# Mechanical and signaling mechanisms that guide pre-implantation embryo movement.

Diana Flores, Manoj Madhavan, Savannah Wright and Ripla Arora
DOI: 10.1242/dev.193490

**Editor**: Patrick Tam

**Review timeline**
Original submission: 3 June 2020
Editorial decision: 24 June 2020
First revision received: 12 October 2020
Editorial decision: 27 October 2020
Second revision received: 27 October 2020
Accepted: 29 October 2020

---

**Original submission**

First decision letter

MS ID#: DEVELOP/2020/193490

MS TITLE: Mechanical and signaling mechanisms that guide pre-implantation embryo movement.

AUTHORS: Diana Flores, Manoj Madhavan, Savannah Wright, and Ripla Arora

I have now received all the referees' reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see, the referees express considerable interest in your work, but have some criticisms and recommend a revision of your manuscript before we can consider publication. If you are able to revise the manuscript along the lines suggested, which may involve further experiments (such as testing the impact of number of embryos/beads on the spacing in utero, and whether the spacing pattern is indicative of productive implantation), I will be happy receive a revised version of the manuscript. Your revised paper will be re-reviewed by one or more of the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' major concerns. Please also note that Development will normally permit only one round of revision.

We are aware that you may currently be unable to access the lab to undertake experimental revisions. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please attend to all of the reviewers' comments and ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion. I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

*Advance summary and potential significance to field*

This ms revisits a problem that has been largely dormant in implantation biology for some decades. The introduction gives a good historical context. The authors use whole mount imaging to show that mouse embryos are deposited at the rostral end of the uterus, move as a group halfway to the caudal end, then disperse under neuromuscular control, to produce even spacing in a process that depends on embryonic-uterine signalling. They choose an informative gene knockout and apply pharmacological inhibitors to demonstrate mechanistic change from a physical stimulus that generates neuromuscular activity in the first phase, to a paracrine embryo-maternal signal in the subsequent dispersal phase. The processes they reveal are likely to be mouse-specific, or at least restricted to a subset of polytocous species. Overall it is an interesting and novel study and I have no major reservations.

*Comments for the author*

The discussion is the weakest part of the ms -- it adds very little. It should be revised to eliminate straightforward repetition of the results.

The authors should make clear that LPAR3 is, at least as far as I know, exclusively found in the myometrium. This raises questions about how the embryonic signal is transmitted across the endometrium, and about its identity. Some comment is required.

For dispersed embryos, 'scattered' is used -- I prefer the former term, as scattering is random. In fact they are social distancing. It might be useful in the discussion to point out what is known about the signalling processes that are involved in embryonic lockdown in the chamber -- there is a mouse genetics literature to draw on.

Did the authors observe any implantation failures?

What happens when the number of embryos or beads is reduced? Is it necessary to have a cohort of a certain size to create a sufficiently strong signal to move the group caudally?

For discussion: embryo migration is known to occur in larger species including horse, pig, cat dog etc. Is there a transition in other species from an initial physical signalling phase (most have larger blastocysts) to one dependent on chemical signalling?

In terms of writing style there are numerous places where words are repeated in the same sentence eg line 11,24,92,114,121 and others.

The word 'biological' in line 94 seems a poor choice as the mechanical forces are also biological.

Should 'fragment' referring to parts of the uterus be 'segment' as eg in lines 237,8?

Reviewer 2

*Advance summary and potential significance to field*

This manuscript utilizes advanced imaging and 3D reconstruction technology to evaluate murine embryo location in the uterus. The analysis revealed distinct pre-implantation stages based on advanced imaging data. Muscle inhibitor and a genetically modified mouse model (Lpar3 null) were used to understand the influence of muscle contractions and Lpar3 signaling on embryo spacing in the mouse.

*Comments for the author*

OVERALL AND MAJOR COMMENTS

The manuscript is well written for the most part and provides some new insights into embryo spacing in mice using a series of carefully executed and designed experiments that are sufficiently powered to substantiate most of the conclusions. The Discussion places the current findings in view of the scientific literature and across different species. Overall the knowledge gained from the studies are incremental in nature.

Major comments:
(1) Please adjust the times to military format, e.g. 0300 h, by removing the :
(2) The Materials and Methods need to include more detail to assist with study reproducibility.
    a. Beads transfer: How was this accomplished? Using what approach and size needle?
(3) Figure 5: Change KO to Lpar3-/-
(4) The studies would be strengthened by using an antibody or reporter gene to specifically visualize the embryos in utero.
(5) A major conclusion is that “Thus, uterine implantation sites in mice are neither random nor predetermined but are guided by the number of embryos entering the uterine lumen.”
What is the rationale for that conclusion? The authors should conduct a study where the number of embryos in the uterus are manipulated using an embryo transfer experiment to test that hypothesis.

SPECIFIC COMMENTS

| Line | Comment |
|---|---|
| 86 | milieu is not an appropriate term…just say uterus |
| 292 | This paragraph has only a single sentence? |

Reviewer 3

*Advance summary and potential significance to field*

Flores and colleagues have explored the mechanisms that lead to the spacing of preimplantation mouse embryos (blastocysts) within the uterine horn. They exploit the confocal/3D image reconstruction method they developed to locate embryos along the longitudinal axis of the uterine horn. A very detailed timing of embryo location suggests 3 phases of embryo location, 1. entry into the uterus, 2. the bulk of the embryos located at the midpoint of the uterus, and 3. embryos spaced along the entire axis. By blocking adrenergic uterine smooth muscle contractions they find that Phase 2 is altered. Utilizing Lpar3 mutant females, they show that embryo spacing is disrupted. Bead transfers result in their location at the midpoint but there is a failure of spacing, suggesting an active embryo-uterine interaction for spacing.

This is a very detailed study of a fundamental developmental process that is essential for the initial steps of mammalian development. New insights are obtained on the mechanisms that result in the spacing of blastocysts for optimal embryo-maternal interactions.

*Comments for the author*

Major comments

I caution the authors about the wording they use. To claim "movement", one has to follow one embryo at time 0 and then the same embryo at time 1. That technology is not available yet. So the authors are making conclusions based on fixed specimens. I suggest using descriptive text rather than active words. This is more apparent in paragraph, line 116. The authors state "to separate", "to disperse", "to move", "to space". Better to describe where the embryos are located.

There are complex movements of preimplantation embryos within the oviduct. The authors should emphasize they are describing the positions of embryos within the uterus. For example, "embryo entry" perhaps uterine entry?

Line 269, I suggest more specific wording. I can't really distinguish between the two possibilities.

I would like to see Supplementary Fig. 1 added as Fig. 1.

Minor comments

Line 101, help the reader by stating when vascular permeability begins.

Line 37, in the mouse till date?

Line 124, does mean distance have units?

Follow mouse nomenclature. Lpar3 (italics) not LPAR3.

Add substrain. C57BL/6 J?

Describe genetic background of Lpar3 mutant strain.

Provide more detail about how many beads were transferred.

Fig. 1. What is the arrow?

Fig. 4. Define syringe needle.

---

**First revision**

Author response to reviewers' comments

**MS ID#: DEVELOP/2020/193490**
**MS TITLE: Mechanical and signaling mechanisms that guide pre-implantation embryo movement.**
**AUTHORS: Diana Flores, Manoj Madhavan, Savannah Wright, and Ripla Arora**

We thank the reviewers for their thoughtful comments and suggestions for clarification. Our responses are indicated below in blue and corresponding changes to the manuscript are highlighted in yellow.

**Editors Comments for the Author**

**Test the impact of number of embryos/beads on the spacing in utero, and whether the spacing pattern is indicative of productive implantation.**
Response: We thank the editor and the reviewers for this suggestion. To answer this question we conducted two sets of embryo transfer experiments into pseudopregnant mice. Either 4-6 embryos were transferred or 10-14 embryos were transferred into a single uterine horn and implantation was assessed using the blue dye reaction and embryo location was assessed using our methodology. In both sets of embryo transfer experiments we observed an inverse correlation between number of embryos at the time of implantation and the EE distance, supporting our hypothesis that implantation sites are not predetermined and are guided by the number of embryos transferred. These data have been included as an additional Figure 4 and the results and discussion sections have been updated to include this analysis.

Line 147 reads: "*To further confirm the relationship between number of embryos and embryo-embryo spacing at implantation, we controlled the number of embryos in the uterine horn using surgical embryo transfer (ET). ET was performed in pseudopregnant recipient females on GD2 (1800h), and embryo location was assessed on GD4 (1800h) (Fig. 4) (McLAREN and MICHIE, 1956). We noted that with ET, the first implantation site tends to be further away from the oviduct compared to natural pregnancy. Consequently, the first segment contains the lowest percentage of embryos - 13% and 23% for smaller numbers (4-6) and higher numbers (10-14) of transferred embryos respectively (Fig. 4A). Sites of wound healing in the uterus, such as placental scars from a prior*

*pregnancy, are refractory to embryo implantation (Momberg and Conaway, 1956). Thus, scarring from the injection site may impact embryo distribution in ET procedures. In uteri with a low number of ET (4-6 embryos), a higher mean EE distance was observed as compared to uteri with a high number of ET (10-14 embryos) where a lower mean EE distance was observed (Fig. 4A, 4B, 4C). Thus, similar to natural pregnancy (Fig. 3D), in a pregnancy with ET, there is an inverse correlation between the number of embryos and the mean EE distance (Fig. 4C, $R^2$=0.78). In the ET experiments, sometimes we had only 3 implantation sites (Fig. 4A, 4C), although 4-6 embryos were transferred. This is likely due to the efficiency of implantation post ET. On the other hand, with our natural pregnancy analysis, the lowest number of implantation sites in a single uterine horn at GD4 was 4 (Fig. 3D), likely due to a larger litter size of CD1 mice (Bechard et al., 2012). To evaluate mice with only 3 embryos in their uterine horn through natural pregnancy, we assessed embryo location at the time of implantation in C57BL/6J females that have a smaller litter size (Bechard et al., 2012). We combined the data for both CD1 and C57BL/6J females and observed that the correlation coefficient ($R^2$) decreases from 0.88 when there are at least 4 embryos in the uterine horn to 0.79 when there are at least 3 embryos in the uterine horn (Fig. 4D) with natural pregnancy. This can be interpreted as a threshold of 4 embryos is required to achieve a strong correlation between the number of embryos and the EE distance. The COV for EE distances obtained from implantation sites at GD4 1800h after ET was 0.35, which is similar to the COV for post-implantation time points of natural pregnancy GD4 0000h, and 1800h (0.34 and 0.22, respectively) and is suggestive of non-random distribution of embryos. Thus, data from both natural pregnancy and ET supports the notion that implantation sites cannot be predetermined in a non-pregnant uterus, but instead, they are guided by the number of embryos present in the uterine horn of the mouse."*

**Reviewer 1 Advance Summary and Potential Significance to Field:**
This ms revisits a problem that has been largely dormant in implantation biology for some decades. The introduction gives a good historical context. The authors use whole mount imaging to show that mouse embryos are deposited at the rostral end of the uterus, move as a group halfway to the caudal end, then disperse under neuromuscular control, to produce even spacing in a process that depends on embryonic- uterine signalling. They choose an informative gene knockout and apply pharmacological inhibitors to demonstrate mechanistic change from a physical stimulus that generates neuromuscular activity in the first phase, to a paracrine embryo-maternal signal in the subsequent dispersal phase. The processes they reveal are likely to be mouse-specific, or at least restricted to a subset of polytocous species. Overall it is an interesting and novel study and I have no major reservations.

**Reviewer 1 Comments for the Author**

1) The discussion is the weakest part of the ms -- it adds very little. It should be revised to eliminate straightforward repetition of the results.
Response: We double checked and removed any straightforward repetition of results from the discussion section.

2) The authors should make clear that LPAR3 is, at least as far as I know, exclusively found in the myometrium. This raises questions about how the embryonic signal is transmitted across the endometrium, and about its identity. Some comment is required.
Response: LPAR3 is expressed in the luminal epithelium on Gestational Day 3 of pregnancy as shown in Fig. 1C, 1D of Ye et. al, 2005. This information has been added to the introduction section when we first mention the known role of LPAR3 signaling in uterine function.

Line 62 reads: *"Lpar3 is expressed in the luminal epithelium before implantation, and when deleted, affects embryo spacing and implantation (Ye et al., 2005)."*

3) For dispersed embryos, 'scattered' is used -- I prefer the former term, as scattering is random. In fact, they are social distancing. It might be useful in the discussion to point out what is known about the signaling processes that are involved in embryonic lockdown in the chamber -- there is a mouse genetics literature to draw on.
Response: Our data shows that embryo location at the beginning of the bidirectional phase is indeed random (COV 1.06, Fig. 3C) thus the authors prefer the term scattered over dispersed.

Upon the reviewer's suggestion, we have update the discussion section to mention signaling processes that are involved in fluid resorption and luminal closure and are thought to lock embryos in place prior to implantation.

Line 307 reads: *"It has been hypothesized that luminal closure occurs due to fluid resorption and plays a major role in locking the embryo in place (Chen et al., 2013; Davidson and Coward, 2016). Thus, factors that regulate fluid resorption including hormones (Progesterone, (Clemetson et al., 1977; Salleh et al., 2005)), genetic factors (FOXO1, (Vasquez et al., 2018)) and ion channels ($Na^+$, (Nobuzane et al., 2008)) might be involved in the switch between the two phases of movement."*

4) Did the authors observe any implantation failures?
Response: When we compared average number (+/- SD) of embryos at GD3 1200h, GD4 0000h and GD4 1800h we did not observe any significant differences in embryo number. Based on this data, there are no implantation failures between the time points assessed. We have included this data in the results section of the manuscript.

Line 113 reads: *"We compared the average number (+/- SD) of embryos per horn for different time points - GD3 1200h (7.5 +/- 2.87 embryos/horn), GD4 0000h (6.17 +/- 1.95 embryos/horn) and GD4 1800h (7.17+/- 0.90 embryos/horn). We did not find any significant differences between the groups (ANOVA p=0.38), suggesting no loss of embryos between embryo movement and implantation time points."*

5) What happens when the number of embryos or beads is reduced? Is it necessary to have a cohort of a certain size to create a sufficiently strong signal to move the group caudally?
Response: In order to evaluate embryo-embryo distances we restricted our location analysis to uterine horns that have at least 3 embryos. Based on our GD3 1200h data for LPAR3 analysis (new Fig. 7), there are uteri that have 3 embryos and these embryos exhibit clustering and a caudal location with respect to the oviductal-uterine junction. With the few uterine horns that did have one or two embryos (data not included in the manuscript), the embryos did display a caudal location at the expected time point. Thus, we do not believe that there is a cohort of a certain size required to create a sufficiently strong signal to move the group caudally, but instead the uterine muscle contractions guide the movement of objects unidirectionally.

6) For discussion: embryo migration is known to occur in larger species including horse, pig, cat dog etc. Is there a transition in other species from an initial physical signaling phase (most have larger blastocysts) to one dependent on chemical signaling?
Response: We have included a discussion of larger mammals that require embryo migration for successful implantation.

Line 389 reads: *"Physical embryo movement is significant not only for small mammals but also for larger animals such as cats, dogs, pigs, and horses. Embryo mobility in the cat (Tsutsui et al., 1989), dog (Tsutsui et al., 2002), and pig (Sittmann, 1973) is essential for transuterine migration. This is because unlike the mouse, embryos in these species can move across the uterine horns to equalize the number of embryos in each horn. In the pig and horse, it has been conclusively shown that embryo movement is also essential for pregnancy success. When embryo mobility is restricted in the pig, either by limiting embryos to one horn or by ligating the uterine horn, the entire pregnancy was lost (Dhindsa and Dziuk, 1968; Dziuk, 1985). Similarly, embryos in mares must be able to travel across at least two-thirds of the endometrial surface as uterine ligatures that reduce this surface cause pregnancy failure (McDowell et al., 1988). Interestingly uterine ligation in the mare reduces the levels of serum progesterone, suggesting a direct link between mechanical stimulus of the embryo and induction of ovarian progesterone essential for signaling. Similar to rodents and rabbits, uterine contractions have also been implicated in embryo mobility in the pig (Dhindsa et al., 1967) and the horse (GINTHER, 1985; Leith and Ginther, 1985). In humans, embryo movement under the influence of uterine contractions and intraluminal uterine fluid flow has shown to be important for embryo survival. Women with hydrosalpinx have an increase in tubal pressure creating a pressure gradient between the fundus and the cervix. This pressure gradient adversely affects the cervix-to-fundus myometrial contractions and is predicted to thrust the embryo away from a viable area for implantation. Thus, even in women, embryo mobility is essential to navigate the site of implantation, and uterine contractions regulate this movement of the embryo (Eytan et al., 2001).*

*Exploring the mechanisms of embryo movement in the mouse model opens up avenues to understanding these events in larger animals and primates."*

7) In terms of writing style there are numerous places where words are repeated in the same sentence eg line 11,24,92,114,121 and others.
Response: We have used alternative words to avoid repetition in the relevant sentences.

8) The word 'biological' in line 94 seems a poor choice as the mechanical forces are also biological.
Response: We have changed 'biological' to 'signaling'.

9) Should 'fragment' referring to parts of the uterus be 'segment' as eg in lines 237,8?
Response: We have changed 'fragment' to 'segment' in the relevant sentences.

**Reviewer 2 Advance Summary and Potential Significance to Field:**
This manuscript utilizes advanced imaging and 3D reconstruction technology to evaluate murine embryo location in the uterus. The analysis revealed distinct pre-implantation stages based on advanced imaging data. Muscle inhibitor and a genetically modified mouse model (Lpar3 null) were used to understand the influence of muscle contractions and Lpar3 signaling on embryo spacing in the mouse.

**Reviewer 2 Comments for the Author:**
OVERALL AND MAJOR COMMENTS

The manuscript is well written for the most part and provides some new insights into embryo spacing in mice using a series of carefully executed and designed experiments that are sufficiently powered to substantiate most of the conclusions. The Discussion places the current findings in view of the scientific literature and across different species. Overall, the knowledge gained from the studies are incremental in nature.

1) Please adjust the times to military format, e.g. 0300 h, by removing the:
Response: We have adjusted the times to military format.

2) The Materials and Methods need to include more detail to assist with study reproducibility.
a. Beads transfer: How was this accomplished? Using what approach and size needle?
Response: We have expanded the materials and methods section to include more details of the beads transfer experiments.

3) Figure 5: Change KO to Lpar3-/-.
Response: The suggested change has been made.

4) The studies would be strengthened by using an antibody or reporter gene to specifically visualize the embryos in utero.
Response: In the article where we first developed this imaging methodology (Figure 1, Arora et. al, 2016, Development), we evaluated embryo specific expression of Cadherin 1 and FoxA2 along with Hoechst to ensure that Hoechst alone identifies embryos at these early pregnancy peri-implantation stages. These embryo specific markers were not used in all the data generated in this manuscript to save on the time required for staining and imaging and to generate image files with reduced sizes. This allowed us to evaluate and present data from ~200 uterine horns in the manuscript.

5) A major conclusion is that "Thus, uterine implantation sites in mice are neither random nor predetermined but are guided by the number of embryos entering the uterine lumen." What is the rationale for that conclusion? The authors should conduct a study where the number of embryos in the uterus are manipulated using an embryo transfer experiment to test that hypothesis.
Response: We have now performed additional embryo transfer experiments (new Fig. 4) - See response to the editors comment.

6) Line 86 milieu is not an appropriate term…just say uterus
Response: This line has been edited per the reviewer's suggestion.

7) Line 292 This paragraph has only a single sentence?

Response: This line has been combined with the previous paragraph per the reviewer's suggestion.

**Reviewer 3 Advance Summary and Potential Significance to Field:**
Flores and colleagues have explored the mechanisms that lead to the spacing of preimplantation mouse embryos (blastocysts) within the uterine horn. They exploit the confocal/3D image reconstruction method they developed to locate embryos along the longitudinal axis of the uterine horn. A very detailed timing of embryo location suggests 3 phases of embryo location, 1. entry into the uterus, 2. the bulk of the embryos located at the midpoint of the uterus, and 3. embryos spaced along the entire axis. By blocking adrenergic uterine smooth muscle contractions they find that Phase 2 is altered. Utilizing Lpar3 mutant females, they show that embryo spacing is disrupted. Bead transfers result in their location at the midpoint but there is a failure of spacing, suggesting an active embryo-uterine interaction for spacing.
This is a very detailed study of a fundamental developmental process that is essential for the initial steps of mammalian development. New insights are obtained on the mechanisms that result in the spacing of blastocysts for optimal embryo-maternal interactions.

**Reviewer 3 Comments for the Author:**

1) I caution the authors about the wording they use. To claim "movement", one has to follow one embryo at time 0 and then the same embryo at time 1. That technology is not available yet. So, the authors are making conclusions based on fixed specimens. I suggest using descriptive text rather than active words. This is more apparent in paragraph, line 116. The authors state "to separate", "to disperse", "to move", "to space". Better to describe where the embryos are located.
Response: We agree with the reviewer and have gone through the manuscript and used descriptors of embryo location to replace terms such as "to separate", "to disperse", "to move", "to space" wherever possible.

2) There are complex movements of preimplantation embryos within the oviduct. The authors should emphasize they are describing the positions of embryos within the uterus. For example, "embryo entry" perhaps uterine entry?
Response: We define the first phase as embryo entry but have now clearly stated that we are referring to entry into the uterus in the results section.

3) Line 269, I suggest more specific wording. I can't really distinguish between the two possibilities.
Response: We have rewritten this sentence in response to the reviewer's suggestion.

4) I would like to see Supplementary Fig. 1 added as Fig. 1.
Response: We have moved Supplementary Fig. 1 as main Fig. 1 and relabeled other figures accordingly.

5) Line 101, help the reader by stating when vascular permeability begins.
Response: Based on the reviewer suggestion we have added information on when vascular permeability begins to the in the discussion section.

6) Line 37, in the mouse till date?
Response: We have rewritten this sentence.

7) Line 124, does mean distance have units?
Response: Since the OE, EE, OB, BB distances were normalized to the horn length they are a ratio and are unitless. This statement has been added to the methods section where we describe the measurements.

8) Follow mouse nomenclature. Lpar3 (italics) not LPAR3.
Response: We have edited the nomenclature based on MGI that suggests gene names to be italicized (*Lpar3*) but protein names to have all uppercase letters (LPAR3).
Source: http://www.informatics.jax.org/mgihome/nomen/short_gene.shtml

9) Add substrain. C57BL/6 J?
Response: The substrain has been added to the methods section.

10) Describe genetic background of Lpar3 mutant strain.
Response: The genetic background of Lpar3 mutant strain is now described in the methods.

11) Provide more detail about how many beads were transferred.
Response: We have provided more detail with respect to our bead transfer experiment including the number of beads in the methods section.

12) Fig. 1. What is the arrow?
Response: We have added the explanation for the arrow in the figure legend.

13) Fig. 4. Define syringe needle.
Response: We have defined the syringe needle in the figure legend.

---

Second decision letter

MS ID#: DEVELOP/2020/193490

MS TITLE: Mechanical and signaling mechanisms that guide pre-implantation embryo movement.

AUTHORS: Diana Flores, Manoj Madhavan, Savannah Wright, and Ripla Arora

I have now received all the referees reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

The overall evaluation is positive and we would like to publish a revised manuscript in Development, provided that the referee #2 specific points are satisfactorily addressed.

Reviewer 1

*Advance summary and potential significance to field*

It's a distinct advance and the revisions And additional data have improved the ms.

*Comments for the author*

Now acceptable for publication.

Reviewer 2

*Advance summary and potential significance to field*

This manuscript utilizes advanced imaging and 3D reconstruction technology to evaluate murine embryo location in the uterus. The analysis revealed distinct pre-implantation stages based on advanced imaging data. Muscle inhibitor and a genetically modified mouse model (Lpar3 null) were used to understand the influence of muscle contractions and Lpar3 signaling on embryo spacing in the mouse.

*Comments for the author*

OVERALL AND MAJOR COMMENTS

The authors have addressed the comments from the initial review by revision and provision of an additional experiment. Overall, the manuscript reads well and provides some interesting new observations.

SPECIFIC COMMENTS
Line Comment
3 In reality, embryo movement has been known in mammals for many years, and the mechanisms governing their movement has already been investigated in many different species with respect to the oviduct and uterus. Thus, the first sentence is not correct and should be removed and replaced.

277 seen should be replaced with observed

Reviewer 3

*Advance summary and potential significance to field*

New insights are obtained on the mechanisms that result in the spacing of blastocysts for optimal embryo-maternal interactions.

*Comments for the author*

The authors have fully addressed my previous comments in a satisfactory manner.

---

**Second revision**

Author response to reviewers' comments

We have edited our manuscript per reviewer 2's suggestions. The changes are highlighted in yellow in the edited manuscript.

Reviewer 2 Comments for the author

OVERALL AND MAJOR COMMENTS
The authors have addressed the comments from the initial review by revision and provision of an additional experiment. Overall, the manuscript reads well and provides some interesting new observations.

SPECIFIC COMMENTS
LineComment
3 In reality, embryo movement has been known in mammals for many years, and the mechanisms governing their movement has already been investigated in many different species with respect to the oviduct and uterus. Thus, the first sentence is not correct and should be removed and replaced.

Response: We have replaced the first sentence in the abstract to read "How a mammalian embryo determines and arrives at its attachment site has been studied for decades but our understanding of this process is far from complete. "

277 seen should be replaced with observed.
Response: We have replaced seen with observed in line 277.

---

Third decision letter

MS ID#: DEVELOP/2020/193490

MS TITLE: Mechanical and signaling mechanisms that guide pre-implantation embryo movement.

AUTHORS: Diana Flores, Manoj Madhavan, Savannah Wright, and Ripla Arora
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.