

Manuscript EMBO-2011-78968

UCP2 regulates energy metabolism and differentiation potential of human pluripotent stem cells

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Review timeline:

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|-----------------------------------|-------------------|
| Submission date: | 27 July 2011 |
| Editorial Decision: | 05 September 2011 |
| Additional Author Correspondence: | 22 September 2011 |
| Editorial Correspondence: | 23 September 2011 |
| Revision received: | 12 October 2011 |
| Accepted: | 14 October 2011 |

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision

05 September 2011

Thank you very much for submitting your research paper proposing UCP2 is modulator of ES-cell metabolism and thus potential regulator of cellular proliferation and differentiation for consideration to The EMBO Journal editorial office.

I received comments from three expert scientists that appreciate the potential (!) novelty and impact of your study. However, they also outline major shortcomings related to functional causality and mechanistic insight into UCP2's presumptive role that essentially preclude publication of this rather preliminary dataset considering the expectations of a more general and strongly molecular-oriented title such as that of The EMBO Journal.

With the challenging demands to mechanistically and causally connect the metabolic with the differentiation part of the study, we are also unable to invite a straightforward and timely limited round of revision, as the outcome of necessary experimentation remains currently uncertain. We would thus understand if you might find it easier to seek more rapid publication elsewhere.

However and recognizing the potential of the study, we would be prepared to assess a significantly improved version at a later time and in case you were able to satisfactorily address these concerns. In case you might entertain such an option, I do urge you to take the referee demands seriously to avoid disappointments much later in the process.

Please do not hesitate to contact me in case of further questions or indeed outlining possible experiments and timeline in case you plan to resubmit (preferably via E-mail).

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS:

Referee #1:

This is a very interesting manuscript and study. It is proposing attractive hypothesis that UCP2 is modulating metabolism and thereby also modulating cell proliferation and differentiation. However, there is a general disconnect between the metabolism part of the study and the differentiation. It is not clear that UCP2 is mediating any of its effects on cell differentiation or proliferation through altering metabolism.

The authors may want to expand on the mechanism by which UCP2 alters metabolism and cell differentiation/proliferation. Previous studies have shown that UCP2 modulates cell differentiation and proliferation, and ES cells have been shown to downregulate glycolysis during differentiation.

This is a large and complex study to review and I apologize to the authors if I missed any important point.

The effect of oligomycin on ATP in the cells is not acceptable as an indication for the source of ATP. The reason stems from the capacity of the cells to compensate by generating ATP from other sources.

There is also a technical issue: the authors have to demonstrate that oligomycin 0.1 μ M was sufficient to completely block mitochondrial ATP synthesis in each of the experimental groups. Partial blockade can produce an artifact where the permeability of oligomycin to the cell impacts the results more than the effect on mitochondria. Therefore, a dose response is required to demonstrate this point.

Figure 3E. I am worried that the PSCs are decaying fast in the respirometry experiment. The post oligomycin respiration is not stable and this can explain the inability to produce FCCP effect. It is not clear why all groups had identical OCR at time=2-31 minutes if differentiated cells were found to consume more oxygen and be dependent on glycolysis. Also, it is not clear if this graph is showing 3 separate experiments of 3 separated cell preparations, or is it showing replicates of wells in the Seahorse XF plate. The FCCP dose response in the supplement was done in the absence of oligomycin and therefore also raises suspicion that these cells are decaying.

The authors indicate reverse complex 5 activity is there to prevent mitophagy of mitochondria that would otherwise lose their potential. The paper cited in the manuscript stresses that this mechanism is true only in cells over expressing Parkin or cells that have high levels of endogenous Parkin. It will be valuable if the authors can cite previous studies that show that PSCs express Parkin or provide a western blot analysis for Parkin.

I am afraid I cannot agree with the interpretation and conclusion that PSC cells run a reverse ATPase activity to energize their mitochondria. Failure to depolarize with antimycin is not an indication that mitochondria charge their membranes through reverse activity of ATP synthase. It is, however, suggesting that in the presence of such toxin, these cells have the capacity to use ATP synthase as ATPase to pump protons out. This is not unique to PSC cells. It is a very common feature in various tissues. The effect of IF1 over expression in the presence of KCN is supporting the claim that when exposed to such toxin the cells become dependent on ATPase activity of complex 5. Thus the conclusion that can be justified per the data is that these cells have reduced IF1 activity.

The result shown in 4E and F is interesting, but could be completely attributed to other effects of IF1 (See Campanella et al. 2008; and 2009). For example IF1 over expression was shown to reduce ROS production, which in turn would reduce proliferation rate.

Figure 4A shows membrane potential data with JC1. I am afraid that although Invitrogen markets JC1 as a membrane potential dye, the scientific community does not accept it as so. JC1 is not a proper dye for membrane potential and it is a consensus within the mitochondria field that JC1 data cannot be used to draw conclusions.

Figure 6 and 7. The results show that UCP2 has an effect on cell differentiation and proliferation. There are examples for that in the literature. (See Chen X et al JBC 2009 PM:19073597 for muscle differentiation, and Elorza et al JBC 2008 for erythroid differentiation). This can be due to UCP2 effect on ROS, not on glucose metabolism. The authors should address the possibility that UCP2-differentiation axis may contribute the effect at 6 days of RA. That means, the effect on differentiation lead to the observed effect on metabolism.

However, the result in day 0 is surprising and interesting. The pentose phosphate pathway is a strong responder to ROS due to its role in glutathione metabolism. Thus, the authors should address the possibility that in the absence of UCP2 there is more ROS and increased need to produce and reduce glutathione, leading to increased flux through the pentose phosphate pathway.

The authors demonstrate that O₂ consumption of the hPSC (per mitochondrial mass) is similar to the differentiated cells. This seems to be inconsistent with UCP2 effect (which is not similar between hPSC and differentiated cells) which should be effective even when normalized to mitochondrial mass, unless the authors interpretation is that UCP2 expression alter genes that modulate extra-mitochondrial metabolism. In other words, if this was all about UCP2, one would expect that normalization to mitochondrial mass will still maintain the difference. This is not supporting the study hypothesis.

The authors should acknowledge that stem cell differentiation was shown to be associated with reduced glycolytic activity (See Chung S. 2009 PM:20045004).

I am also surprised that the authors did not mention the finding by Schieke SM et al. JBC 2008 that have demonstrated that ES cells differ in their capacity to differentiate based on mitochondrial metabolism. Within the ES cell population, the cells that can be mobilized to differentiate have increased mitochondrial oxidative capacity.

Minor:

The authors should not use the term "per mitochondrion" when normalizing to mitochondrial mass

Referee #2:

This is an important paper showing that the transition from undifferentiated to differentiated cells is at least partially controlled by the metabolic state of mitochondria. The stem cells are shown to contain mitochondria, whose membrane potential is sustained by ATP from glycolysis. As long as mitochondria are unable to oxidatively metabolise glucose, these cells rely on glycolysis and remain undifferentiated. The key finding is the identification of UCP2 as the key component regulating the mitochondrial state and thus glucose metabolism. UCP2 is critical for maintaining the glycolysis and thus the undifferentiated state.

The findings and conclusions are very well supported with a plethora of experimental data. The paper is well written, but sometimes too condensed vis-avis the large spectrum of data shown. The authors avoid discussing the important but treacherous question by what mechanism UCP2 causes the preference for glycolysis. The referee thinks that their data are consistent with a regulated uncoupling and concomitant decrease of membrane potential by UCP2. The difference to UCP1 may reside in the way how the uncoupling, i.e. H⁺ transport is regulated.

Referee #3:

General:

In this paper the groups of Koehler and Teitell combine efforts and show that human pluripotent stem cells (hPSCs) in culture mainly rely on glycolysis, although they contain functional mitochondria. They conclude this from comparing extensively the metabolic phenotype of several stem cells model lines to fibroblasts as well as to cells differentiated from these hPSC lines. Manipulating the expression levels of UCP-2 shows that UCP-2 expression in such stem cells seems to be important for this specialized metabolic phenotype.

This is a very extensive and experimentally well done paper. Very recently, the metabolism of stem cells within tissues, but also in cell culture, has become a true hot topic in the field. The results are novel and highly interesting to a broad community of researchers interested in stem cell biology as well as mitochondrial metabolism.

Unfortunately, the authors are unable to come even close to a mechanism which would answer the question how UCP-2 shifts glucose metabolism from oxidation to CO₂ to conversion to lactate. Also, although UCP-2 seems to be truly important, other mechanisms are equally likely and have to be seriously considered. E.g. the group of T. Suda has shown, using k.o. mice, that it is the constitutive expression of hypoxia inducible factor HIF-1 α which drives a similar phenotype of hematopoietic stem cells in vivo. Also, there is a huge amount of literature on the Warburg effect in tumor cells, which have been shown to possess this metabolic phenotype in the 1920s, and inactivation of pyruvate dehydrogenase by modulating its kinase or binding of hexokinase to mitochondria are just two examples how this might be achieved. All this has to be considered and at least be discussed.

Major Points:

1. One important problem is that the differences explaining the metabolic phenotype, i.e. the differences between hPSCs, hESCs and hiESCs, which are all used in this paper, compared to more differentiated cell types are very moderate. This is true when it comes to differences in oxygen consumption, lactate production, UCP expression, effect of the lactate dehydrogenase inhibitor oxamate on glycolytic flux etc. Thus, I am not fully convinced that these differences are truly important for maintaining stemness.
2. UCPs are thought to be anion transporters and many researchers think that they transport fatty acids and at the same time protons through the membrane, which explains its uncoupling role. However, it's unclear if the UCP-2/3 are truly uncouplers! Could it be, that UCP-2 transports pyruvate and thus expels it from the mitochondrial compartment? Since the authors have sophisticated methods in their hands using tracers, pulse chase experiments with mitochondria isolated from different cell types with different levels of UCP-expression may help to test this hypothesis.
3. Page 7, second paragraph: I do not understand why electron transport chain activities were not compared to fibroblasts and differentiated cells as well, since this is one of the major points of the paper. Why only use HEK293 tumor cells here?
4. Probably, it would be more appropriate to relate all data per cell (or per cell volume) to make useful comparisons.
5. Page 9, second paragraph: The fact that OCR could not be fully inhibited by oligomycin could also be due to other oxygen consuming reactions in those cells. Has this been tested? Even if this would not be the case, this experiment does not show that hPSCs are less dependent on OXPHOS. It just could mean that they have an unusual ATPase which is insensitive to oligomycin. It is also strange that increasing the glucose concentration increases ECAR. This means that experiments under "normal glucose concentrations" have been performed under limiting concentrations - if this is true, they are probably meaningless!
In the same line: Why are you surprised in other instances that an increase of glucose or insulin does not influence OCR or ECAR? Please keep in mind, energy demand pulls flux through energy metabolism and is not pushed by energy supply (with fi-cells probably being an exception)!
Also, I do not agree with the interpretation of the results with 2-deoxyglucose(2-DG): The results just mean that the concentration of the inhibitor was not sufficient to fully block glycolysis, and they do not mean that the hPSCs are more dependent on glycolysis.
6. In the introduction, the authors quote work from other labs that UCP-2 augments fatty acid oxidation. However, their own data show that hPSCs expressing UCP-2 do not prefer to oxidize fatty acids - how is this discrepancy explained?

Minor Points:

Page 2:

Wouldn't it be better to say hPSCs have functional respiratory complexes that are able to consume oxygen at maximal capacity?

Page 3:

First paragraph:

"Differences in energy and biosynthetic demands...." In my opinion these are nonsense sentences, please be specific.

Second paragraph:

"Morphologic studies suggest that there are few mitochondria"

The reference Prigione et al. is not correct.

Page 5:

First paragraph:

"...are punctuate staining..." strange language

"NHDFs" : Is this and all other abbreviations necessary and useful? I find it hard to follow when why which cell line was used. Why not call at least the NHDFs simply fibroblasts?

Second paragraph:

I disagree that inner membrane cristae folding is "regulated" by the F1F0 ATP synthase.

Page 6:

Middle paragraph:

Would it not make more sense to give the calculated volume instead of the diameter of the cells, assuming the cells are spheres.

Page 7:

Second paragraph:

What are HSF1 cells? These have not been introduced before.

Why did authors not determine the respiratory control ratio, which is not perfect, but still a good indicator of coupling of electron flow to ATP synthesis?

Third paragraph:

First line: Do you mean differentiating cell types or different cell types?

Lower paragraph:

I would prefer to have the oxygen consumption given per cell, which would make it easier to compare these values to other values in the literature - this would be in the femtomol range.

Page 8:

Second paragraph:

Why now use fetal-derived NHDFs? Or have fetal-derived NHDFs been used all the time?

Again, I do not understand the reason for the experiment using different concentrations of glucose or insulin. Glucose should be present in the cell culture medium at concentrations which saturate the transporter and also insulin is provided by the serum.

Last sentence: end with "respectively..." and discuss shortly what GLUT isoform expression means.

What happens to other cell types if you inhibit glycolysis with 2-DG? I guess they will also arrest.

Page 9:

Second paragraph:

What is the difference between this experiment and the experiment described on page 8? Why is now supplementary glucose driving ECAR?

Why do hPSCs react differently than HSF1 and hIPS cells?

Why is it not possible to choose a 2-DG concentration which completely inhibits glycolysis? The uptake of 2-DG may be different, thus explaining the time lag.

Bottom: The higher ATP-level is very probably simply due to the higher cell volume.

Lower paragraph: It would be important to inhibit OXPHOS with other inhibitors as well, e.g. KCN, rotenone, malonate ect.

Last paragraph:

The two-fold higher steady state ATP level is probably a consequence of the two-fold higher cell mass!

Last sentence: "< 5% affected..." this is strange language.

Page 10:

Upper paragraph:

It is surprising that the difference in ECAR after blockade of glycolysis by sodium oxamate is so small.

Page 11:

First paragraph:

What does addition of extracellular carnitine do? Are cells carnitine depleted in culture ? carnitine is sold in fitness studios to improve fat burning, but there is no solid evidence that this works!

This experiment also shows that HSF1 cells do not have the enzymatic equipment (transporters, fatty acid binding protein, CPTs etc) to oxidize fatty acids, but it does not tell anything about their general reliance on OXPHOS.

Second paragraph:

What is the "strength" of ?

Page 12:

Upper paragraph:

It is interesting that the shift induced by FCCP is larger in HSF1 and hiPS2 cells compared to fibroblasts. Can the authors comment on that?

Lower paragraph: It would be nice to see the raw gel data used to measure the IF1 proteins, since this is a very small protein, probably hard to detect on Western blots.

Page 13:

End of the first paragraph: UCP1 is expressed only in brown adipose tissue. This should be clearly stated.

Page 14:

First paragraph:

"Pattern of hPSCs glucose metabolism..." Is pattern the right term?

Although this is a beautiful experiment, the effects of UCP-2 silencing on metabolism are very small and thus not extremely convincing..

Page 15:

First paragraph:

"During which time..." this is strange language.

"Measure the glucose contributed to mitochondrial oxidation..." this is strange language.

Also, a 13 % decrease in 13C incorporation is not very convincing.

What do the letters within brackets after the word glutamate mean?

Page 16:

First paragraph:

The main question remains: How does UCP-2 control access of substrate to oxidation in mitochondria? Maybe its blockade of pyruvate dehydrogenase.

Second paragraph:

If ROS increases during differentiation, this probably goes in parallel with the increasing oxygen consumption. Steady state levels of ROS measured by various methods may simply reflect OCR, if they are a constant byproduct of electron flux.

Page 20:

End of first paragraph:

I am confused why the authors talk about oxygen concentrations. I do not see that any experiments modulating oxygen concentrations have been done in this paper, or did I overlook something?

Figure 4C:

This is not a very convincing Western Blot. Why are bands for OCT4 and IF1 so broad, while beta-tubulin bands are so small?

Figure 7: The abbreviations used in panel C are not very helpful.

Additional Author Correspondence

22 September 2011

Thank you for your interest in our study and for your willingness to engage in a conversation about making a revised submission to The EMBO Journal suitable for publication.

We can address all of the specific reviewer comments with clarifications and additional work. The role of UCP2 in regulating human pluripotent stem cell (hPSC) metabolism is novel and important to report, a view shared in all 3 reviewers' comments.

We can address the mechanism by which UCP2 regulates stem cell metabolism, as suggested by Reviewer 3. In agreement, the data indicate that UCP2 regulates hPSC energy metabolism by pyruvate shunting away from the mitochondria (Figures 6H, 6I) instead of using a mitochondrial

uncoupling mechanism (Figure S6F). This mechanism of action for UCP2 is a hotly debated area in metabolism research.

Our data also indicate that changes in metabolism by UCP2 manipulation are causative for disturbed hPSC differentiation and exclude Reviewer 3 suggestions that changes in differentiation or changes in reactive oxygen species (ROS) induce the changes in metabolism that we measure. The key for this causative link is found in the sequential order of events that occur with manipulations of the level of UCP2 expression. hPSC glycolysis and steady-state ATP concentrations change (Figure 6B, 6C) with UCP2 manipulations before hPSCs lose expression of OCT4 (Figure 6A), a main controller of pluripotency, or before expression of differentiation genes in all germ lineages (Figure 7E). Changes in ROS levels from UCP2 alterations (Figure 7A, 7C) do not affect metabolism, and increased or decreased ROS does not cause changes in OCT4 or differentiated gene expression when changes in metabolism have already occurred. Combined, the data indicate that changes in UCP2 regulation of hPSC metabolism are causative for changes in hPSC differentiation. Our data address one of the main conceptual advances required by the Editor, that of causation.

We recognize our study is detailed and complex with many intricate findings that can be linked in ways beyond what we have spelled out in the text by critically evaluating the data presented. In this regard, we did not show that UCP2 had any effect on pluripotency or "stemness" as Reviewer 1 stated we did, nor did we show that UCP2 had any direct role on cell proliferation, as Reviewer 3 stated we did. Our data only indicate a role for UCP2 regulated metabolism as causative for hPSC differentiation, and not the maintenance of pluripotency or altered proliferation. In a revised and clarified submission, we will further explain these and other key points of our study.

However, to provide an exact mechanism for how UCP2-regulated metabolism controls differentiation is a very difficult question that will take many years to determine. Only Reviewer 1 inquired into how UCP2-regulated metabolism affects hPSC differentiation. Reviewer 2 did not make this comment. And Reviewer 3 was more interested in the mechanism for how UCP2 "shifts glucose metabolism from oxidation to CO₂ to conversion to lactate" and not a mechanism for how UCP2-metabolism regulates hPSC differentiation. Reviewer 3 even proposed a mechanism for UCP2 shunting pyruvate out of mitochondria, which is exactly what the isotope tracing studies (Figure 6H, 6I) and tight mitochondrial coupling data (Figure S6F) fully support. Other investigators have not shown causation for hPSC metabolism in controlling hPSC differentiation, although there are large efforts trying to show metabolic causation for differentiation through metabolite-mediated epigenetic modifications in more specialized and further differentiated cell settings than hPSCs, such as in myeloid cell differentiation (Xu et al., 2011). So far, no studies can go beyond parallel associations between metabolism and differentiation. Therefore, we feel and the published data support that this standard for publication is currently excessively high for a paper or even the field and is mainly supported by only one of three reviewers. This standard negates all the provided, detailed mechanistic and causative data in our study, which we hope to provide to the community in a timely manner.

Given this situation, we can provide a mechanism for UCP2 regulation of hPSC metabolism and a causative link between proper metabolic regulation and hPSC differentiation. However, we cannot (nor can any lab so far) provide the exact mechanism(s) for how metabolism controls hPSC differentiation. We hope you would consider a revised manuscript that addresses all reviewer comments and is re-written to more clearly indicate the data supporting the mechanism for UCP2 regulation of hPSC metabolism and causation for a role in hPSC differentiation. We believe this pushes the field further than anyone has so far and, while the study cannot address everything in one paper, sets the stage for screens to determine exactly how UCP2 regulated metabolism controls differentiation for many future years of work.

Thank you once again for engaging in this dialogue on our study. We eagerly await your decision on whether we can proceed.

Thank you for your detailed letter. I very much appreciate your explanations re-emphasizing the advance provided by your study. I am also glad for the illustration of possible issues with the perception, caused by a relatively dense manuscript.

Based on your reasoning, I am perfectly fine with your proposal, though will have to insist that some definitive insight into UCP2's role in metabolic control (as requested by both refs#2 and #3) is provided. Further, the intriguing results on Oct4 regulation upon UCP2-modulation (distinguishing it from ROS-effects and thus implying a causal relationship to the pluripotency state) should appropriately be highlighted, a task relatively easy following your suggestion to clarify key findings in a revised manuscript.

Please note that I would run a revised version minimally with either referee#2 or #3 for final assessment before reaching a final decision on the study.

I am very much looking forward to your revisions and constructive interactions in the future.

Yours sincerely,

Editor
The EMBO Journal

Thank you for allowing a thoroughly revised manuscript, EMBOJ-2011-78968, entitled "UCP2 Regulates Energy Metabolism and Differentiation Potential of Human Pluripotent Stem Cells", for continued consideration as a research article in *The EMBO Journal*.

We also thank the three expert reviewers whose comments we have carefully and fully considered, and for which we provide clarifications and additional focused studies to appropriately address each issue raised. We believe these insightful and helpful comments have led to a markedly strengthened manuscript, with the changes we have made detailed as follows:

Referee #1:

Comment 1: This is a very interesting manuscript and study. It is proposing an attractive hypothesis that UCP2 is modulating metabolism and thereby also modulating cell proliferation and differentiation. However, there is a general disconnect between the metabolism part of the study and the differentiation. It is not clear that UCP2 is mediating any of its effects on cell differentiation or proliferation through altering metabolism.

The authors may want to expand on the mechanism by which UCP2 alters metabolism and cell differentiation/proliferation. Previous studies have shown that UCP2 modulates cell differentiation and proliferation, and ES cells have been shown to downregulate glycolysis during differentiation.

This is a large and complex study to review and I apologize to the authors if I missed any important point.

Response 1: We thank expert Referee #1 for the positive comment on the interest of the study. We agree that the study is large and complex, which can lead to some misunderstandings of the data and associated text as we originally presented it. We also recognize that it rests with us to make the complicated things clear, and we have done so in a point-by-point response to the specific issues raised by the reviewer, as follows.

Comment 2: The effect of oligomycin on ATP in the cells is not acceptable as an indication for the source

of ATP. The reason stems from the capacity of the cells to compensate by generating ATP from other sources.

Response 2: ATP is generated from multiple substrates by glycolysis and oxidative phosphorylation (OXPHOS). Figure 3C showed that ATP in human pluripotent stem cells (hPSCs) decreases less with 0.1-1.0 μ M oligomycin (<5%), a specific inhibitor of the ATP synthase that blocks OXPHOS, than it does in fibroblasts (~30%) ($p < 0.05$). By contrast, Figure 3D shows that ATP in hPSCs decreases more with 50mM sodium oxamate (>60%), a glycolysis inhibitor, than it does in fibroblasts (<40%) ($p < 0.05$). The potential for glycolytic compensation with oligomycin was also evaluated. The extracellular acidification rate (ECAR) increases ~5% in oligomycin-inhibited hPSCs (Figure S3A, lane 0), whereas ECAR increases by 3-fold more, or by ~15%, in oligomycin-inhibited fibroblasts (Figure S3A, NHDF lane). ATP production is directly linked to lactic acid production in glycolysis and changes in ECAR indicate glycolytic compensation. Even with a 5-15% compensatory increase in glycolysis, the total ATP concentration for fibroblasts and hPSCs still decreases (Figure 3C), indicating that oligomycin is blocking ATP production from OXPHOS. Although the compensatory increase in glycolysis in fibroblasts (15%) is more than that in hPSCs (5%), the decrease in total ATP concentration by oligomycin inhibition of OXPHOS is greater in fibroblasts than in hPSCs (Figure 3C), further indicating that fibroblasts are more dependent on OXPHOS. From these data we conclude that the relative ATP concentration in hPSCs is less sensitive to OXPHOS inhibition and conversely more sensitive to the inhibition of glycolysis than it is in fibroblasts.

Comment 3: There is also a technical issue: the authors have to demonstrate that oligomycin 0.1 μ M was sufficient to completely block mitochondrial ATP synthesis in each of the experimental groups. Partial blockade can produce an artifact where the permeability of oligomycin to the cell impacts the results more than the effect on mitochondria. Therefore, a dose response is required to demonstrate this point.

Response 3: An artifact of oligomycin-induced cell permeability would likely harm hPSC and/or fibroblast growth and survival. We analyzed apoptosis and cell cycle parameters for 1 μ M oligomycin at 45 min, which is the length of treatment time in Figure 3 studies, and little to no change in apoptosis or cell cycle parameters were detected (Figure S2F, S3C). This lack of toxic effects up to 1 μ M oligomycin supports the well accepted action of oligomycin as a specific complex V ATP synthase inhibitor. In addition, we did provide a 0.1 – 5 μ M oligomycin dose-response study in Figure 3C, as suggested. Since fibroblasts compensate for lost ATP production with a stronger glycolytic response than do hPSCs (Figure S3A), 1 μ M oligomycin was chosen as an acceptable study concentration because a higher concentration (eg., 5 μ M) suggested potential toxicity for the cells.

Comment 4: Figure 3E. I am worried that the PSCs are decaying fast in the respirometry experiment. The post oligomycin respiration is not stable and this can explain the inability to produce FCCP effect. It is not clear why all groups had identical OCR at time=2-31 minutes if differentiated cells were found to consume more oxygen and be dependent on glycolysis. Also, it is not clear if this graph is showing 3 separate experiments of 3 separated cell preparations, or is it showing replicates of wells in the Seahorse XF plate. The FCCP dose response in the supplement was done in the absence of oligomycin and therefore also raises suspicion that these cells are decaying.

Response 4: There is little to no change in apoptosis or cell cycle parameters after 1 μ M oligomycin treatment for 45 min (Figure S2F, S3C), which is the length of the assay. Therefore, the cells are not decaying. On page 11, top paragraph in the revised text we add that “No significant changes in cell viability were detected during the course of oligomycin exposure (Supplementary Figure S3C)” to allay this concern. The time between addition of 1 μ M oligomycin and FCCP is 38 min, which did not alter cell growth or survival. There is a pronounced FCCP effect in both the hPSCs and fibroblasts from the oligomycin inhibited OCR level, which demonstrates hPSC coupling. For hPSCs, there is a return to the pre-oligomycin baseline OCR level, whereas fibroblasts show a higher maximum than their baseline. This is a major point, that hPSCs in room air consume oxygen at maximal capacity, whereas fibroblasts have a higher unused respiration capacity at baseline growth conditions in room air. The small decrease with time of OCR in hPSCs post-oligomycin addition between 41-69 minutes suggests a slower rate of settling to the

oligomycin-inhibited level of respiration for hPSCs than for fibroblasts, and not hPSC cellular decay. This continuing decrease of post-oligomycin OCR has also been reported in other studies in other cell contexts as well (Gurumurthy et al, 2011; Kimmelman et al, 2011), and may reflect distinct drug response kinetics in different cell types. No mention or data in these additional papers and others suggest that the oligomycin-exposed cells are decaying within the short (<1 hour) experimental timeframe.

Fibroblasts consume ~2-fold more oxygen than hPSCs on a per-cell basis (Figure 2A), although hPSC and fibroblast mitochondria consume oxygen at similar rates when normalized to mitochondrial mass, as discussed on the top of page 7 in the text. In Figure 3E, OCR measurements were plotted for hPSCs and fibroblasts normalized to protein content and set to 0 at the first time point in order to compare the changes between these cell types during the course of the experiment. Prior to the addition of oligomycin at 41 min, therefore, hPSC and fibroblast mitochondria exhibit an identical rate of oxygen consumption and therefore show an identical, normalized OCR tracing.

Figure 3E shows 3 separate wells in the same representative experiment. This same experiment was repeated multiple times using an additional hPSC line with the same and with a 2-fold increased pyruvate carbon source concentration, yielding identical results (Figure S3E).

In general, single drug dose-titration inhibitor curves were performed for oligomycin (Fig. 3C), sodium oxamate (Fig. 3D), and FCCP (Fig. S3D). These titrations cover the informative inhibitors used in our studies and they were not paired with other drugs to avoid complex interpretations. Because OCR rises for oligomycin-treated hPSCs and fibroblasts exposed to FCCP (79 min time point in Figure 3E), there is no evidence of decaying cells in these studies. Further evidence for a lack of cellular decay is provided by the rapid OCR decrease to antimycin and rotenone electron transport chain blockade (108 min time point in Figure 3E).

Finally, studies that use a series of standard drug exposures, as we have in Figure 3E and in other work in the paper, is a well-accepted approach to assessing mitochondrial respiration activity. Almost the same experimental design has been reported in many influential studies (Brinton et al, 2009; Foskett et al, 2010; Gurumurthy et al, 2011; Kimmelman et al, 2011; Mootha et al, 2010; Tang et al, 2011; Zhang et al, 2011), which use 0.25 – 2 μ M oligomycin and a lag time between oligomycin and FCCP exposure that ranges from 20 – 40 min. No study has thus far reported that the cellular FCCP response is affected by oligomycin exposure.

Comment 5: The authors indicate reverse complex 5 activity is there to prevent mitophagy of mitochondria that would otherwise lose their potential. The paper cited in the manuscript stresses that this mechanism is true only in cells over expressing Parkin or cells that have high levels of endogenous Parkin. It will be valuable if the authors can cite previous studies that show that PSCs express Parkin or provide a western blot analysis for Parkin.

Response 5: We did not study mitophagy at any point in this work. We used inhibitors of the electron transport chain (ETC), glycolysis, and complex V ATP hydrolase activity to tease apart contributions to maintaining the mitochondrial membrane potential ($\Delta\Psi$) in hPSCs and fibroblasts. While membrane depolarization can lead to mitophagy, and the PINK1/PARKIN system seems to have a role in regulating this process, this is not the focus of our studies as mitochondrial quality control is a separate issue that requires significant attention and studies on its own merits. However, as the reviewer requested, we did perform western blot analysis, which revealed PARKIN expression in hPSCs and their differentiated derivatives, which is consistent with the need to maintain $\Delta\Psi$ in hPSCs. This data is provided in new Figure S4A along with the following sentence and references in the revised manuscript text at the top of page 12: “A reduction in $\Delta\Psi$ has been linked to mitochondrial dysfunction and possible mitophagy. The presence of PARKIN protein expression in HSF1 cells that increases with RA-induced differentiation is

consistent with the need to maintain $\Delta\psi$ in hPSCs (Supplementary Figure S4A), although this suggestion requires further investigation”.

Comment 6: I am afraid I cannot agree with the interpretation and conclusion that PSC cells run a reverse ATPsy activity to energize their mitochondria. Failure to depolarize with antimycin is not an indication that mitochondria charge their membranes through reverse activity of ATP synthase. It is, however, suggesting that in the presence of such toxin, these cells have the capacity to use ATPsynthase as ATPase to pump protons out. This is not unique to PSC cells. It is a very common feature in various tissues. The effect of IF1 over expression in the presence of KCN is supporting the claim that when exposed to such toxin the cells become dependent on ATPase activity of complex 5. Thus the conclusion that can be justified per the data is that these cells have reduced IF1 activity.

Response 6: We partially agree with the reviewer’s knowledgeable interpretation of the data. In isolation, the low level of IF1 in hPSCs (Figure S4D) could allow complex V to act as an ATPase in the presence of antimycin and pump protons out, whereas fibroblasts and differentiated hPSCs have a higher level of IF1 (Figure S4D) and therefore complex V cannot function as efficiently as an ATPase, so $\Delta\Psi$ collapses with antimycin (Figure 4A). However, blockade of ATP production from glycolysis with sodium oxamate (and no manipulation of the ETC) also partially collapses the $\Delta\Psi$ in hPSCs but not in fibroblasts (Figure 4B). This suggests a component of $\Delta\Psi$ in hPSCs is maintained by ATPase activity in the absence of antimycin

Comment 7: The result shown in 4E and F is interesting, but could be completely attributed to other effects of IF1 (See Campanella et al. 2008; and 2009). For example IF1 over expression was shown to reduce ROS production, which in turn would reduce proliferation rate.

Response 7: Another effect of IF1 expression is to reduce ROS, as the reviewer astutely indicates (Campanella et al, 2009). ROS has also been shown to function as a signaling molecule that can impact cell fate (Toledano & D’Autreaux, 2007). Generally, high levels of ROS cause cell death (Martin & Barrett, 2002) including in embryonic stem cells (Han et al, 2008), whereas low levels or physiological levels of ROS stimulate proliferation in many cell types (Black et al, 2001; Chain et al, 1999; Chatterjee et al, 2002; Choi et al, 2009; Chung et al, 2003; Chung et al, 2001; Djordjevic et al, 2006; Dunning et al, 2006; Kawada et al, 2008; Liu et al, 2002; Ogita et al, 2010; Rodriguez-Puyol et al, 2000). However, this growth stimulatory role for even low levels of ROS has never been reported in embryonic stem cells, probably because embryonic stem cells are exquisitely sensitive to oxidative stress, which causes them to die (Saretzki et al, 2008). Therefore, if IF1 overexpression in hPSCs reduced ROS, the main predicted effects would be to reduce cell death, which is the opposite of what was observed and not consistent with the increase in cell death presented in Figures 4D – 4F.

Comment 8: Figure 4A shows membrane potential data with JC1. I am afraid that although Invitrogen markets JC1 as a membrane potential dye, the scientific community does not accept it as so. JC1 is not a proper dye for membrane potential and it is a consensus within the mitochondria field that JC1 data cannot be used to draw conclusions.

Response 8: Actually, Figures 4A and 4B use TMRM staining and not JC-1 staining, as stated in the Figure 4 legend and Methods section. We agree that JC-1 fluorescence intensity/emission spectra are not dependent only on Nernstian accumulation but bind and quench anomalously and are therefore not consistent or reliable measures of mitochondrial membrane potential, although we did include JC-1 data in the supplement (Figure S4C), which is in agreement with the TMRM staining data in Figures 4A and 4B.

Comment 9: Figure 6 and 7. The results show that UCP2 has an effect on cell differentiation and proliferation. There are examples for that in the literature. (See Chen X et al JBC 2009 PM:19073597 for muscle differentiation, and Elorza et al JBC 2008 for erythroid differentiation). This can be due to UCP2 effect on ROS, not on glucose metabolism. The authors should address the possibility that UCP2-differentiation axis may contribute the effect at 6 days of RA. That means, the effect on differentiation lead to the observed effect on metabolism.

Response 9: Our data indicate that changes in metabolism by UCP2 manipulations are causative for disturbed hPSC differentiation and not that changes in differentiation induce the changes in metabolism that we measure. The key for this causative link is found in the sequential order of events that occur with manipulations of the level of UCP2 expression. hPSC glycolysis and steady-state ATP concentrations change (Figure 6B, 6C) with *shUCP2* knockdown before hPSCs lose expression of OCT4 (Figure 6A), a main controller of pluripotency, or before expression of differentiation genes with ectopic *UCP2* expression in trilineage differentiation (Figure 7E, day 0). The data also did not show that UCP2 regulates hPSC proliferation, only that hPSCs that differentiate slow down in their growth (Figure S7A and S7D). *shUCP2* knockdown increased ROS in hPSCs (Figure 7A) and ectopic UCP2 expression reduced ROS in hPSCs (Figure 7C). Importantly, despite changes in ROS levels, OCT4 expression is unaffected by *shUCP2* knockdown and trilineage differentiation genes are not induced with ectopic UCP2 expression (Figure 7E, day 0). Therefore, the effect of UCP2-induced changes in ROS does not change the expression of pluripotency or differentiated gene expression in the absence of a differentiating stimulus. Combined, the data indicate that changing UCP2 expression in hPSCs temporally causes changes first in metabolism, which are followed by changes in hPSC differentiation, and these changes are not dependent on UCP2-induced alterations in ROS levels. To strengthen this point, two ROS scavengers, N-acetylcysteine (NAC) and glutathione, were used during hPSC differentiation with or without ectopic UCP2 expression and the data provided in new Supplementary Figures S7B and S7C, along with added text on page 18 of the revised manuscript. With NAC or glutathione dosing levels high enough to scavenge ROS (Figure S7C), ectopic UCP2 expression impairs FBS-induced differentiation gene expression in hPSCs (Figure S7B). These results strongly support that the effect of ectopic UCP2 expression on differentiation is not due to ROS.

From the combined data, we proposed that changes in UCP2 expression first affect glucose metabolism by the substrate shunting mechanism we provided in Figure S7G. Support for this shunting mechanism comes from metabolic flux tracing data shown in Figure 6H and 6I. This change in metabolism would then have an active role in regulating differentiation, as detailed above. Importantly, several recent studies strongly support the mechanism by which changes in metabolism regulate differentiation, in these instances by metabolite concentrations (such as α -ketoglutarate) controlling epigenetic changes that then regulate differentiation gene expression (Figuroa et al, 2010; Teperino et al, 2010; Xu et al, 2011). A recent review from the Cantley lab also emphasizes that alterations in metabolic flux regulate cell signaling mechanisms and dramatically affect cell fate decisions (Locasale & Cantley, 2011). Our data is consistent with these studies and provides a molecular link to one physiologically-relevant (Figure 5) regulator of hPSC metabolism, UCP2.

Comment 10: However, the result in day 0 is surprising and interesting. The pentose phosphate pathway is a strong responder to ROS due to its role in glutathione metabolism. Thus, the authors should address the possibility that in the absence of UCP2 there is more ROS and increased need to produce and reduce glutathione, leading to increased flux through the pentose phosphate pathway.

Response 10: Actually, the data show that UCP2 reduction leads to an increase in ROS in hPSCs (Figure 7A), whereas ectopic UCP2 expression leads to a decrease in ROS in hPSCs (Figure 7C). Ectopic UCP2 expression also leads to an increase in flux through the pentose phosphate pathway (Figure 6I). Although quite sensible, this flux result is the opposite of the reviewer's prediction.

Comment 11: The authors demonstrate that O_2 consumption of the hPSC (per mitochondrial mass) is similar to the differentiated cells. This seems to be inconsistent with UCP2 effect (which is not similar between hPSC and differentiated cells) which should be effective even when normalized to mitochondrial mass, unless the authors interpretation is that UCP2 expression alter genes that modulate extra-mitochondrial metabolism. In other words, if this was all about UCP2, one would expect that normalization to mitochondrial mass will still maintain the difference. This is not supporting the study hypothesis.

Response 11: Our studies focus on UCP2-regulated glucose metabolism because of the differential expression of this candidate metabolism regulating gene identified in 3 separate gene expression profiling studies (Figure 5). However, multiple pathways can regulate glucose metabolism and other carbon sources can contribute to oxygen consumption, including fatty acids (Ellis et al, 2010; Lee et al, 2011; Pecqueur et al, 2008; Zaugg et al, 2011) and amino acids (Kovacevic & Morris, 1972; Mares-Perlman & Shrago, 1988; Peng et al, 2007; Rapoport et al, 1971; Stanisiz et al, 1983). We do not claim that UCP2 is the only molecule contributing to oxygen consumption and these additional pathways and mechanisms may compensate for the effect of UCP2 expression, thereby maintaining an overall similar oxygen consumption rate between hPSCs and fibroblasts or differentiated hPSC derivatives. Additional approaches beyond our in silico gene expression data mining work will be required to determine what pathways and mechanisms beyond UCP2 regulate hPSC metabolism and oxygen consumption.

Comment 12: The authors should acknowledge that stem cell differentiation was shown to be associated with reduced glycolytic activity (See Chung S. 2009 PM:20045004).

I am also surprised that the authors did not mention the finding by Schieke SM et al. JBC 2008 that have demonstrated that ES cells differ in their capacity to differentiate based on mitochondrial metabolism. Within the ES cell population, the cells that can be mobilized to differentiate have increased mitochondrial oxidative capacity.

Response 12: We have corrected these oversights on our part and cite the listed papers on page 3 (Chung, et al., 2009) and page 12 (Schieke SM et al., 2008) of the revised text, as suggested.

Comment 13: Minor: The authors should not use the term "per mitochondrion" when normalizing to mitochondrial mass

Response 13: We agree, and have used the phraseology "normalized to mitochondrial mass" on page 7 of the revised text, as suggested.

Referee #2:

Comment 1: This is an important paper showing that the transition from undifferentiated to differentiated cells is at least partially controlled by the metabolic state of mitochondria. The stem cells are shown to contain mitochondria, whose membrane potential is sustained by ATP from glycolysis. As long as mitochondria are unable to oxidatively metabolise glucose, these cells rely on glycolysis and remain undifferentiated. The key finding is the identification of UCP2 as the key component regulating the mitochondrial state and thus glucose metabolism. UCP2 is critical for maintaining the glycolysis and thus the undifferentiated state.

Response 1: We thank the reviewer for the positive comments on the importance of our study.

Comment 2: The findings and conclusions are very well supported with a plethora of experimental data. The paper is well written, but sometimes too condensed vis-avis the large spectrum of data shown.

The authors avoid discussing the important but treacherous question by what mechanism UCP2 causes the preference for glycolysis. The referee thinks that their data are consistent with a regulated uncoupling and concomitant decrease of membrane potential by UCP2. The difference to UCP1 may reside in the way how the uncoupling, i.e. H⁺ transport is regulated.

Response 2: We thank the reviewer for positive comments on the quality of the experimental data. We agree that the area is complex and the study large, and have simplified and de-condensed the text appropriately to improve clarity. As explained on page 16 of the revised text and data shown in Figure S6F, we do not have evidence for an uncoupling mechanism but rather the data fit very well with a mechanistic role in hPSC metabolism for UCP2 in shunting oxidative substrates, such as pyruvate, out of the mitochondria, thereby facilitating glycolysis and the pentose phosphate pathway. Data supporting this alternative mechanism is provided by metabolic flux studies using [U¹³C₆] glucose tracing analysis on pages 15-16 in the revised text and in Figures 6H and 6I. Also, we have no evidence for the expression of UCP1 in hPSCs (Figure 5F), which is consistent with UCP1 expression being restricted to brown adipose tissue.

Referee #3:*General Comment 1:*

In this paper the groups of Koehler and Teitell combine efforts and show that human pluripotent stem cells (hPSCs) in culture mainly rely on glycolysis, although they contain functional mitochondria. They conclude this from comparing extensively the metabolic phenotype of several stem cells model lines to fibroblasts as well as to cells differentiated from these hPSC lines. Manipulating the expression levels of UCP-2 shows that UCP-2 expression in such stem cells seems to be important for this specialized metabolic phenotype.

This is a very extensive and experimentally well done paper. Very recently, the metabolism of stem cells within tissues, but also in cell culture, has become a true hot topic in the field. The results are novel and highly interesting to a broad community of researchers interested in stem cell biology as well as mitochondrial metabolism.

Response 1: We thank the reviewer for comments on experimental approach, novelty of the results, and interest of the field.

General Comment 2: Unfortunately, the authors are unable to come even close to a mechanism which would answer the question how UCP-2 shifts glucose metabolism from oxidation to CO₂ to conversion to lactate.

Response 2: Actually, our data fully supports this reviewer's insightful comment (below) that the main mechanism of UCP2 action is to shunt pyruvate out of mitochondria, reducing pyruvate oxidation and thereby increasing glycolytic and pentose phosphate pathway flux. This mechanism was revealed by [¹³C₆] glucose isotope tracing analysis, which showed that UCP2 expression in hPSCs reduced TCA cycle carbon flux (Figure 6H) and increased pentose phosphate pathway carbon flux (Figure 6I). An alternative mechanism for UCP2 action, as a respiration uncoupler, was excluded in studies that evaluated endogenous, *shUCP2* knockdown, and ectopic UCP2 levels of expression in hPSCs with and without ETC blockade or uncoupling compound exposures (Figure S6F). In these studies, the native, reduced, or enhanced expression of UCP2 had no effect on OCR, indicating mitochondrial coupling independent of UCP2 dosage, thereby strongly arguing against a respiration uncoupling role for UCP2 in hPSCs. Combined, the shift in metabolic flux and lack of an effect on uncoupling with changes in UCP2 expression indicates that the UCP2 mechanism of action is as a substrate shunt that shifts glucose metabolism away from respiration and toward glycolysis.

Comment 3: Also, although UCP-2 seems to be truly important, other mechanisms are equally likely and have to be seriously considered. E.g. the group of T. Suda has shown, using k.o. mice, that it is the constitutive expression of hypoxia inducible factor HIF-1 α which drives a similar phenotype of hematopoietic stem cells in vivo. Also, there is a huge amount of literature on the Warburg effect in tumor cells, which have been shown to possess this metabolic phenotype in the 1920s, and inactivation of pyruvate dehydrogenase by modulating its kinase or binding of hexokinase to mitochondria are just two examples how this might be achieved. All this has to be considered and at least be discussed.

Response 3: We do not disagree with the reviewer comment that other molecular mechanisms will have a role in regulating hPSC metabolism, which may or may not be coordinated with UCP2, to establish or modify an overall metabolic program. However, in our study UCP2 was identified amongst all candidate metabolic regulators through surveys of 3 independent gene expression profiling studies as the best candidate gene that was differentially expressed in hPSCs and differentiated cells, as described in the text and shown in Figure 5 and S5. We did not identify differential expression of HIF 1 α , pyruvate dehydrogenase, or hexokinase, as well as many other candidate metabolic regulators in these profiling studies, which is why the mechanistic analyses focused on UCP2. In the revised text Discussion section, this point is made much clearer, as suggested.

Comment 4: Major Points: 1. One important problem is that the differences explaining the metabolic phenotype, i.e. the differences between hPSCs, hESCs and hiESCs, which are all used in this paper, compared to more differentiated cell types are very moderate. This is true when it comes to differences in oxygen consumption, lactate production, UCP expression, effect of the lactate dehydrogenase inhibitor

oxamate on glycolytic flux etc. Thus, I am not fully convinced that these differences are truly important for maintaining stemness.

Response 4: Overall, it is very common that changes in metabolic function are less extreme than changes that can be observed in gene expression. Although there are many examples for this, a seminal study on the role of pyruvate kinase muscle isozymes M1/M2 (PKM1/2) in the well-known Warburg cancer effect showed that the change in oxygen consumption or lactate production after manipulating PKM2 or PKM1 isoforms was a modest ~1.5 – 2-fold (Christofk et al, 2008). Also, studies similar to those reported here that utilize carbon isotope tracers to analyze changes in metabolic flux, modest (~10%) metabolic pathway changes in TCA cycle flux or pentose phosphate pathway flux are often reported (Boren et al, 2003). In a recent Cantley lab review, it was discussed that even a 0.1 – 1.0% change in glucose could profoundly affect downstream metabolic pathways and subsequent cell fate decisions (Locasale & Cantley, 2011). In sum, although the changes in metabolic function may not seem large, even seemingly small alterations (when compared to gene expression changes) can have profound effects on cell function, as documented for hPSC differentiation with ectopic UCP2 expression (Figure 7D, 7E). An important last response is that our study did not reveal a role for UCP2 in maintaining “stemness” or pluripotency. Our data suggests against a role for UCP2 in maintaining stemness- for example, *shUCP2* knockdown alters hPSC metabolism (Figure 6B, 6C) without altering expression of a main pluripotency regulator, OCT4 (Figure 6A). Instead, our study shows a role for UCP2 silencing in facilitating a metabolic transition that supports proper hPSC differentiation.

Comment 5: 2. UCPs are thought to be anion transporters and many researchers think that they transport fatty acids and at the same time protons through the membrane, which explains its uncoupling role. However, its unclear if the UCP-2/3 are truly uncouplers! Could it be, that UCP-2 transports pyruvate and thus expels it from the mitochondrial compartment? Since the authors have sophisticated methods in their hands using tracers, pulse chase experiments with mitochondria isolated from different cell types with different levels of UCP-expression may help to test this hypothesis.

Response 5: The experimental data is in complete agreement with the reviewer’s astute comment on the mechanism of action for UCP2 in hPSCs. This is clearly explained in Response 2 to Referee #3 above and we have also commented on this mechanism in several places within the revised text (text pages 15-16 and Figures 6H and 6I). The data also show no role for UCP2 as a respiration uncoupler in hPSCs (revised text page 16 and Figure S6F), in further agreement with this reviewer’s insightful comments, as explained in detail above. Finally, two references describing the isotope tracing analysis methodologies (Boren et al, 2003; Lee et al, 2010) were also added on page 16 of the revised text. We and others did not use isolated mitochondria because labeled carbon was traced in pathways outside of mitochondria, such as the pentose phosphate pathway (Figure 6I).

Comment 6: 3. Page 7, second paragraph: I do not understand why electron transport chain activities were not compared to fibroblasts and differentiated cells as well, since this is one of the major points of the paper. Why only use HEK293 tumor cells here?

Response 6: HEK293 cells were chosen as a comparator cell type for this study for two main reasons: 1) they have a similar mitochondrial content (Figure 1B) and size (Figure S1E) compared to hPSCs, providing roughly equivalent experimental parameters and 2) our studies showed that feeding ETC substrates to NHDF cells was difficult. The most important point from this data was to show that the ETC was functional in hPSCs; more physiologically-relevant studies of ETC function (respiration) and fibroblast or differentiated cells were made with intact cell studies throughout the paper (for examples, please see Figures 2A, 2B, S2B, S2C, S2E), as suggested.

Comment 7: 4. Probably, it would be more appropriate to relate all data per cell (or per cell volume) to make useful comparisons.

Response 7: The problem with providing values on a per cell basis is that the mitochondrial content is not equivalent on a per cell basis. This led the field to erroneously conclude that there are few mitochondria in hPSCs and that there is a disproportionate increase in mitochondrial content during early hPSC differentiation. This cell-based data was a very strong motivator for evaluating mitochondrial content in stem cells as a function of the mass of the cell (Figure 1B, 1C, S1C, S1D), as this is the only way to know whether hPSCs disproportionately increase mitochondrial content during lineage non-specific differentiation. Our data clearly show that hPSCs contain mitochondria proportionate to their overall mass, which is ~equivalent to the proportional mitochondrial content in fibroblasts. Data presented in this manner helps dispel the notion that there is a developmental trigger mechanism that increases mitochondrial mass disproportionate to the overall increase in mass with hPSC differentiation, as was previously thought. Of course, for specialized cells such as adult cardiomyocytes there is a disproportionate increase in mitochondrial mass relative to the overall cell mass in comparison with other less energetic cell types, but the regulation of this increased commitment (the mitochondrial mass “set-point”) for cells has never been identified, despite much progress in identifying the regulators of mtDNA replication. We did provide the sizes of the different cell types used in this comparison (Figure S1E), so the data can be converted to a per cell basis, and in text on the top of page 7, using this data, we did indicate that there are ~1.5 – 2-fold more mitochondria in NHDF cells than there are in hPSCs.

Comment 8: 5. Page 9, second paragraph: The fact that OCR could not be fully inhibited by oligomycin could also be due to other oxygen consuming reactions in those cells. Has this been tested? Even if this would not be the case, this experiment does not show that hPSCs are less dependent on OXPHOS. It just could mean that they have an unusual ATPase which is insensitive to oligomycin.

Response 8: This insightful question was addressed in Figure 3E studies. Addition of rotenone (complex I inhibitor) and antimycin (complex III inhibitor) is known to block oxygen consumption by mitochondria. The remainder of the rotenone plus antimycin insensitive OCR is contributed by other non-mitochondrial oxygen consuming reactions, which in Figure 3E is the distance between the rotenone + antimycin curves at 127 – 146 min and the abscissa. The amount of non-mitochondrial oxygen consumption in hPSCs is reproducibly <5% of the starting OCR before adding oligomycin. hPSCs are also less dependent on OXPHOS than glycolysis for the reasons provided in a detailed response to Referee #1, Comment 2 above (please refer to that response). In short, although they are quite sensitive to oligomycin (Figure 3E, S3E), the overall cellular ATP content, even with a compensatory increase in ATP production by increased glycolysis (Figure S3A), still falls less in hPSCs than in fibroblasts (Figure 3C). Oligomycin sensitivity (Figure 3E, S3E), coupled with no reports of an oligomycin insensitive variant ATP synthase in hPSCs (or human cells?), suggests against an insensitive ATP synthase in hPSCs.

Comment 9: It is also strange that increasing the glucose concentration increases ECAR. This means that experiments under "normal glucose concentrations" have been performed under limiting concentrations - if this is true, they are probably meaningless!

In the same line: Why are you surprised in other instances that an increase of glucose or insulin does not influence OCR or ECAR?

Response 9: The circulating glucose concentration in humans is ~5 mM. However, for the field of hPSC biology the standard supportive culture concentration of glucose is between 17.5 mM and 25 mM. This is the concentration range used for major studies in hPSC signaling (Kattman et al, 2011), genetics and epigenetics (Boyer et al, 2005), and reprogramming to pluripotency (Lowry & Plath, 2008; Lowry et al, 2008; Takahashi et al, 2007; Yu et al, 2007). We considered this situation and felt that it would be critical to retain the carbon sources and concentrations relevant to the vast majority of labs studying all facets of hPSC biology. In fact, one recent study showed that low glucose concentrations inspire pluripotent ESCs to differentiate (Mochizuki, 2011), which could impede studies of hPSC metabolism in low glucose over extended time periods. Almost all experiments in our current studies use 2.5 mM glucose. The glucose concentration was also titrated between 0 – 25 mM at increments of 5 mM (Figure S2H). The data show that from 0 mM to 5 mM glucose, or below to physiologic glucose concentration, that there is an increase of ECAR with added glucose, whereas above 5 mM glucose ECAR is maximal and statistically constant. In Figure 3B, the study was began in the non-maximized glucose range (2.5 mM) to establish a limiting condition, followed by an increase to 25 mM glucose to induce maximal ECAR. We slightly modified the

text to address these points on page 9 of the revised manuscript. In sum, glucose was not limiting in these studies, nor was it surprising that glucose (Figure S2H) or insulin (Figure S2I) increases did not increase OCR or ECAR under these feeding conditions for these and additional reasons cited below.

Comment 10: Please keep in mind, energy demand pulls flux through energy metabolism and is not pushed by energy supply (with ß-cells probably being an exception)!

Also, I do not agree with the interpretation of the results with 2-deoxyglucose(2-DG): The results just mean that the concentration of the inhibitor was not sufficient to fully block glycolysis, and they do not mean that the hPSCs are more dependent on glycolysis.

Response 10: We agree with the reviewer that energy metabolism is not pushed by energy supply but by the “pull” of energy demand. We and many other investigators have used 2-DG to competitively inhibit glucose uptake at GLUT1 (Wu et al, 2007). Cells that rely more heavily on glycolysis for energy production will be most affected by glucose uptake blockade and show the largest decrease in ECAR. In Figure 3B, blocking glucose uptake with 2-DG caused a greater decrease in ECAR for hPSCs than for fibroblasts. Importantly, hPSCs and fibroblasts express equivalent levels of the 2-DG target, *GLUT1* (Figure S2J, S2K), indicating a similar extent of competitive blockade. In Figure 3B, during the first 9 min after 2-DG addition, hPSC ECAR drops significantly, whereas NHDF ECAR is unchanged. Over a longer period, hPSC ECAR drops by 65%, whereas NHDF ECAR drops by 45%, indicating that for an equivalent level of GLUT1 block, hPSCs are more dependent on glucose and glycolysis than are NHDFs.

Comment 11: 6. In the introduction, the authors quote work from other labs that UCP-2 augments fatty acid oxidation. However, their own data show that hPSCs expressing UCP-2 do not prefer to oxidize fatty acids - how is this discrepancy explained?

Response 11: This is an interesting observation brought forward by the reviewer. We provide new Figure 3F, showing that fibroblasts increase OCR up to 35% in parallel with increasing doses of external palmitate, whereas hPSCs show only a modest 5% dose-independent increase in OCR with added palmitate. However, internal fatty acids can be synthesized from glucose, and lipids can be liberated to make fatty acids from internal sources as well. For example, it has been reported that monoacylglycerol lipase (MAGL) liberates internal lipids to support tumor growth (Nomura et al, 2010) and that tumor cells and mouse embryonic stem cells have been reported to rely on β -oxidation of liberated internal fatty acids (Pike et al, 2010; Zaugg et al, 2011). Notably, *CPT1*, *ACACL*, *ACADM*, *ACADS*, and *HADHA* genes required for β -oxidation are expressed in hPSCs and human fibroblasts at similar levels (data not shown). We propose, but cannot prove with current technologies, that liberation and oxidation of internal fatty acids in hPSCs could have already saturated the ETC, such that adding external fatty acids would not further increase OCR. To clarify this point, the following text was added to the top of page 12 in the revised manuscript “The lack of a robust free fatty acid response in HSF1 cells could be from ETC saturation with glucose or internally-produced fatty acids. Notably, the essential enzymes for fatty acid oxidation are equally expressed in HSF1 cells and NHDFs.”

Comment 12: Minor Points: Page 2: Wouldn't it be better to say hPSCs have functional respiratory complexes that are able to consume oxygen at maximal capacity?

Response 12: Yes, but as grown routinely in the lab, hPSCs do respire at their maximal capacity, as shown in Figure 3E inhibitor studies. We have changed the Abstract to the phraseology suggested by the reviewer in the revised text.

Comment 13: Page 3: First paragraph: "Differences in energy and biosynthetic demands...." In my opinion these are nonsense sentences, please be specific.

Response 13: We have changed the wording to “energy status and biosynthesis” on page 3 of the revised text, as suggested.

Comment 14: Second paragraph: "Morphologic studies suggest that there are few mitochondria" The reference Prigione et al. is not correct

Response 14: The reference in the text was corrected to (Prigione et al, 2010) as it was in the references cited list, as suggested. We thank the reviewer for catching this mistake.

Comment 15: Page 5: First paragraph: "...are punctuate staining..." strange language "NHDFs" : Is this and all other abbreviations necessary and useful? I find it hard to follow when why which cell line was used. Why not call at least the NHDFs simply fibroblasts?

Response 15: We removed the word "staining" and changed the sentence to read "punctate, or fragmented". Punctate is defined as spotted; marked with points or punctures, to describe the appearance of mitochondria in hPSCs. We also defined NHDFs as fibroblasts the first time it was used in the text and have made that change throughout the text, as requested by the reviewer.

Comment 16: Second paragraph: I disagree that inner membrane cristae folding is "regulated" by the F1F0 ATP synthase.

Response 16: It has been suggested that the F1F0 ATP synthase plays a scaffolding role in the inner mitochondrial membrane, determining cristae structure (Buzhynskyy et al, 2007; Campanella et al, 2008; Giraud MF et al, 2002; Minauro-Sanmiguel F et al, 2005; Paumard P et al, 2002; Strauss et al, 2008). We have added these 6 references to the revised manuscript text on page 5 and have changed the wording to "The F1F0 ATP synthase appears to play a scaffolding role for the cristae structure of the mitochondrial inner membrane...." to eliminate the possible misinterpretation that the ATP synthase has an active regulatory role, akin to an enzyme, in this process, as suggested.

Comment 17: Page 6: Middle paragraph: Would it not make more sense to give the calculated volume instead of the diameter of the cells, assuming the cells are spheres.

Response 17: We provide the calculated cell volume, assuming a sphere, in an additional column in Figure S1E and in the revised manuscript text on page 6, as requested.

Comment 18: Page 7: Second paragraph: What are HSF1 cells? These have not been introduced before.

Response 18: We thank the reviewer for catching this omission and have added that HSF1 cells are a hESC line in the revised manuscript text on page 5, as requested.

Comment 19: Why did authors not determine the respiratory control ratio, which is not perfect, but still a good indicator of coupling of electron flow to ATP synthesis?

Response 19: We agree with the reviewer that measuring state III vs state IV respiration can provide a good indication of coupling efficiency. However, the delivery of ADP across intact cell membranes in XF24 Extracellular Flux Analyzer (aka "Seahorse machine") studies is inefficient. Also, the ratio of OCR with FCCP exposure to OCR with oligomycin exposure indicates electron flow coupling for hPSCs.

Comment 20: Third paragraph: First line: Do you mean differentiating cell types or different cell types?

Response 20: Different cell types- this has been made clear in the revised text, as suggested.

Comment 21: Lower paragraph: I would prefer to have the oxygen consumption given per cell, which would make it easier to compare these values to other values in the literature - this would be in the femtomol range.

Response 21: The data provided in Figure 2A is the oxygen consumption rate per 5×10^6 cells for a Clark-type oxygen electrode experiment. As requested, in the revised text at the top of page 7, we provide the oxygen consumption per cell, which is within the femtomol range, as expected.

Comment 22: Page 8: Second paragraph: Why now use fetal-derived NHDFs? Or have fetal-derived NHDFs been used all the time?

Response 22: There is much current discussion within the field of stem cell biology regarding the differences between cells within tissue lineages based upon their developmental age, especially in terms of changes in gene expression and epigenetic state of the chromatin. Almost all of the work using NHDFs in the current study is from fetal-derived NHDFs, which is also the source cell type for reprogramming hiPSC lines 2 and 18. To check that OCR and ECAR values are relatively constant independent of fibroblast development time, a single OCR/ECAR ratio was provided in Figure S2E.

Comment 23: Again, I do not understand the reason for the experiment using different concentrations of glucose or insulin. Glucose should be present in the cell culture medium at concentrations which saturate the transporter and also insulin is provided by the serum.

Response 23: Referee # 3 Comment 10, above, asked whether glucose was saturating as used in this study. The titration curve for glucose was required to show that this is the case (Figure S2H). All of the hPSC lines are grown in StemPro SFM medium, a proprietary serum-substitute that helps to maintain hPSC pluripotency and is not known to contain insulin. In fact, serum causes hPSCs to differentiate and therefore cannot be used for hPSC maintenance. Insulin is known to stimulate glycolytic capacity in many mammalian cell types by driving glucose uptake. Insulin titrated over a 0 to 17.5 mM range failed to increase ECAR and did not statistically alter OCR (Figure S2I), suggesting that hPSCs are at their maximal glycolytic and respiratory capacities.

Comment 24: Last sentence: end with "respectively..." and discuss shortly what GLUT isoform expression means.

Response 24: There are 13 glucose transporter isoforms. Each glucose transporter isoform plays a specific role in glucose metabolism determined by its pattern of tissue expression, substrate specificity, transport kinetics, and regulated expression in different physiological conditions. The two main isoforms are GLUT1 which is responsible for the low-level of basal glucose uptake required to sustain respiration in all cells, and GLUT4 which is an insulin-regulated glucose transporter. We measured the expression of these two main isoforms in hPSCs and differentiated cells to exclude the possibility that it is the level of glucose transporter that is responsible for differences in metabolic profiles between cell types and conditions used in these studies. *In silico* analysis of available gene expression profiling (GEP) data and QPCR to examine the mRNA levels of GLUT1 and GLUT4 glucose transporters was performed. GEP and QPCR showed that GLUT1 was highly expressed at similar levels in hPSCs and human fibroblasts, in contrast to GLUT4, whose expression was almost undetectable in all lines examined (Figure S2J, S2K). We modified the sentence in question to "Also, glucose transporters GLUT1 (widely expressed) and GLUT4 (insulin-regulated) are expressed at ~equivalent high or barely detectable levels in hPSCs and fibroblasts, respectively (Supplementary Figure S2J, S2K), indicating an equivalent ability to bind and uptake glucose" in the revised text on the top of page 9, as suggested.

Comment 25: What happens to other cell types if you inhibit glycolysis with 2-DG? I guess they will also arrest.

Response 25: As shown in Figure 2D, hPSCs arrest in G2 phase of the cell cycle more robustly than do fibroblasts from glycolysis inhibition with 2-DG, again indicating to the relatively higher dependence that hPSCs have on glycolysis compared with differentiated cells.

Comment 26: Page 9: Second paragraph: What is the difference between this experiment and the experiment described on page 8? Why is now supplementary glucose driving ECAR?

Response 26: Please see Response to Referee #3 Comment 10, above.

Comment 27: Why do hPSCs react differently than HSF1 and hiPS cells?

Response 27: hPSCs is an abbreviation for human pluripotent stem cells. As defined by the stem cell community and in our study here, human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs) are subsets of hPSCs. HSF1 is a specific hESC line, and therefore it is also a hPSC. We apologize to the reviewer for the somewhat complicated designations that we did not devise but are required to use to communicate clearly and specifically with colleagues in stem cell biology.

Comment 28: Why is it not possible to choose a 2-DG concentration which completely inhibits glycolysis? The uptake of 2-DG may be different, thus explaining the time lag.

Response 28: 2-DG is a competitive inhibitor of glucose with similar Km values. The concentration of 2-DG used (150 mM) is 6-fold higher than the 25 mM glucose present in the media for optimal competition at the GLUT receptors. During the first 9 min after 2-DG addition, ECAR in hPSCs has a significant drop, whereas the ECAR in fibroblasts remains unchanged (Figure 3B). This is not due to a difference in glucose or 2-DG uptake because there is no statistical difference in glucose transporter expression between hPSCs and fibroblasts (Figure S2J, S2K). Rather, the data indicate that hPSCs are more dependent on glucose uptake for glycolysis as indicated by the the differential changes in ECAR.

Comment 29: Bottom: The higher ATP-level is very probably simply due to the higher cell volume.

Response 29: We agree with the reviewer comment and have changed the sentence in question at the top of page 10 in the revised text to read “The steady-state ATP level is ~2-fold higher per cell in fibroblasts than in hPSCs, reflecting ATP production, consumption, and the size difference between these two cell types (Supplementary Figure S3B)”, as suggested.

Comment 30: Lower paragraph: It would be important to inhibit OXPHOS with other inhibitors as well, e.g. KCN, rotenone, malonate ect.

Response 30: Some of these inhibitors only inhibit one ETC complex and therefore do not completely block the ETC. For example, rotenone or malonate only block complex I or complex III, respectively, and their use still allows complex III substrates, such as glycerol-3-phosphate, to deliver electrons to the ETC. The inhibitors and inhibitor combinations we have used in all studies in this work are those that have been routinely adopted by multiple investigators for examining the function of the ETC and respiration in additional cellular contexts as well.

Comment 31: Last paragraph: The two-fold higher steady state ATP level is probably a consequence of the two-fold higher cell mass!

Response 31: We agree with the reviewer comment and have changed the sentence in question at the top of page 10 in the revised text to read “The steady-state ATP level is ~2-fold higher per cell in fibroblasts than

in hPSCs, reflecting ATP production, consumption, and the size difference between these two cell types (Supplementary Figure S3B)”, as suggested.

Comment 32: Last sentence: "< 5% affected..." this is strange language.

Response 32: We agree and have changed the sentence in question in the revised text at the top of page 10 to “OXPHOS inhibition with 0.1 μ M oligomycin caused a ~30% ATP drop in fibroblasts, whereas the ATP drop in hPSCs was less than 5% (Figure 3C)”, as suggested.

Comment 33: Page 10: Upper paragraph: It is surprising that the difference in ECAR after blockade of glycolysis by sodium oxamate is so small.

Response 33: Please see the detailed Response to Referee #3 Comment 4, above.

Comment 34: Page 11: First paragraph: What does addition of extracellular carnitine do? Are cells carnitine depleted in culture? carnitine is sold in fitness studios to improve fat burning, but there is no solid evidence that this works!

Response 34: hPSCs are grown in defined conditions supplemented with a proprietary medium called StemPro SFM. We do not know (but doubt) whether L-carnitine is present in StemPro SFM medium. The experiment in question was to determine whether hPSCs can respond to external fatty acids by fatty acid oxidation. Fatty acid oxidation requires the enzyme carnitine palmitoyltransferase (CPT), which catalyzes the rate-limiting step of transporting fatty acids into the mitochondrial matrix. L-carnitine is the substrate for this CPT and thus is essential for fatty acid oxidation. L-carnitine was therefore added for this experiment alone and the expression of fatty acid oxidation enzymes evaluated by real-time RT-PCR (QPCR) to make sure that there was no unknown limitation in the ability of hPSCs to transport fatty acids into the mitochondrial matrix. By doing this, any difference in OCR between hPSCs and fibroblasts would only reflect the OXPHOS status, as shown in Figure 3F. As detailed in Responses 12 (above) and 36 (below) to Referee #3 comments, the data suggest and we propose in the revised text on the bottom of page 11 that the OXPHOS apparatus is more highly saturated in hPSCs than in fibroblasts, possibly due to a reduced handling capacity and the presence of internally manufactured free fatty acids.

Comment 35: This experiment also shows that HSF1 cells do not have the enzymatic equipment (transporters, fatty acid binding protein, CPTs etc) to oxidize fatty acids, but it does not tell anything about their general reliance on OXPHOS.

Response 35: Our QPCR data actually shows that HSF1 cells express all of the essential enzymes to oxidize fatty acids, at a level of expression similar to NHDFs, which robustly oxidize fatty acids as shown in Figure 3F. These hPSC-expressed enzyme genes include *CPT1*, *ACACL*, *ACADM*, *ACADS*, and *HADHA*, all genes required for β -oxidation by QPCR. To clarify this point, the following text was added to the top of page 12 in the revised manuscript “The lack of a robust free fatty acid response in HSF1 cells could be from ETC saturation with glucose or internally-produced fatty acids. Notably, the essential enzymes for fatty acid oxidation are equally expressed in HSF1 cells and fibroblasts by QPCR.”

Comment 36: Second paragraph: What is the "strength" of the mitochondrial membrane potential?

Response 36: The cited study showed that the level of $\Delta\Psi$ that can have an impact on hPSC differentiation potential was an arbitrary value determined by the level of TMRM dye staining (Schieke et al, 2008) In accordance, we have changed the word “strength” to “relative level” when discussing this work on page 12 of the revised manuscript, as suggested.

Comment 37: Page 12: Upper paragraph: It is interesting that the shift induced by FCCP is larger in HSF1 and hiPS2 cells compared to fibroblasts. Can the authors comment on that?

Response 37: In the context of all studies in this paper, the data suggest that hPSCs without FCCP treatment are already respiring at the maximum capacity in the basal state of growth in ambient culture.

Comment 38: Lower paragraph: It would be nice to see the raw gel data used to measure the IF1 proteins, since this is a very small protein, probably hard to detect on Western blots.

Response 38: The reviewer is correct in that ATP1F1 is a ~10 kd protein and is hard (but not impossible) to reproducibly identify and measure on western blots. Although reproducible, the western blots are not attractive and therefore we have quantified the IF1/ATP synthase beta subunit ratio using densitometry and have provided these ratios in Figure S4D, as done previously (Campanella et al, 2008).

Comment 39: Page 13: End of the first paragraph: UCP1 is expressed only in brown adipose tissue. This should be clearly stated.

Response 39: We have changed the sentence in question at the top of page 14 in the revised text to read “UCP2 and UCP3 typically account for 0.01 to 0.1% of mitochondrial inner membrane proteins, whereas UCP1, whose expression is confined to brown adipose tissue cells, accounts for up to 10% of the inner membrane protein mass”, as suggested.

Comment 40: Page 14: First paragraph: "Pattern of hPSCs glucose metabolism..." Is pattern the right term?

Response 40: We have changed the sentence in question to read “UCP2 expression in hPSCs suggests that it could regulate or influence glucose metabolism” on page 14 of the revised text, as suggested.

Comment 41: Although this is a beautiful experiment, the effects of UCP-2 silencing on metabolism are very small and thus not extremely convincing.

Response 41: Please see the detailed Response to Referee #3 Comment 4, above.

Comment 42: Page 15: First paragraph: "During which time..." this is strange language. "Measure the glucose contributed to mitochondrial oxidation..." this is strange language.

Response 42: The first sentence in question was changed to “during which” and the second sentence in question was changed to “the amount of labeled glucose that contributed to mitochondrial oxidation”, in the revised text on page 15, as suggested.

Comment 43: Also, a 13 % decrease in 13C incorporation is not very convincing.

Response 43: Please see the detailed Response to Referee #3 Comment 4, above.

Comment 44: What do the letters within brackets after the word glutamate mean?

Response 44: $\sum mn$ means the added sum of the number of carbon 13 molecules in each isotopomer multiplied by the probability of that isotopomer. Alternatively, it can represent the average number of carbon 13 molecules in the pool of all isotopomers.

Comment 45: Page 16: First paragraph: The main question remains: How does UCP-2 control access of substrate to oxidation in mitochondria? Maybe its blockade of pyruvate dehydrogenase.

Response 45: One proposed mechanism is that UCP2 functions as an anion transporter to shunt pyruvate (an anion) out of the mitochondria, limiting this carbon substrate for OXPHOS (Jezek & Borecky, 1998). The isotope tracing experiments provided in Figure 6H, 6I clearly show that UCP2 expression reduces glucose-derived pyruvate from entering into the mitochondria for oxidation and thereby augments glycolysis and the pentose phosphate pathway by providing additional carbon substrates. This mechanism is consistent with a role for UCP2 as an anion transporter. Other data argues that UCP2 does not function as a respiration uncoupler (Figure S6F and please see detailed Response to Referee #3 Comment 2, above). In sum, our data indicate that, in hPSCs, UCP2 limits the availability of the substrate of pyruvate dehydrogenase and does not block pyruvate dehydrogenase enzymatic activity itself.

Comment 46: Second paragraph: If ROS increases during differentiation, this probably goes in parallel with the increasing oxygen consumption. Steady state levels of ROS measured by various methods may simply reflect OCR, if they are a constant byproduct of electron flux.

Response 46: ROS may not be a constant byproduct of electron flux because the level of ROS is not only determined by the production of ROS, but also by the degradation or scavenging of ROS. ROS scavengers, such as SOD, GPX1, and catalase may change in expression and activity during hPSC differentiation (Cho et al, 2006). Therefore, changes in ROS levels can not be simply equated to changes in oxygen consumption or electron flux through the ETC.

Comment 47: Page 20: End of first paragraph: I am confused why the authors talk about oxygen concentrations. I do not see that any experiments modulating oxygen concentrations have been done in this paper, or did I overlook something?

Response 47: The reviewer did not overlook anything in regard to experiments with oxygen. Our point was to indicate that the metabolic phenotype of hPSCs is independent of the origin of hESCs, which are derived from the inner cell mass of blastocysts, which in the physiological setting of the female reproductive tract are hypoxic. In contrast, the hiPSCs used in this study were derived from fibroblasts in ambient (21%) oxygen. Since oxygen content has a large role in regulating metabolism, through hypoxia inducible factors (HIFs) and other mechanisms, we wanted to indicate that the metabolic phenotype reported here is independent of an influence from oxygen content because both hESCs and hiPSCs show the same overall phenotype.

Comment 48: Figure 4C: This is not a very convincing Western Blot. Why are bands for OCT4 and IF1 so broad, while beta-tubulin bands are so small?

Response 48: A new western blot was used to replace original Figure 4C in this revised version, as requested.

Comment 49: Figure 7: The abbreviations used in panel C are not very helpful.

Response 49: The revised manuscript text on page 17 was changed from “20% KSR → 20% FBS” to “20% KSR → 20% FBS, or spontaneous differentiation day 2 (SPD2), to indicate what exactly the abbreviations in Figure 7C are, as suggested.

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