nature portfolio

Peer Review File

Adipocytes signal to recruit specific mRNAs from surrounding cells to restore expression deficits

Corresponding Author: Dr Clair Crewe

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Crewe et al. report on a surprising observation after adipocyte-specific knock-out of the gene encoding neutral sphingomyelinase (SMPD3). They found a more than 100-fold increase in Smpd3 mRNA in adipocytes from adipocyte-specific SMPD3 knock-out mice compared to control adipocytes. The authors suggest that this phenomenon may be due to a communication between adipocytes and other cells of their microenvironment which respond to deficiency of Smpd3 mRNA in adipocytes by transferring more Smpd3 mRNA via extracellular vesicles back to adipocytes. Although this is an interesting observation, which may have broader implications, in its present form, the manuscript is very preliminary. Much more rigor is required to substantiate this observation. The analysis of the phenomenon often relies on only one method. In addition, the underlying mechanisms remain completely obscure, and the authors have not put much effort into understanding at least some of the fundamental processes underlying this phenomenon.

1. The huge, more than 100-fold upregulation of Smpd3 mRNA after knock-out of the corresponding gene is indeed very surprising. However, it is difficult to imagine any of the proposed mechanisms can account for this. A much more robust analysis of this upregulation is required in addition to the presented data based on qPCR. Is all of the signal representing full-length mRNA including non-translated regions? Do alternative methods (RNA-seq, Northern blotting, etc.) produce the same results? To what degree are the protein levels of SMPD3 affected?

2. If inter-cellular transfer of mRNA via EVs really occurs, one should be able to detect this. In Fig. 2G, the authors show that Smpd3 mRNA is only slightly and non-significantly increased in EVs from the knock-outs. This alone would not be sufficient to account for the huge upregulation of adipocyte Smpd3 mRNA. This can only be explained by a tremendous increase in the flux of EVs from the donor to the recipient cell type. Can the authors provide evidence for this?

3. The authors used rather indirect methods to support the model that Smpd3 mRNA from other cells of the adipose tissue is packaged into EVs and transferred to adipocytes. If indeed immune cells are the source, one would expect considerable changes in the EV-producing machinery in these immune cells as well as other changes reflecting the specific adaptation of the cells to loss of Smpd3 expression in adipocytes. Is there evidence for this? In addition, knock-out of Smpd3 in immune cells (in addition to KO in adipocytes) should abolish the upregulation of Smpd3 mRNA in adipocytes.

4. The mechanisms underlying the observed phenomenon remain largely unknown because the authors do not try to address even some obvious questions. For instance, the authors show that conditioned medium from adipocytes can carry the information of the Smpd3 knock-out in adipocytes to the donor cells. What are the physicochemical properties of this mediating agent? What initiates the signal in adipocytes? Loss of protein, loss of mRNA? The conditional knock-out leads to the deletion only of exon 3 of the gene encoding SMPD3. This usually allows the genes to be transcribed. Is there Smpd3 mRNA lacking the sequence encoded by exon 3 formed? Is it upregulated? Has a knock-down of Smpd3 using siRNA the same effect? What are the dynamics and mechanisms of Smpd3 upregulation in the donor cells? Which EV properties make sure that the EVs loaded with Smpd3 mRNA are delivered specifically to adipocytes? These and many other obvious questions answerable with standard methods should be addressed.

Reviewer #2

(Remarks to the Author)

The manuscript by Scherer et al highlights the intriguing observation that SMPD3 KO in adipocytes leads to intercellular transfer of Smpd3 mRNA from immune cells, most likely via EVs. The observation is substantiated via a set of well-designed in vitro and in vivo data which support the claim. While the observation remains descriptive and little mechanistic insight is provided, the subject is of interest to readers from the EV and adjacent fields, but is also of relevance to preparation of knockouts in general. The work would benefit from additional insights as follows:
- Figure 1A: Can the authors also demonstrate genomic deletion of Smpd3 by PCR amplifying the endogenous gene?
- The authors should check how SMPD3 protein is affected in adipocytes in the KO mice and whether it is upregulated in KO cells as a consequence of mRNA transfer.

Minor points:
- Abstract: Consider replacing "double-membraned" by "membraned/membraneous"

Reviewer #3

(Remarks to the Author)
This manuscript reports the transfer of Smpd3 mRNA from SVF to Smpd3-deficient adipocytes. The authors propose that Smpd3-deficient adipocytes send undetermined signals to CD45+ cells, which secrete extracellular Smpd3 mRNA and send them to adipocytes via EVs. This is an interesting finding. However, the authors did not demonstrate that EVs are the source of the Smpd3 mRNA. The authors need to address the following concerns to improve the manuscript.
Major concerns:
1) The authors propose that CD45+ cells send Smpd3 mRNA to adipocytes via EVs. However, they did not consider the possibility of other extracellular RNA sources. Ribonucleoprotein (RNP) complexes and lipoproteins are also carriers of extracellular RNA. The authors are suggested to design experiments to show that EVs, but not other vehicles are the source of mRNA. One experiment that could be done is to test whether the inclusion of RNase in the co-culture medium inhibits Smpd3 mRNA transfer from SVF/CD45+ cells to adipocytes.
2) In Fig.1, Smpd3 mRNA was increased 100-fold in Smpd3-deficient adipocytes, whereas not increased in SVF from the same mice. This is contradictory to the result of Fig.3A, where SVF Smpd3 was increased. Why did the signals from Smpd3-deficient adipocytes not induce Smpd3 expression in vivo, but did in vitro? On the other hand, if the Smpd3 expression was not induced in SVF cells in vivo, where did the Smpd3 mRNA in Smpd3-deficient adipocytes come from? The authors indicated that this was a snapshot and did not reflect turnover. If the Smpd3 mRNA increase was the result of an accumulation of continuous input over long-term, then the authors should demonstrate that the Smpd3 mRNA is very stable in these cells.
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4) Is the Smpd3 mRNA transfer restricted to SVF/CD45+ cells and adipocytes? In Fig3F, how about replacing the adipocytes with other cell types, but using Smpd3-Ko condition medium?
5) The authors are suggested to isolate EVs from co-culture system as Fig.3F, and analyze extracellular RNA by RNA-seq. Then they can compare the co-culture EV RNA data with that of Fig.2H, 2I.
Minor Comments:
Line 44: Please include citations for the 3 levels that EV can signal.
Line 53: Please include the complete name for SMPD3, sphingomyelin phosphodiesterase 3. And is the SMPD3 protein an alternative name for nSMase2? If so, can you specify that (“SMPD3 also known as nSMase2”).
Line 71: Epididymal white adipose tissue (eWAT)
Line 73: Subcutaneous white adipose tissue (sWAT)
Fig.2G: please define IEVs.

Reviewer #4

(Remarks to the Author)
This manuscript reports the transfer of Smpd3 mRNA from SVF to Smpd3-deficient adipocytes. The authors propose that Smpd3-deficient adipocytes send undetermined signals to CD45+ cells, which secrete extracellular Smpd3 mRNA and send them to adipocytes via EVs. This is an interesting finding. However, the authors did not demonstrate that EVs are the source of the Smpd3 mRNA. The authors need to address the following concerns to improve the manuscript.

Major concerns:

The authors propose that CD45+ cells send Smpd3 mRNA to adipocytes via EVs. However, they did not consider the possibility of other extracellular RNA sources. Ribonucleoprotein (RNP) complexes and lipoproteins are also carriers of extracellular RNA. The authors suggest designing experiments to show that EVs, but not other vehicles are the source of mRNA. One experiment that could be done is to test whether the inclusion of RNase in the co-culture medium inhibits Smpd3 mRNA transfer from SVF/CD45+ cells to adipocytes.

In Fig.1, Smpd3 mRNA was increased 100-fold in Smpd3-deficient adipocytes, whereas not increased in SVF from the same mice. This is contradictory to the result of Fig.3A, where SVF Smpd3 was increased. Why did the signals from Smpd3-deficient adipocytes not induce Smpd3 expression in vivo, but did in vitro? On the other hand, if the Smpd3 expression was not induced in SVF cells in vivo, where did the Smpd3 mRNA in Smpd3-deficient adipocytes come from? The authors indicated that this was a snapshot and did not reflect turnover. If the Smpd3 mRNA increase was the result of an

accumulation of continuous input over long-term, then the authors should demonstrate that the Smpd3 mRNA is very stable in these cells.

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Line 71: Epididymal white adipose tissue (eWAT)

Line 73: Subcutaneous white adipose tissue (sWAT)

Fig.2G: please define IEVs.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
None.

Reviewer #2

(Remarks to the Author)
The authors have adequately addressed my previous concerns. Although the exact mechanism underlying the reported phenomenon remains somewhat elusive, I believe it would be of value to the research community to get this work out.

Reviewer #3

(Remarks to the Author)
The authors have adequately addressed by concerns.

Reviewer #4

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

REVIEWER COMMENTS

We thank the reviewers for their time and thorough evaluation of our manuscript. In response to their comments, we have incorporated numerous new experiments into the revised version, which we believe substantially strengthen the work. Please see our point-by-point response to reviewer's concerns below.

Reviewer #1 (Remarks to the Author):
Crewe et al. report on a surprising observation after adipocyte-specific knock-out of the gene encoding neutral sphingomyelinase (SMPD3). They found a more than 100-fold increase in Smpd3 mRNA in adipocytes from adipocyte-specific SMPD3 knock-out mice compared to control adipocytes. The authors suggest that this phenomenon may be due to a communication between adipocytes and other cells of their microenvironment which respond to deficiency of Smpd3 mRNA in adipocytes by transferring more Smpd3 mRNA via extracellular vesicles back to adipocytes. Although this is an interesting observation, which may have broader implications, in its present form, the manuscript is very preliminary. Much more rigor is required to substantiate this observation. The analysis of the phenomenon often relies on only one method. In addition, the underlying mechanisms remain completely obscure, and the authors have not put much effort into understanding at least some of the fundamental processes underlying this phenomenon.

1. The huge, more than 100-fold upregulation of Smpd3 mRNA after knock-out of the corresponding gene is indeed very surprising. However, it is difficult to imagine any of the proposed mechanisms can account for this. A much more robust analysis of this upregulation is required in addition to the presented data based on qPCR. Is all of the signal representing full-length mRNA including non-translated regions? Do alternative methods (RNA-seq, Northern blotting, etc.) produce the same results? To what degree are the protein levels of SMPD3 affected?

We appreciate the reviewer's point about orthogonal methods. While we did show mostly qPCR data for adipocyte smpd3 expression in the original submission, we also used a hybridization method (molecular beacon) to show that Smpd3 mRNA is transferred to adipocytes. In the revised manuscript, we added RNA sequencing to demonstrate that the isolated knockout adipocytes contain the full-length Smpd3 mRNA, including all exons (Figure 1J). Interestingly, Smpd3 intronic reads were significantly reduced in ad-SMPD3KO adipocytes, suggesting that the *de novo* transcribed Smpd3 mRNA is reduced, but mature mRNA remains abundant. This is consistent with Figure 2C where we show nuclear Smpd3 mRNA is reduced. Both experiments suggest the adipocyte is not making its own Smpd3 mRNA. Protein levels are not changed in the knockout vs WT cells (Figure 1H).

The dramatic increase in adipocyte Smpd3 mRNA with the knockout has diminished over generations of breeding the ad-SMPD3KO line (Fig. 1I). Over the course of the last 5 years of breeding the ad-SMPD3 KO, we could no longer detect the 100-fold increase in Smpd3 mRNA in adipocytes between wild-type and knockout. However, there is clearly still mRNA transfer occurring because Smpd3 mRNA is not reduced with an adipocyte-specific Cre but it is with whole body Cre (Figure 2A). Furthermore, our *in vitro* data continues to validate the robust transfer of Smpd3 mRNA seen *in vivo*. We have still included the 100-fold increase in the manuscript because we think it demonstrates the robustness of this mechanism of mRNA recruitment.

2. If inter-cellular transfer of mRNA via EVs really occurs, one should be able to detect this. In Fig. 2G, the authors show that Smpd3 mRNA is only slightly and non-significantly increased in EVs from the knock-outs. This alone would not be sufficient to account for the huge upregulation of adipocyte Smpd3 mRNA.

This can only be explained by a tremendous increase in the flux of EVs from the donor to the recipient cell type. Can the authors provide evidence for this?

We agree with the reviewer that there must be an explanation for how Smpd3 becomes so strongly upregulated. Although this phenotype is less dramatic now (please see response to 1), an important point is that the Smpd3 mRNA level in the stromal vascular fraction (SVF) of adipose tissue is about 15-fold higher than in adipocytes (Fig. 2H) and is upregulated in the ad-SMPD3KO mice SVF (Fig. 1L) . Therefore, transfer of a small amount of the Smpd3 mRNA pool in SVF may have a larger relative effect on adipocyte mRNA levels. Furthermore, adding a larger number of animals, we do see a significant 2-fold increase in Smpd3 mRNA in tissue EVs (Fig. 2G). So, within a 2-week period, replenishment of adipocyte mRNA to levels higher than WT is plausibly achievable.

Our new results suggest there is no increase in the flux of EV release and uptake by SVF and adipocytes, respectively. We found no change in the amount of EVs released from SVF in response to adipocyte conditioned media (CM), and no change in adipocyte uptake of SVF EVs *in vitro* (Fig. S2C). However, knockout adipocyte CM strongly induces Smpd3 mRNA in $CD45^+$ cells and increases the amount of Smpd3 mRNA in $CD45^+$ cell EVs compared to WT CM (Fig. 5). This suggests that the amount of mRNA in EVs, not the flux, explains the phenomenon. Overall, *in vivo*, it is challenging to detect because the $CD45^+$ positive donating cell type is a minority of the SVF. In addition it is expected that any increase in SVF Smpd3 mRNA would be particularly hard to capture if the mRNA was indeed being exported from the cell. However, we were able to detect a significant increase in SVF Smpd3 mRNA early after induction of the knockout (Fig. 1L).

3. The authors used rather indirect methods to support the model that Smpd3 mRNA from other cells of the adipose tissue is packaged into EVs and transferred to adipocytes. If indeed immune cells are the source, one would expect considerable changes in the EV-producing machinery in these immune cells as well as other changes reflecting the specific adaptation of the cells to loss of Smpd3 expression in adipocytes. Is there evidence for this? In addition, knock-out of Smpd3 in immune cells (in addition to KO in adipocytes) should abolish the upregulation of Smpd3 mRNA in adipocytes.

We agree with this comment that our conclusions would be stronger with a concomitant knockout of Smpd3 in adipocytes and the donating cells. At the time of the initial manuscript, we did not know which immune cell type the donating cell was, making it impossible to do this double knock-out approach. The original reason we considered immune cells (potentially B and T cells) as the donating cell is that ad-SMPD3KO tissue EVs had a massive immune cell signature (mostly B and T cells signaling), more B and T cells were positive for Smpd3 mRNA after the knockout in adipocytes (scRNAseq data), and $CD45^+$ cells transferred Smpd3 mRNA *in vitro*. However, new experiments have revealed that the donating cell type is a sub-population of adipocyte progenitor cells (APCs) that highly upregulate CD45 in response to adipocyte Smpd3 KO, close to the levels seen in immune cells (Fig. 4D). These cells have a highly inflammatory expression profile (Fig. S4). APCs have the highest expression of Smpd3 among tissue cells (Fig. 4B), but in the initial submission, we demonstrated that sorted APCs did not transfer Smpd3 mRNA to adipocytes. To capture adipocyte progenitors and messechymal cells of interest we sorted to obtain CD45 negative cells. Thus, we were discarding the true APC population donating SMPD3 mRNA (CD45+;PDGFRa+). By scRNAseq, the APC population only showed significant expression changes in immune and fibrosis markers (Fig. S4; no changes in EV biogenesis genes). After confirming these immune cell-like APCs are the donating cell type we crossed our ad-SMPD3KO mouse with a pdgra-cre to knockout SMPD3 in mature adipocytes and APCs simultaneously. Figure 4G demonstrates the double

knockout resulted in a significant decrease in Smpd3 mRNA compared to control mice, in contrast to an increase in mRNA with the adipocyte-specific KO alone. This data confirms *in vivo* that APCs contribute to the transfer of Smpd3 mRNA.

4. The mechanisms underlying the observed phenomenon remain largely unknown because the authors do not try to address even some obvious questions. For instance, the authors show that conditioned medium from adipocytes can carry the information of the Smpd3 knock-out in adipocytes to the donor cells. What are the physicochemical properties of this mediating agent? What initiates the signal in adipocytes? Loss of protein, loss of mRNA? The conditional knock-out leads to the deletion only of exon 3 of the gene encoding SMPD3. This usually allows the genes to be transcribed. Is there Smpd3 mRNA lacking the sequence encoded by exon 3 formed? Is it upregulated? Has a knock-down of Smpd3 using siRNA the same effect? What are the dynamics and mechanisms of Smpd3 upregulation in the donor cells? Which EV properties make sure that the EVs loaded with Smpd3 mRNA are delivered specifically to adipocytes? These and many other obvious questions answerable with standard methods should be addressed.

We agree with this reviewer that understanding the mechanism of mRNA recruitment is important. In the revised manuscript, we have done experiments to gain a better understanding, although the mechanism still eludes us, especially due to the selectivity of the mechanism for Smpd3 mRNA recruitment. We report in the revised manuscript that the signal sent from adipocytes is likely in a high-density EV (Fig. 3B). We do not know what initiates the signal in adipocytes, but it is likely a reduction in the transcription of the Smpd3 gene. We harvested adipocytes at acute timepoints after the doxycycline-induced knockout and could only detect a reduction in pre-mRNA, never a change in mRNA (Fig. 1K) or protein (Fig. 1H). Likewise, siRNA knockdown of Smpd3 *in vitro* did not stimulate Smpd3 mRNA transfer when co-cultured with SVF (Fig. S2A-B). These data suggest that the transfer mechanism may be initiated in the nucleus with a reduction in Smpd3 transcription.

With regards to a transcriptional product in adipocytes, our sequencing experiment demonstrates all exon sequences are present at the same abundance (Fig. 1J). So, there is likely no transcript produced that lacks exon 3 or is truncated. Finally, we found that the signal adipocytes send to CD45+ preadipocytes induces the *transcriptional* upregulation of Smpd3 in the receiving cells. CD45+ cells incubated in adipocyte conditioned media results in a 4-fold increase in Smpd3 mRNA which is almost completely prevented if cells were co-treated with actinomycin D to block transcription (Fig. 5A). The same was seen with Smpd3 pre-mRNA (Fig. 5B). Lastly, we found that Smpd3 mRNA is likely transported back to adipocytes in EVs, as the size of particles carrying Smpd3mRNA is ~1um (Fig 5D), likely too large for a ribonuclear protein complex.

<u>Reviewer #2 (Remarks to the Author):</u>

The manuscript by Scherer et al highlights the intriguing observation that SMPD3 KO in adipocytes leads to intercellular transfer of Smpd3 mRNA from immune cells, most likely via EVs. The observation is substantiated via a set of well-designed in vitro and in vivo data which support the claim. While the observation remains descriptive and little mechanistic insight is provided, the subject is of interest to readers from the EV and adjacent fields, but is also of relevance to preparation of knockouts in general.

We thank this reviewer for these positive comments. We would like to direct your attention to our response to Reviewer 1 #3 and 4, where we have done new mechanistic experiments. Although we have not fully elucidated the mechanism, the new experiments solidify our conclusions that adipocytes are recruiting Smpd3 mRNA.

The work would benefit from additional insights as follows:

1) Figure 1A: Can the authors also demonstrate genomic deletion of Smpd3 by PCR amplifying the endogenous gene?

In Figure 1A, we amplified a PCR product that would only be present if Smpd3 exon 3 was cut out and the DNA recombined. Additionally, sequencing demonstrates a loss of Smpd3 pre-mRNA (Fig. 1J), suggesting the knockout worked to suppress Smpd3 transcription, although the mature mRNA remains abundant.

2)The authors should check how the SMPD3 protein is affected in adipocytes in the KO mice and whether it is upregulated in KO cells as a consequence of mRNA transfer.

We thank the reviewer for this comment. Despite the increase in mRNA, we did not detect any changes in Smpd3 protein in knockout adipocytes (new Fig.1H).

Minor points:
- Abstract: Consider replacing "double-membraned" by "membraned/membraneous". We have now changed it to “membrane-delimited” to make this sentence clearer.

Reviewer #3 (Remarks to the Author):

This manuscript reports the transfer of Smpd3 mRNA from SVF to Smpd3-deficient adipocytes. The authors propose that Smpd3-deficient adipocytes send undetermined signals to CD45+ cells, which secrete extracellular Smpd3 mRNA and send them to adipocytes via EVs. This is an interesting finding. However, the authors did not demonstrate that EVs are the source of the Smpd3 mRNA. The authors need to address the following concerns to improve the manuscript.
Major concerns:
1) The authors propose that CD45+ cells send Smpd3 mRNA to adipocytes via EVs. However, they did not consider the possibility of other extracellular RNA sources. Ribonucleoprotein (RNP) complexes and lipoproteins are also carriers of extracellular RNA. The authors are suggested to design experiments to show that EVs, but not other vehicles are the source of mRNA. One experiment that could be done is to test whether the inclusion of RNase in the co-culture medium inhibits Smpd3 mRNA transfer from SVF/CD45+ cells to adipocytes.

This is an excellent experiment suggestion. We repeated the Transwell molecular beacon transfer experiment with or without 100µg/ml RNaseI. Interestingly, RNAseI had no effect on transfer for Smpd3 mRNA to WT adipocytes, but it suppressed transfer to KO adipocytes by 50% (Fig. 5E). This suggests that there may be two mechanisms of transfer. One that is constitutive, and one that is induced by the knockout in adipocytes. This RNAse-sensitive, induced mechanism could be RNPs, but also could be RNA on the outside of EVs. Evidence for the latter is that Smpd3 mRNA is associated with large, ~1 µm-sized particles (Fig. 5D), and our EM images in Figure 2 suggest no structures that look like RNPs in tissue EVs that we confirmed have Smpd3 mRNA. We have discussed this point in the revised manuscript.

2) In Fig.1, Smpd3 mRNA was increased 100-fold in Smpd3-deficient adipocytes, whereas not increased in SVF from the same mice. This is contradictory to the result of Fig.3A, where SVF Smpd3 was increased. Why did the signals from Smpd3-deficient adipocytes not induce Smpd3 expression in vivo, but did in

vitro? On the other hand, if the Smpd3 expression was not induced in SVF cells in vivo, where did the Smpd3 mRNA in Smpd3-deficient adipocytes come from? The authors indicated that this was a snapshot and did not reflect turnover. If the Smpd3 mRNA increase was the result of an accumulation of continuous input over long-term, then the authors should demonstrate that the Smpd3 mRNA is very stable in these cells.

We agree that this needs to be reconciled. Please see the discussion in response to Reviewer 1, comment #2. The Smpd3 mRNA level is ~15 fold higher in SVF compared to adipocytes, making it more likely that any Smpd3 transfer could result in high levels in adipocytes without the need for the mRNA to be long lived.

With regards to Smpd3 upregulation of Smpd3 *in vivo*, 2 weeks after induction of the knockout, the timepoint we have conducted most of the experiments, we did not detect an increase in Smpd3 mRNA in the donating cell *in vivo* by qPCR or the scRNAseq data. So we instead harvested SVF and adipocytes after acute induction (4-day dox exposure), and we could detect a ~50% increase in Smpd3 mRNA in SVF (Fig. 1L). We have shown with RNA scope that Smpd3 mRNA is transferred to adipocytes *in vivo*, and so we may not expect to see an increase in the donating cell if it is exporting the mRNA. The transfer may not be as efficient *in vitro*, which allows us to more easily detect an increase in the donating cell.

3) In Fig.3A, the authors observed that the KO of Smpd3 in adipocytes induced SVF Smpd3. Why was there no Smpd3 mRNA transfer to adipocytes in this setting?

This is an important point. We repeated this *in vitro* experiment in the new Figure 3A, but instead of culturing the cells with doxycycline for 2 days (as in the original manuscript), we incubated them for 4 days. When we harvested the adipocytes and undifferentiated SVF, we saw a greater than 2-fold increase in Smpd3 mRNA in both adipocytes and SVF. The temporal aspect of this experiment also strengthens our conclusion: 2 days of culture results in only Smpd3 mRNA increase in SVF, whereas 4 days results in an increase in both the “knockout” adipocytes and SVF. Ths, in addition to other experiments in the manuscript, supports the conclusion that the Smpd3 mRNA is transferred from SVF cells.

4) Is the Smpd3 mRNA transfer restricted to SVF/CD45+ cells and adipocytes? In Fig3F, how about replacing the adipocytes with other cell types, but using Smpd3-Ko condition medium?

Thank you for this interesting thought. Although it is possible that the transfer occurs to cells other than adipocytes, a screen would likely be required to find these cells. This is not feasible as there is no experimental design that would allow us to identify these cells outside of manually switching out one cell type for another. At least, we know that Smpd3 is not transferred to hepatocytes, as a hepatocyte-specific knockout results in efficient loss of Smpd3 mRNA (Fig. 2B).

5) The authors are suggested to isolate EVs from co-culture system as Fig.3F, and analyze extracellular RNA by RNA-seq. Then they can compare the co-culture EV RNA data with that of Fig.2H, 2I.

This would be an interesting experiment; however, it is not feasible for our culture system. Generally, we would need between 100 to 200 ml of conditioned media to isolate enough EVs for an RNAseq experiment. Our co-culture is done in Transwell plates where 500 µL of media is exposed to both cell types. Even if we tried to increase the plate size, it would be unlikely that we could get enough $CD45^+$

cells. The cells that survive the culture conditions are rare compared to the total SVF and only participate in the transfer of Smpd3 mRNA to adipocytes up to passage 1.

Minor Comments:
Line 44: Please include citations for the 3 levels that EV can signal.
Line 53: Please include the complete name for SMPD3, sphingomyelin phosphodiesterase 3. And is the SMPD3 protein an alternative name for nSMase2? If so, can you specify that ("SMPD3 also known as nSMase2").
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Fig.2G: please define IEVs.

These corrections have been made.

REVIEWER COMMENTS

We appreciate the time the reviewers took to assess the revision.

Reviewer #1
None.

Reviewer #2
The authors have adequately addressed my previous concerns. Although the exact mechanism underlying the reported phenomenon remains somewhat elusive, I believe it would be of value to the research community to get this work out.

We continue to work on the mechanism and hope to have a follow-up paper describing it in detail.

Reviewer #3
The authors have adequately addressed by concerns.

Reviewer #4:
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.