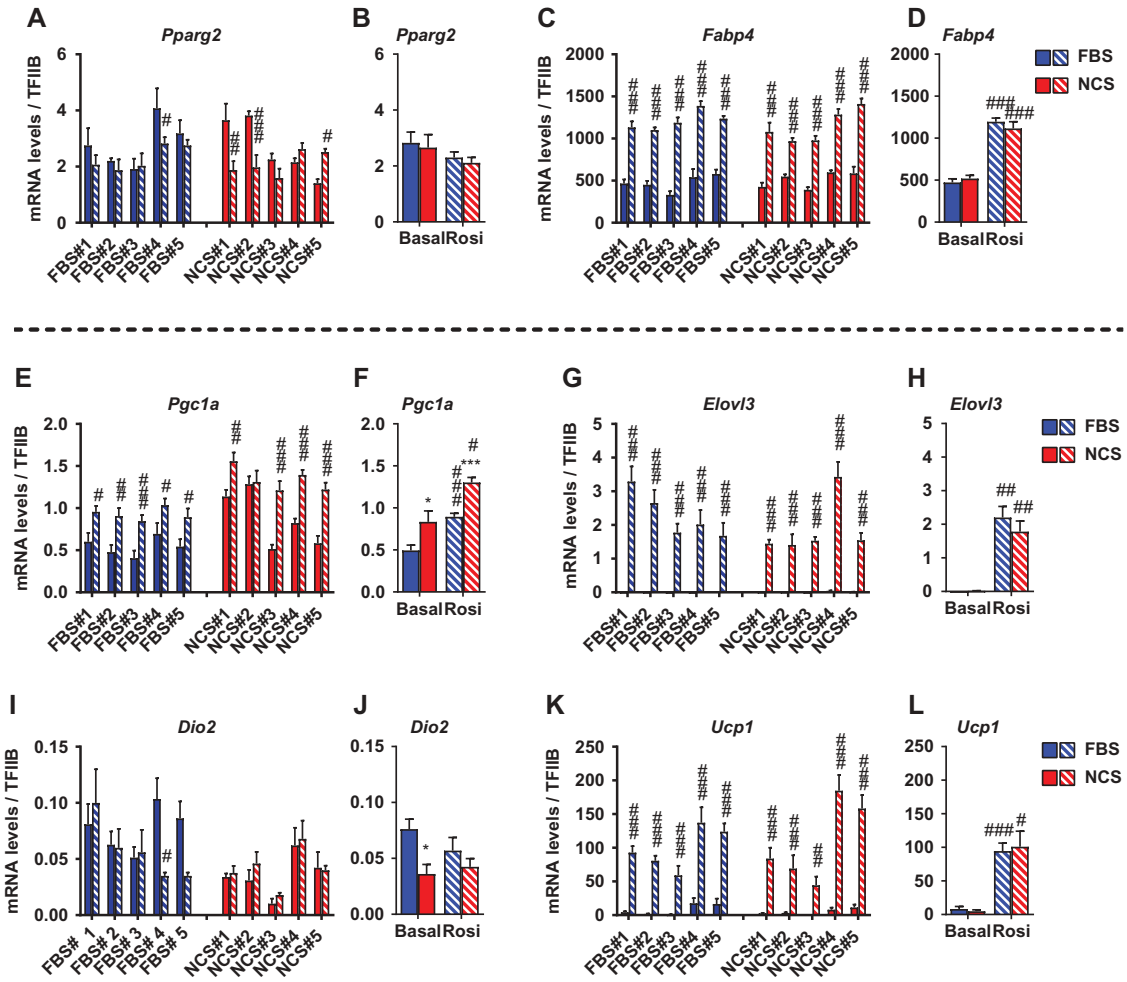

Figure 4. Rosiglitazone-induced gene expression in brown adipose primary cultures in different serum type conditions. Brown adipose primary cultures were differentiated for 7 days in 5 different batches of FBS (blue bars) or NCS (red bars) in the presence of ethanol (Basal, solid bars, same data as presented in Figure 2) or 1 μM rosiglitazone (Rosi, hatched bars). Expression levels of the indicated genes are presented per individual serum batch (n = 4–5 independent cell cultures) and as means of FBS and NCS. Note that in G, the control values are so low compared to the rosiglitazone-induced values that they are not visible in this representation (cf. Figure 2G). Statistical significance of rosiglitazone-effect in individual batches was calculated by multiple *t*-tests using the Holm-Sidak method to correct for multiple comparisons. Statistical significance of differences between mean values was calculated by Student's *t*-tests. * indicates serum-effect; & indicates rosiglitazone-effect. */& $P < 0.05$, && $P < 0.01$, ***/&&& $P < 0.001$.

The development of thermogenic competence

In culture, the differentiation of brown adipocytes is characterized by a successive manifestation of the ability to express thermogenesis-related genes, particularly *Ucp1*, as an effect of acute stimulation with norepinephrine or similar adrenergic agents.[41] The *Ucp1* gene thus does not become spontaneously active during differentiation, and the acquirement of this thermogenic ability is only manifested after norepinephrine stimulation. We refer to this "concealed" alteration in gene expression control as acquirement of "thermogenic competence". We examined whether serum type affected the occurrence and magnitude of this thermogenic competence, i.e. to which degree norepinephrine could induce gene expression after maturation of the cells in the two serum types.

In general, we found that the brown adipocytes – irrespective of serum type – had consistently acquired thermogenic competence. Thus, we saw large norepinephrine-induced increases in *Pgc1a* expression (Figure 5EF) (principally in agreement with earlier observations[16,27]), in *Elovl3* expression in the NCS serum types (Figure 5GH) (in agreement with[28]), in *Dio2* expression (Figure 5IJ) (in agreement with[42]) and in *Ucp1* expression (Figure 5KL) (in agreement with[41] and many others). However, the magnitude of the norepinephrine-induced gene expression was markedly higher in brown adipocytes that had differentiated

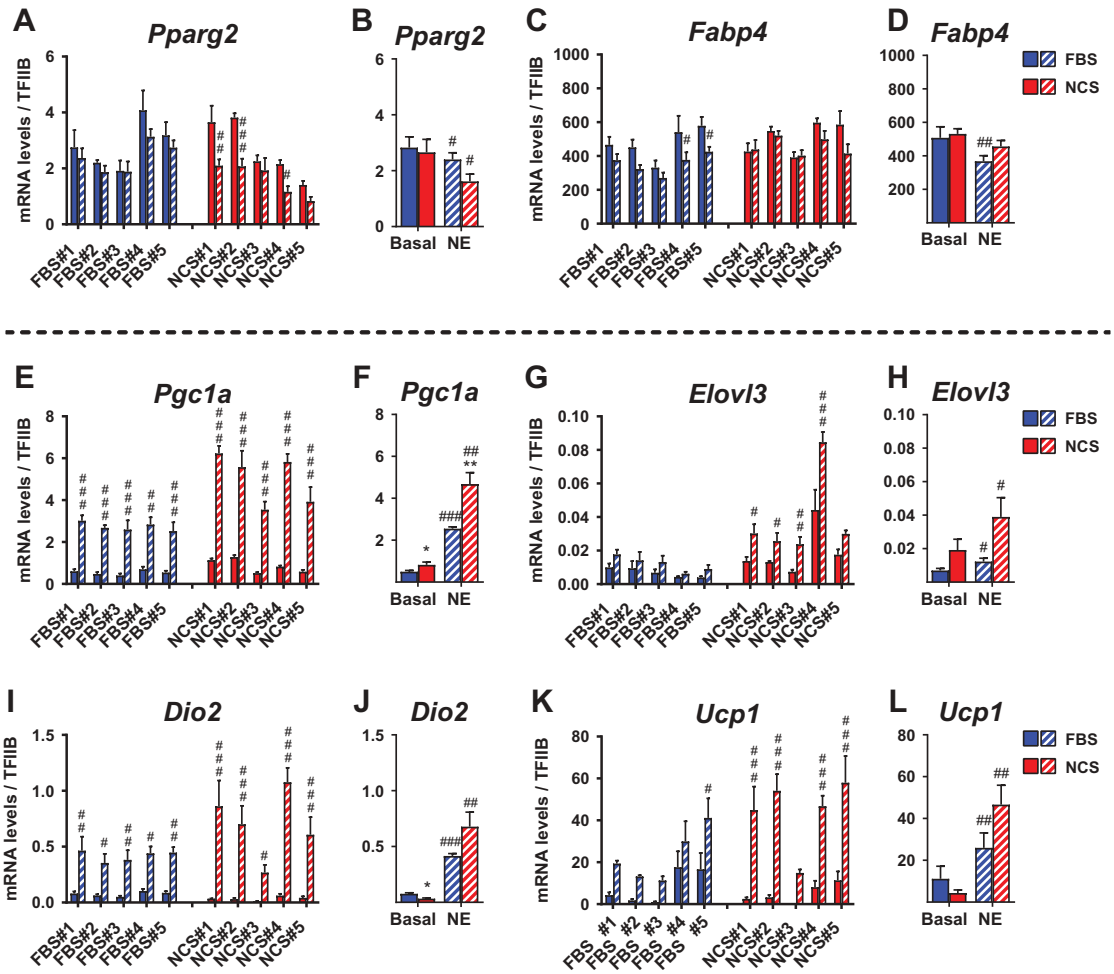

Figure 5. Norepinephrine-induced gene expression in brown adipose primary cultures in different serum type conditions. Brown primary adipose cultures grown for 7 days in the indicated batches of FBS (blue bars) or NCS (red bars) were treated for 2 h with either water (Basal, solid bars, same as presented in Figure 2) or 1 μM norepinephrine (NE, hatched bars). Expression levels of the indicated genes are presented per individual serum batch (A, C, E, G, I, K; n = 4–5) and as means of FBS and NCS (B, D, F, H, J, L) (n = 5 different batches of each serum type). For statistics see legend to Figure 4. * indicates serum effect; */& indicates NE effect. & $P < 0.05$, **/&& $P < 0.01$, &&& $P < 0.001$.

in NCS than in FBS (Figure 5FHJL). Thus, clearly, the cells in the NCS sera had proceeded further in acquiring thermogenic competence than those in FBS. Concerning *Ucp1*, it may also be noted that the fold-increase over basal was robustly higher in cells cultured in NCS than in FBS (11-fold and 2-fold, respectively).

We additionally examined whether this effect was an acute effect of the presence of a particular serum type specifically during the norepinephrine-induced *Ucp1* gene expression time period or whether the effect had become persistent in the genome. For this, we switched the serum type used during the norepinephrine stimulation to the opposite type. As seen (Figure 6A), it was not the acute presence of either serum that affected thermogenic competence but thermogenic competence was a property acquired before the brief period of norepinephrine stimulation.

Thus, the overall NE-induced expression of genes related to cellular thermogenic competence was consistently more pronounced in brown adipocytes that were cultured in the presence of NCS as compared to FBS.

Newborn versus fetal serum promotes a brown versus a white adipocyte phenotype

For in vitro studies of the acquisition of thermogenic ability, transition from albeit mature but unstimulated brown adipocytes (as in Figure 1) to brown adipocytes that strongly express thermogenic genes after norepinephrine stimulation is a very relevant condition. It may be noted from the above that the effect of norepinephrine is not only to induce thermogenic gene expression but also to somewhat repress conventional adipocyte markers. Therefore, it may be considered

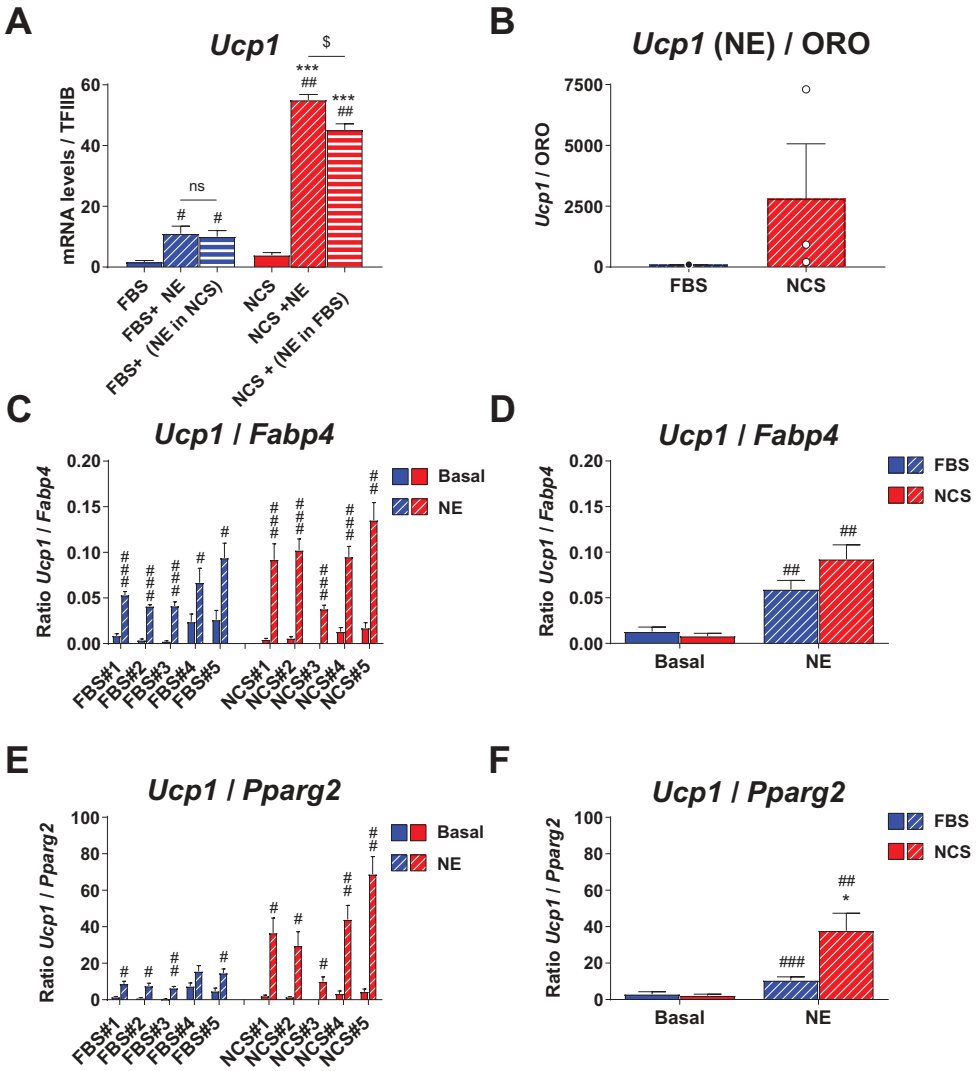

Figure 6. Promotion of thermogenic competence versus adipose conversion by NCS versus FBS. A: Effects of acute serum change on *Ucp1* gene expression. Brown adipocytes were grown for 7 days as above, in either FBS &2 or NCS &2. On day 7, the cultures were treated as follows: with fresh medium (same as they had had before) and treated with norepinephrine or water for 2 h, or changed into the opposite medium and treated with norepinephrine for 2 h (all cultures were washed twice in the medium they were to receive); n = 4 independent experiments. & and && indicate the effect of norepinephrine treatment ($P < 0.05$ and 0.01), *** the effect of chronic serum type ($P < 0.001$), and $ and ns the effect of acute change of serum type ($P < 0.05$ and > 0.05); Student's *t*-tests. B: Promotion of thermogenic competence (as norepinephrine-induced *Ucp1* gene expression) versus adipose conversion in FBS versus NCS; n = 3 (adipose conversion from Figure 1 and the corresponding *Ucp1* gene expression data from Figure 5K). Individual serum batch results are indicated as circles. CD: Promotion of thermogenic competence versus adipose conversion (as norepinephrine-induced *Ucp1* gene expression divided by *Fabp4* gene expression after norepinephrine treatment) in FBS versus NCS; data from Figure 5K divided by the corresponding data in Figure 5C. & indicates effect of norepinephrine treatment. & $P < 0.05$; && $P < 0.01$; &&& $P < 0.001$. EF: As CD, except that the adipose conversion was followed as *Pparg2* gene expression (Figure 5A). & indicates effect of norepinephrine treatment; & $P < 0.05$; && $P < 0.01$; &&& $P < 0.001$.* indicates effects of serum type ($P < 0.05$). In CE, statistical significance of norepinephrine-effect in individual batches was calculated by multiple *t*-tests using the Holm-Sidak method to correct for multiple comparisons. In DF, statistical significance of differences between mean values was calculated by Student's *t*-test. & $P < 0.05$, **/&& $P < 0.01$, ***/&&& $P < 0.001$.

whether indices of brown adipocyte conversion should not only include enhanced thermogenic gene expression but also encompass the effect of adrenergic stimulation on adipogenesis-related genes.

In Figure 6B-F we have therefore constructed such indices, all based on *Ucp1* gene expression but expressed relative to different adipogenic markers. This includes total lipid content (Figure 6B), *Fabp4* gene expression (Figure 6CD) and *Pparg2* gene expression (Figure 6EF). From this compilation, it becomes very apparent that NCS is superior to FBS in promoting the differentiation of brown adipocytes

in culture towards a thermogenically competent brown phenotype.

Discussion

For examination of the possibilities to modify the thermogenic capacity of brown adipocytes in in vitro systems, it is of paramount importance that the cell culture conditions used allow for optimal manifestation of the brown adipocyte phenotype. Many technical and biological factors may affect the outcome of such cell culture experiments (temperature, partial oxygen pressure etc.). We examine here the significance of two serum types for the acquirement of the brown phenotype. We find that in accordance with general acceptance and routine procedures, the use of fetal bovine serum (FBS) promotes morphological adipose conversion, even in cell cultures developed from precursors obtained from classical brown adipose tissue depots. However, we find that the use of newborn calf serum (NCS) is superior to the use of FBS for promoting the development of the brown phenotype and the acquisition of thermogenic competence in these cells. These observations can be of importance for giving an understanding of brown adipocyte development and for examination of possibilities to promote brown adipocyte differentiation for therapeutic purposes. The observations also point to the existence of serum-type specific but distinct innate external factors (in the sera) and internal factors (in the cells) that constitute and convey the effects of the serum types.

Fetal bovine serum promotes adipose conversion

The broad use of FBS (fetal bovine serum) in adipose cell culture media appears to have resulted primarily from observations made in 3T3 adipose cell cultures in which FBS was reported to promote adipose conversion to a greater extent than did newborn calf serum (NCS).[8,9] Only one batch of each serum type was compared in each of those studies. To be able to draw conclusions about the effects of one serum type compared to another (e.g. newborn versus fetal), it is important to compare multiple serum batches, as done here. We here show that serum type consistently batch-independently did affect adipose conversion (lipid accumulation) of primary brown adipocytes (Figure 1). As lipid accumulation is often considered a measure of adipose conversion, this can lead to the conclusion that fetal serum more potently enhances this process than does newborn serum. However, since expression levels of *Pparg2* and *Fabp4* – two well-established markers of adipocyte differentiation – did not overall depend on serum type, adipose conversion molecularly was not affected by the type of serum. In the presence of the thiazolidinedione rosiglitazone, FBS was still associated with enhanced lipid accumulation (Figure 3), despite the fact that serum type again did not affect expression of adipocyte genes (Figure 4). Thus, there is no simple relationship between promoting the morphological features of lipid accumulation and promoting inherent adipocyte conversion.

From the present studies, it is not possible to specify the nature of the lipid-accumulation-promoting effect of FBS. Basically, it could either be that any serum type affects the expression of genes related to the formation of lipid droplets, or that FBS includes more triglyceride precursors, and cellular lipid droplets are then more easily formed than in the presence of NCS.

Newborn calf serum promotes thermogenic competence

A commonly used measure to define the functional potential (thermogenic competence) of adipocytes is by analyzing their response to norepinephrine.[41] Norepinephrine acutely induces the expression of genes involved in brown adipocyte thermogenic function. As shown here, primary brown adipocytes cultured in NCS display higher levels of norepinephrine-induced thermogenesis-related genes than when cultured in FBS (Figure 5E-L). One earlier report showed a similar positive effect of a calf serum (as compared to a fetal serum) on norepinephrine-induced *Ucp1* expression in cultured brown adipocytes.[43] At the same time, NCS promotes the ability of norepinephrine to somewhat decrease the expression of adipose-related genes (Figure 5A-D). Thus, NCS in general directs brown adipocytes in culture towards their cognate phenotype. Again it should not be surprising that acquirement of thermogenic competence is not paralleled by promotion of adipose conversion, as the brown adipocytes studied here are of a different lineage from those giving rise to white adipocytes and are more related to muscle cells.[19,20,44,45]

Serum as a source of regulators of brown adipocyte formation and function

In our observations, fetal serum consistently is less potent for the induction of thermogenic competence characteristics in brown adipocytes than is newborn serum. This difference could be the result of the presence of stimulatory factors in NCS or inhibitory factors in FBS. Identification of such factors (as has been done for white adipocytes[10,11,46,47]) and of the

substances with which they interact on the target cells, can further our understanding of how circulatory factors influence brown adipose development and function. However, stimulatory factors putatively present in NCS may impede the identification of additional stimulatory factors, as differentiation may be already advanced. It may concordingly particularly be pointed out that when FBS is used, factors that have been suggested to promote thermogenic competence in brown adipocytes may essentially not do this but may rather be inhibitors of the factors in the FBS that counteract the acquirement of the brown phenotype. Thus, only by promoting non-inhibitory conditions for brown adipocyte cultures can genuine pro-thermogenic factors be identified.

Materials and methods

Cell culture

The experiments were approved by the Animal Ethics Committee of the North Stockholm region. Male Naval Medical Research Institute (NMRI) mice of 3–4 weeks of age (purchased from Scanbur, Sweden) were used. The mice were acclimated in our facility for at least 24 h at room temperature on a 12:12-h light-dark cycle with free access to chow food (Labfor R70; Lantmännen, Södertälje, Sweden) and water. The mice were then euthanized using CO_2, followed by cervical dislocation. The interscapular (iBAT), cervical (cBAT) and axillary brown adipose tissue (aBAT) depots were pooled (see description in[35]). Cells were prepared for cell culture as described in.[16] Prior to seeding, cells were resuspended in DMEM (Sigma-Aldrich, D6429) and then seeded in wells with pre-warmed (37°C) medium containing the specified sera (Table 1). Full medium formulation was as follows: DMEM (Sigma-Aldrich, D6429) supplemented with 10 mM HEPES (Sigma-Aldrich, H0887), 4 mM glutamine (Sigma-Aldrich, G7513), 50 U/ml penicillin, 50 µg/ml streptomycin (Sigma-Aldrich, P0781), 25 µg/ml sodium L-ascorbate (Sigma-Aldrich, A4034), 3 nM insulin (Sigma-Aldrich, I9278) and 10% (v/v) of the specified serum. Medium was changed on the day after seeding and then on day 3 and 5. Cells were treated from seeding either with ethanol (basal control; final concentration 0.5%) or 1 µM rosiglitazone (Enzo Life Sciences, ALX-350–125) dissolved in ethanol (to same final concentration as controls). Where indicated, on day 7 (seeding is considered day 0), cells were treated for 2 h either with water (control) or 1 µM norepinephrine (Sigma-Aldrich, A9512). In a separate experiment (Figure 6A), on day 7, medium was changed either from FBS to NCS or from NCS to FBS (cells were washed twice in the new medium) directly before being treated for 2 h with 1 µM norepinephrine. After treatment, cells were harvested in TRI Reagent (Sigma-Aldrich, T9424) and kept at −80°C until further processing.

Microscopy

For microscopy purposes, cells were grown on glass coverslips (Thermo Fisher Scientific, 1014355118NR1). Differentiated cells (day 7) were washed with PBS and fixed for 10 min with 4% paraformaldehyde solution. Cells were stained for 30 min with LipidTox (Life Technologies, H34475) (1:200 in PBS) and for 10 min with Hoechst 33,258 (Sigma-Aldrich, B2883) (5 µg/ml in PBS). Cells were washed with PBS before and after the staining steps and then mounted in ProLong Gold antifade reagent (Invitrogen, P36934) onto microscopy slides (Solveco, 1212). Cells were photographed in a Zeiss LSM 780 confocal microscope with a 20x objective. Image background was changed from black to white in Adobe Photoshop CS4 using the "replace color" option.

Oil red O quantification

Differentiated cells (day 7) were washed with PBS and fixed for 30 min in 4% paraformaldehyde. Cells were then washed with 60% isopropanol and stained with Oil red O (Sigma-Aldrich, O0625) solution for 15 min. Cells were then washed with tap water until the wash-off was clear. The Oil red O stain was extracted with 100% isopropanol (750 µl per well in a 24-well plate) for 10 min. Absorbance of the extracts was measured in an EnSpire® plate reader (Perkin-Elmer) at 520 nm. Values were corrected for the background signal and for the signal obtained from Oil red O extracted from an empty well in the cell culture plate.

DNA quantification

Differentiated cells (day 7) were washed with PBS, and the culture plates were frozen until further processing. Cells were lysed in lysis buffer (0.5% SDS, 0.1 M NaCl, 50 mM Tris/base, 2.5 mM EDTA) with 1 mg/ml Proteinase K (Sigma, P6556) at 55°C for 3 h. Lysate was centrifuged for 15 min at 19 900 x g to remove debris. An equal volume of 100% isopropanol was added to the supernatant. Samples were centrifuged for 15 min at 19 900 x g at 4°C. The pellet was washed

Table 2. Primer sequences.

Gene	Forward (5′–3′)	Reverse (5′–3′)
Dio2	CTGCGCTGTGTCTGGAAC	GGAATTGGGAGCATCTTCAC
Elovl3	GCCTCTCATCCTCTGGTCCT	TGCCATAAACTTCCACATCCT
Fabp4/aP2	CGCAGACGACAGGAAGGT	TTCCATCCCACTTCTGCAC
Pgc1a	GCCTGCAGGAACTTATCAGC	GTAAATCACACGGCGCTCTT
Pparg2	GCATGGTGCCTTCGCTGA	TGGCATCTCTGTGTCAACCATG
TFIIB	TGGAGATTTGTCCACCATGA	GAATTGCCAAACTCATCAAAACT
Ucp1	GGCCTCTACGACTCAGTCCA	TAAGCCGGCTGAGATCTTGT

in 75% ethanol, and DNA was then dissolved in 8 mM NaOH. Aliquots of this were incubated in TNE buffer (10 mM Tris base, 1 mM EDTA, 0.2 M NaCl) with 100 ng/ml Hoechst 33,258 (Sigma-Aldrich, B2883) for 15 min in the dark. Fluorescence was measured in an EnSpire® plate reader (PerkinElmer) at excitation wavelength 354 nm and emission wavelength 480 nm. A standard curve of calf thymus DNA was used to determine DNA concentrations.

Gene expression analysis

RNA was isolated using the chloroform-isopropanol method (according to the TRI Reagent manufacturer's (Sigma-Aldrich) instructions). 500 ng total RNA was reverse-transcribed with the High Capacity cDNA kit (Life Technologies, no. 4368814) in a total volume of 20 µl. Gene-specific primers (listed in Table 2) were mixed with SYBR Green JumpStart Taq Ready Mix (Sigma-Aldrich, S4438) to a final concentration of 0.3 µM. 2 µl aliquots of 10 times diluted cDNA were added to 11 µl of SYBR Green/primer mix. qPCR reactions were run in triplicate. Thermal cycling conditions were 2 min at 50°C, 10 min at 95°C, and 40 cycles of 15 s at 95°C and 1 min at 60°C, followed by melting curve analysis on a Bio-Rad CFX Connect Real-Time system. The ΔC_t ($2^{-\Delta Ct}$) method was used to calculate relative changes in gene expression. TFIIB was used as a reference gene to adjust for variability in cDNA synthesis. TFIIB expression data are presented in Figure S1. None of the variation observed in TFIIB expression affected any of the conclusions.

Acknowledgments

B.C. and J.N. are members of the COST Action MITOEAGLE. The authors would like to thank Dr. David Herron for initial observations.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

Supported by grants from the Vetenskapsrådet (SE) (the Swedish Research Council).

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