Lysophosphatidic acid acts as a nutrient-derived developmental cue to regulate early hematopoiesis

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Editor: Andrea Leibfried

1st Editorial Decision 13 September 2013

Thank you for submitting your manuscript entitled 'Lysophosphatidic acid acts as a nutrient-derived developmental cue to regulate early hematopoiesis'. I have now received the reviews from all referees.

The referees think that your analysis is of value to the field of hematopoiesis and development. However, and as you can see in the reviewers' comments below, they think that your data do not sufficiently support your conclusions and they raise strong technical concerns. Given these opinions from trusted experts in the field, I am sorry to say that we are unable to offer publication in The EMBO Journal.

I thank you in any case for the opportunity to consider this manuscript. I am sorry I cannot be more positive on this occasion, but I hope nevertheless that you will find our referees' comments helpful.
REFEREE COMMENTS

Referee #1

Review: Lysophosphatidic acid acts as a nutrient-derived developmental cue to regulate early hematopoiesis.

Li et al describe the importance of ATX/LPA signaling through the LPAR1 receptor in the in vitro generation of hemangioblasts from mouse embryoid bodies as well as in in vivo primitive hematopoiesis in the zebrafish. They also use the in vitro model to determine that LPA signaling leads to phosphorylation of Akt and SMAD 1/5/8. Their studies represent a new investigation into the role of small lipids in regulating hematopoiesis and development, a field that has not been well studied. Their studies are quite thorough, with a few exceptions, especially regarding the molecular mechanisms presented.

Major Concerns

1. The authors should verify that LPA signals only via LPAR1/3, as well as the specificity of the inhibitors of these receptors. The authors should perform combined treatment on EBs using LPA and the two LPAR inhibitors, which should prevent the effect of LPA treatment. So far they've done this only in the context of additional stimulation with BMP4 (Fig. S3D). To ensure that LPA signals via the LPAR1 receptor, the authors should treat LPAR1 siRNA EBs with LPA, and no effect should be seen.

2. In all cases of combined drug treatments, the authors treat with LPA alone as well as with LPA and a second drug, but never the second drug alone, omitting an important control. For instance, in Fig. 5B, the authors compare a combined treatment of LY294002 and LPA to LPA treatment alone, without treating with LY294002 alone. As is, it is impossible to say whether inhibition of PI3K reduces only LPA-induced changes in the number of CD41+ cells, or PI3K inhibition in general reduces the CD41+ population.

3. In the literature there is little evidence for crosstalk between PI3K/Akt and SMAD 1/5/8 signaling pathways, as the authors here claim. The one paper they cite (Segrelles et al 2008) in fact describes an inhibitory influence of PI3K on SMAD phosphorylation, while the present authors claim the opposite. It is quite surprising that LPA could induce SMADs directly, since most SMAD signaling occurs through the BMP receptors. The authors should treat EBs with LPA and a BMP receptor inhibitor, and evaluate the impact on p-SMAD and maybe a target gene (or SMAD reporter). They should also treat with BMP and the Ki16425 to look for interaction, and disruption of SMAD signaling.

Minor Concerns

1. The authors found that the expression level of gata2 increased upon treatment with LPA (Fig. S3A), as well as with knockdown of LPAR1/3 (Fig. 2C). These results appear contradictory and should be discussed.

2. For completeness, the authors should describe the timing of expression of LPAR3 in the zebrafish. In addition, the late onset of expression of LPAR3 in differentiating embryoid bodies likely explains its lack of importance in LPA signaling in this assay. This should be mentioned in the text.

3. The qPCR data in figure 3c should include a pan-mesoderm marker to determine whether LPAR1/3 inhibition specifically impairs blood development or inhibits general mesodermal development.

4. Given that knockdown or inhibition of LPAR1/3 reduces hemangioblast formation, it is surprising that LPA itself does not stimulate hemangioblast formation (Fig. S5). Was a dose curve attempted in this experiment? Perhaps a higher concentration of LPA is necessary for stimulation at this earlier stage.

5. Minor grammatical errors throughout, including figure legends. In Fig. S1, the graph axis is mislabeled. Additionally, side-by-side FACS plots for treated and untreated samples should be presented with the same axes (for example, Fig. 1A and 3A).
Referee #2

The ATX-LPA signaling pathway has previously been shown to be involved in vascular development. In this report, Li et al investigate the role played by ATX-LPA signaling in vertebrate primitive hematopoiesis. The authors initially observed that the expression of LPA receptors, LPAR1 and LPAR3, in differentiating embryoid bodies (EBs) peaked at the time hemangioblasts are specified, suggesting that this signaling pathway may be involved in hematopoietic differentiation. Pharmacological and genetic inhibition of the ATX-LPA pathway in the EB system resulted in a decrease of hematopoietic output which could not be attributed to apoptosis, confirming that this pathway influences blood differentiation. Importantly they show that LPAR1 and not LPAR3 is required to mediate ATX signaling and that the number of Flk1+ cells and BL-CFCs is decreased further suggesting that this pathway is required for hemangioblast differentiation. These results were supported in vivo using the zebrafish system. In addition, the authors show that inhibition of the ATX-LPA pathway in EBs led to decreased levels of phospho-Smad and phospho-Akt and that Akt pharmacological inhibition impaired CD41+ cell induction by LPA. Thus, an Akt-Smad pathway appears to mediate ATX-LPA signaling in primitive hematopoiesis.

This work presents evidence suggesting the involvement of ATX-LPA signaling in primitive hemangioblast differentiation which is of interest in the field of developmental hematopoiesis. However, the experimental design lacks sufficient strength to support the main claims of the paper.

Apart from Figure 3, the whole study is based on the analysis of whole EBs and, therefore, the results are difficult to interpret. For example, the authors conclude that an Akt-Smad signaling cascade mediates ATX-LPA action but whether decreased Smad and Akt phosphorylation is taking place in the same cells, i.e. hemangioblasts or hematopoietic cells, is not known. Because whole EBs were used in these analyses, Akt and Smad phosphorylation could be happening in completely different cell populations. These studies need to be performed on Flk1+ cells, which are enriched for the hemangioblasts. Also, to support the authors’ claim of cross-talk with BMP signalling, rescue of BMP inhibition with activated Akt and vice versa needs to be performed. It would also be interesting to investigate the effects of Akt and BMP inhibition on their own.

BL-CFC and hematopoietic output should be monitored using Flk1+ cells. Similarly, all pharmacological and genetic inhibitions should be performed on Flk1+ cells as well as Akt and Smad phosphorylation assays. Clear expression differences are observed when ATX signalling is inhibited on whole EBs (Figure 1 and 2) when compared to inhibition on purified Flk1+ cells (Figure 3), particularly for Gata2, highlighting further the need to perform experiments on purified Flk1+ cells.

Are the ATX receptors expressed by Flk1+ cells or hematopoietic cells at all? The authors observed that LPAR3 expression in EBs peaked at the time hemangioblasts are specified but transfection with Lpar3 RNAi has no effect on hematopoiesis, suggesting that this receptor is not expressed in hematopoietic cells.

The authors claim that their zebrafish experiments indicate that ATX signaling is required for hemangioblast specification, but this contradicts published results. In the zebrafish, a lack of hemangioblast specification results in the absence of differentiated blood and endothelial cells. Nevertheless, in ATX morphants the axial vessels develop normally and only defects in segmental artery sprouting is observed (Yukiura et al). Moreover, Yukiura et al report red cells in circulation, indicating that hemangioblast specification is not affected. These issues need clarification. The authors should analyse the expression of endothelial and myeloid genes in addition to more hemangioblast markers.

The authors claim that LPA signaling regulates zebrafish primitive hematopoiesis through LPAR1 and not LPAR3; however, Chiang et al reported a deficiency in red cell differentiation in LPAR3 morphants. This discrepancy requires clarification.

The authors use Dorsomorphin as a BMP inhibitor when this compound has been demonstrated to inhibit the VEGF receptor, Flk1, which could explain the massive effect on CD41+ cell production. Also, Akt is known to mediate VEGFA-Flk1 signaling.
The morpholino oligonucleotide used should be referenced and the concentration indicated.

Appeal 23 September 2013

Thank you for editing our manuscript entitled "Lysophosphatidic acid functions as a nutrient-derived developmental cue to regulate early hematopoiesis" (EMBOJ-2013-86613). We are grateful to the reviewers for their comments and constructive suggestions that will greatly improve the quality of this manuscript.

Apparently, Reviewer #1 has carefully read our manuscript and given positive feedbacks towards our work. The questions he/she raised are mainly about the mechanistic part, which are all doable and could be finished within a very short period of time. In contrast, Reviewer #2 seemed to briefly go through the manuscript without carefully evaluating our work. The following are just a few examples:

1) We performed all the experiments in whole EBs to determine the effect of ATX-LPAR1 signaling on hematopoietic differentiation from figure 1 to figure 4, as shown in our figure legend, but Reviewer #2 thought that we analyzed purified Flk1+ cells in figure 3. The reason why we are analyzing whole EBs at the beginning is to establish a solid phenomenon using both genetic and pharmacological approach. As we mentioned in the manuscript, mESC first give rise to Flk1+ hemangioblasts which then further differentiate into CD41+ hematopoietic progenitor cells. The finding of CD41+ differentiation defects in figure 1 and 2 led us to analyze whether Flk1+ hemangioblast has any defects in figure 3 and 4. We do agree that the following mechanistic study would have been more informative if we analyzed the Flk1+ cells (we are now actually repeating all the experiments in figure 5 using Flk1+ cells), but it is unreasonable to ask us to analyze Flk1+ cells only at the beginning.

2) Reviewer #2 especially asked us to do the BL-CFC assay using Flk1+ cells. However, BL-CFC is a functional assay that quantifies the absolute number of Flk1+ hemangioblasts within the whole EBs (Kennedy et al, 1997). If we sort Flk1+ cells first and then do the assay, there would be no differences at all.

3) Reviewer #2 asked whether ATX receptor is expressed on Flk1+ cells or hematopoietic cells. However, we made it very clear in both the abstract and introduction section that ATX is a secretory lysophosphalipase that catalyzes LPA production, which doesn't have a receptor at all.

4) Reviewer #2 questioned that we observed LPAR3 expression in EBs peaked at the time hemangioblasts are specified but transfection with Lpar3 RNAi has no effect on hematopoiesis, suggesting that this receptor is not expressed in hematopoietic cells. However, we clearly mentioned in the manuscript that Flk1+ hemangioblast is specified between day 3 and day 4 during EB differentiation, while Lpar3 expression was not detected until day 6 and day 8 (Figure S1). In contrast, Lpar1 expression was detected as early as day 4 (Figure S1), which actually explained why Lpar1 but not Lpar3 regulates hemangioblast formation.

Taken together, we believe that Reviewer #2 is giving a biased and unfair evaluation towards our paper, and thus we kindly ask for a third referee to review our manuscript again. We have previously published high quality paper in The EMBO Journal, and we keep looking forward to publishing the most innovative findings in this journal.

2nd Editorial Decision 27 September 2013

Thank you for your recent correspondence regarding your manuscript entitled 'Lysophosphatidic acid acts as a nutrient-derived developmental cue to regulate early hematopoiesis'. In light of your comments, I have carefully reassessed your manuscript and the referees' reports.
I noticed that there might be some misinterpretations regarding the report from referee #2 and I thus understand your concerns.

However, both referees raised even further reaching issues, which in our view preclude at least immediate proceedings at our very selective title. Specifically, in light of conflicting previous publications, the functional evidence for LPA-signaling via the proposed LPAR-PI3K-Smad axis would need definitive validation, including rather genetic than chemical approaches and distinguishing these from potential VEGF-effects.

That being said, as you are proposing to perform significant amendments of your manuscript along these critical points raised by both expert scientists, I would be prepared to re-assess such an improved version of your manuscript. Please note however, that this would have to be treated as a new submission, being evaluated afresh - also in light of novelty at time of submission - and, if applicable, I would tend to go back to referee #1 while also involving a fresh referee for an unbiased opinion.

Please do consider the amount of necessary efforts and substantial further experimentation carefully, also to avoid possible disappointment much later in the process. If you find it too demanding to address these concerns raised appropriately, I believe it would be in your best interest to seek rapid and possibly immediate publication at a less selective title.

Resubmission 04 December 2013

For the resubmission, we have performed a number of new experiments and revised the manuscript accordingly. The following major revisions have been made:

1) We have tested the specificity of LPAR1 in mediating LPA effect by combining LPA treatment with LPAR1/3 inhibitor treatment or lpar1 knockdown, which demonstrated that LPA promotes hematopoietic differentiation via LPAR1.

2) We have tested the effect of PI3K or Akt inhibitor alone on CD41^+ cell percentage by flow cytometry analysis. The data showed that PI3K or Akt inhibition alone does not affect the CD41^+ cell percentage.

3) We have further validated the crosstalk between PI3K/Akt and BMP/Smad pathway through analyzing Smad phosphorylation, which demonstrated that LPA signaling regulates hematopoietic differentiation via LPAR1-PI3K/Akt-Smad axis.

4) We have assessed the effect of different concentrations of LPA on hemangioblast formation by analyzing flk1^+ cell percentage, which showed that higher concentrations of LPA (10-30 µM) did promote hemangioblast formation.

5) We have analyzed primitive myeloid markers l-plastin and mpo at 26 hpf after LPAR inhibition, which showed that there was a significant defect of primitive myelopoiesis.

6) We have tested whether VEGFR inhibitor abolished the effect of LPA treatment on CD41^+ cell percentage by flow cytometry analysis, and demonstrated that there is no crosstalk between LPA signaling and Flk1 signaling.

Our point-to-point responses to the reviewer are included in the following page. As stated in our previous rebuttal letter, we found that reviewer #2 gave a rather biased evaluation on our manuscript.
and we prefer a third reviewer to carefully judge our manuscript. Thank you very much for the chance of resubmission and we hope to get a positive feedback by *The EMBO Journal*.

Referee #1

We thank the reviewer for the helpful advice that have greatly improved the quality of our manuscript.

**Major Concerns**

1. *The authors should verify that LPA signals only via LPAR1/3, as well as the specificity of the inhibitors of these receptors. The authors should perform combined treatment on EBs using LPA and the two LPAR inhibitors, which should prevent the effect of LPA treatment. So far they've done this only in the context of additional stimulation with BMP4 (Fig. S3D). To ensure that LPA signals via the LPAR1 receptor, the authors should treat LPAR1 siRNA EBs with LPA, and no effect should be seen.*

We performed the experiments as suggested. Our data showed that LPAR1 inhibition by either LPAR1/3 inhibitors treatment, or siRNA-mediated knockdown abolished the effect of LPA, indicating that LPA promotes hematopoietic differentiation via LPAR1 (Supplementary Figure 3D and 3E).

2. *In all cases of combined drug treatments, the authors treat with LPA alone as well as with LPA and a second drug, but never the second drug alone, omitting an important control. For instance, in Fig. 5B, the authors compare a combined treatment of LY294002 and LPA to LPA treatment alone, without treating with LY294002 alone. As is, it is impossible to say whether inhibition of PI3K reduces only LPA-induced changes in the number of CD41+ cells, or PI3K inhibition in general reduces the CD41+ population.*

We repeated the experiment in Figure 5B by adding the suggested second drug alone controls. The new results showed that PI3K inhibitor or Akt inhibitor treatment alone does not reduce the CD41+ cell percentage. In addition, we also tested the effect of Gi, ROCK, Rac1, and PKC inhibitors, and found that these inhibitors treatment alone do not decrease the CD41+ population (Supplementary Figure 7D).

3. *In the literature there is little evidence for crosstalk between PI3K/Akt and SMAD 1/5/8 signaling pathways, as the authors here claim. The one paper they cite (Segrelles et al 2008) in fact describes an inhibitory influence of PI3K on SMAD phosphorylation, while the present authors claim the opposite. It is quite surprising that LPA could induce SMADs directly, since most SMAD signaling occurs through the BMP receptors. The authors should treat EBs with LPA and a BMP receptor inhibitor, and evaluate the impact on p-SMAD and maybe a target gene (or SMAD reporter). They should also treat with BMP and the Ki16425 to look for interaction, and disruption of SMAD signaling.*
We carried out new experiments by treating EBs with LPA and BMP inhibitor Dorsomorphin, or with BMP and LPA1/3 inhibitor VPC32183 to demonstrate that LPAR1-PI3K/Akt pathway does crosstalk with BMP pathway through Smad (Supplementary Figure 7E and 7F). Interestingly, our observation is consistent with previously finding that ectopic expression of constitutively active form of AKT enhances Smad phosphorylation (Jin et al, 2011). The discrepancy between our data and Segrelles’s report is probably due to the fact that these experiments are carried out in different cells types. We have deleted this misleading citation in the revised manuscript and cited the paper by Jin et al.

Minor Concerns

1. The authors found that the expression level of gata2 increased upon treatment with LPA (Fig. S3A), as well as with knockdown of LPAR1/3 (Fig. 2C). These results appear contradictory and should be discussed.

The increased gata2 expression after lpar1 or lpar3 knockdown was very mild (40%-60% increase), although they were statistically significant. Together with the fact that simultaneous knockdown of lpar1 and lpar3 didn’t significantly increase gata2 expression (Figure 2C), and that Lpar1/3 inhibitors didn’t increase gata2 expression (Figure 1C), we believe that the discrepancy was due to experimental variations.

2. For completeness, the authors should describe the timing of expression of LPAR3 in the zebrafish. In addition, the late onset of expression of LPAR3 in differentiating embryoid bodies likely explains its lack of importance in LPA signaling in this assay. This should be mentioned in the text.

We do agree with the reviewer. To address the issue, we examined the expression profile of lpar3 during zebrafish embryonic development by qPCR analysis. The data showed that lpar3 expression first appeared at 6 hpf and basically stay constant after 24 hpf (Supplementary Figure 1E). We also mentioned the late onset of lpar3 and the potential correlation with its lack of importance in regulating hematopoiesis in the text (Page 9 line15).

3. The qPCR data in figure 3c should include a pan-mesoderm marker to determine whether LPAR1/3 inhibition specifically impairs blood development or inhibits general mesodermal development.

To address the issue, we evaluated the expression level of pan-mesoderm marker Brachyury after LPAR1/3 inhibition with qPCR. The data showed that LPAR1/3 antagonism or Lpar1 siRNA do not decrease the expression level of Brachyury (Figure 3C and 4B). In addition, the expression level of Brachyury was not down-regulated after ATX inhibition (Figure 3F and 4E). These results suggested that LPA signaling inhibition does not generally affect mesodermal development.

4. Given that knockdown or inhibition of LPAR1/3 reduces hemangioblast formation, it is surprising that LPA itself does not stimulate hemangioblast formation (Fig. S5). Was a dose curve attempted in
this experiment? Perhaps a higher concentration of LPA is necessary for stimulation at this earlier stage.

As suggested, we examined the effect of different concentrations of LPA on Flk1+ cell percentage by flow cytometry. Interestingly, we found that higher concentrations of LPA (10-30 µM) significantly promoted hemangioblast formation (Supplementary Figure 5A).

6. Minor grammatical errors throughout, including figure legends. In Fig. S1, the graph axis is mislabeled. Additionally, side-by-side FACS plots for treated and untreated samples should be presented with the same axes (for example, Fig. 1A and 3A).

We have carefully looked through the manuscript and tried our best to correct all these errors.

3rd Editorial Decision 07 January 2014

Thank you for re-submitting your manuscript entitled 'Lysophosphatidic acid acts as a nutrient-derived developmental cue to regulate early hematopoiesis'. As discussed previously, I have involved a new referee in order to have an arbitrating opinion on your resubmitted work. I have now received the report on your paper, which you can find below. Please excuse the delay in getting back to you, which was due to the christmas holiday season.

As you can see, the referee appreciates your work. However, a few additional experiments are still required to better support your data and claims. Please include a validation/reference of all morpholinos used. We would also appreciate the addition of the proposed test for an in vivo control of hemangioblast expansion via LPA signaling.

Given the clear comments provided, I would like to invite you to submit a revised version of the manuscript, addressing the remaining concerns of the referee.

I should also add that it is EMBO Journal policy to allow only a single round of revision and that it is therefore important to address the concerns raised at this stage.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

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REFEREE COMMENTS

Referee #1:

The authors have investigated the role of lysophosphatidic acid (LPA) a metabolite derived from phosphatidylcholines (PC), in primitive hematopoiesis combining in vitro and in vivo approaches in mouse EBs and zebrafish embryos.

LPA can act as a signaling molecule through G protein-coupled receptors LPA receptors 1 to 6. Hydrolyzation of PC into LPA is catalyzed by the lysosphosphalipase ATX. Using small molecule inhibitors and siRNA targeting LPA receptors 1 and 3 and ATX, the authors showed that disruption of LPA/LPAR1/ATX signaling pathway decreases the number of CD41+ cells which likely results from the downregulation of transcription factors involved in blood formation more than from an increase of apoptosis. Methylcellulose assays revealed a reduced number of Ery-P, CFU-E and CFU-G/M/GM confirming that LPA pathway supports hematopoietic differentiation. Further analysis showed that LPA/LPAR1/ATX signaling pathway is also required at early stage of hemangioblast development, by modulating the number of BL-CFCs and flk1+ precursors and their molecular program without affecting earlier mesoderm, endoderm or ectoderm specification.
Finally, the authors showed that the effect on CD41+ cells seen upon LPA/LPAR1/ATX signaling pathway modulation is mediated through PI3K/Akt which regulates Smad1 phosphorylation without affecting Erk1/2 activity. This finding is interesting as it reveals an unconventional regulation of smad independently of bmp receptor.

Overall, the data presented in this paper are solid, and the authors combined several approaches in two systems to analyze LPA/LPAR1/ATX signaling pathway requirements during primitive hematopoiesis using small chemicals, siRNA or by adding LPA in serum-free culture systems. To validate their findings in vivo, they knocked down LPAR1 and 3 and ATX in zebrafish embryos. To do so, the authors used morpholinos which overall phenocopy what they observed in EB cultures. One concern about their approach is the lack of validation of the morpholinos used in this study (at this exception of LPAR3 which was previously published in Chiang et al., 2011). The authors should either reference the morpholinos used or show their validation in the supplemental figures.

Although it is not essential, it would have also been interesting to treat zebrafish embryos with LPA to observe whether or not LPA signaling also controls expansion of hemangioblasts in vivo.

Finally, the authors addressed the constructive concerns raised by referee 1. However, most of the figures are dense, which will probably affect the clarity of the presentation. It would likely help to transfer data obtained with the compound VPC32183 to supplemental data and fuse the data from Ki6425 and HA130 experiments on the same chart whenever possible in order to facilitate clarity.

Revision - authors' response 24 March 2014

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We thank the reviewer for this helpful advice. To validate the specificity of Atx and Lpar1 morpholinos (MOs), we first coinjected Atx MO or Lpar1 MO with a reporter mRNA containing the MO binding site and immediately followed by the EGFP gene. The results showed that MO co-injection dramatically inhibited EGFP expression (Supplementary Figure 9A-H). In addition, we carried out lpar1 mRNA rescue experiment, and found that the hematopoietic defects caused by Lpar1 MO injection could be rescued by co-injection of lpar1 mRNA (Supplementary Figure 12).

Although it is not essential, it would have also been interesting to treat zebrafish embryos with LPA to observe whether or not LPA signaling also controls expansion of hemangioblasts in vivo.

To address this question, we carried out WISH analyses after different doses of LPA treatment. The results showed that lower dose of LPA (10 µM) did not promote primitive hematopoiesis (figure not included in this file), while higher dose of LPA (100 µM) treatment is embryonic lethal (data not shown). The fact that LPA treatment did not further promote primitive hematopoiesis in zebrafish is possibly due to the abundant phosphatidylcholines in the yolk sac region, which can produce excess amount of LPA required for activating LPAR1.

Finally, the authors addressed the constructive concerns raised by referee 1. However, most of the figures are dense, which will probably affect the clarity of the presentation. It would likely help to transfer data obtained with the compound VPC32183 to supplemental data and fuse the data from Ki6425 and HA130 experiments on the same chart whenever possible in order to facilitate clarity. As suggested, we deleted the VPC32183 data from old Figures 1 and 3, and incorporated them into the new Supplementary Figure 2.

4th Editorial Decision 10 April 2014

I have now received comments (see below) from the original referee of your resubmitted manuscript who is satisfied with the amount of revisions and supports publication.

I am thus pleased to accept your manuscript for publication in the EMBO Journal.

Thank you for contributing to the EMBO Journal!
Referee #1:

We are pleased with the revised version. All of our concerns have been addressed satisfactorily.