nature portfolio

Peer Review File

Early transcriptional divergence underlies cell fate bias in bovine embryos

Corresponding Author: Dr Satoshi Sugimura

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)
In this ms, the authors explore transcriptional variation among blastomeres of preimplantation bovine embryos. They carefully selected embryos at the 1-, 2-, 4- and 8-cell stage, where they could collect a complete set of isolated blastomeres from the same embryo, and then performed single cell RNA-seq on all the isolated blastomeres. They found uniform expression between 2-cell blastomeres, but increasing levels of divergence between blastomeres at the 4- and particularly at the 8-cell stage. This divergence was most noted in genes associated with trophectoderm differentiation, such as Cdx2, and the MAPK signaling pathway. This bias towards TE-associated gene expression was most obvious in the larger blastomeres of 4- and 8-cell sets. To assess whether this variation in gene expression might correlate with later lineage bias, they cultured isolated size-classified blastomeres through to the blastocyst stage equivalent and observed more cavity formation in the cultures isolated from larger blastomeres. These structures showed Cdx2+ cells by immunofluorescence but also expressed Sox2+ ICM-like cells.

Overall, this is a carefully carried out set of experiments designed to see whether it is possible to observe early bias in gene expression in bovine embryos, as has been reported in mouse and somewhat in human embryos (Junyent et al Cell. 2024 May 23;187(11):2838-2854.e17. doi: 10.1016/j.cell.2024.04.029). Although they see evidence of lineage bias in gene expression, they do not observe actual lineage restriction in the outcomes of the later development of individual blastomeres. Larger blastomeres were more likely to form cavities and to have enough cells to undertake antibody staining for Cdx2 and Sox2. But they did contain roughly the expected proportion of Cdx2 to Sox2 positive cells. Smaller blastomeres apparently failed to progress but whether this is related to lineage bias or lack of competency for any further cell division in isolated small blastomeres is not clear. An important experiment that might determine this would be to produce full-sized embryos by reaggregating all large or all small blastomeres or a mixture, so that the general growth insufficiency of individual small blastomeres would be compensated. In this situation, would there be a true bias towards TE vs ICM fate in ‘all large’ embryos versus ‘all small’ embryos?

Other comments:

1. There is a focus on MAPK signaling as likely involved in TE vs ICM fate. However, it is known that in mouse and human, the HIPPO signaling pathway is critical for this lineage decision. Assessment of the localization of nuclear YAP in embryos derived from isolated embryos should be performed. This is particularly important because YAP localization is highly dependent on physical signals, which may underly effect of the blastomere size differential here.
2. Use of the appearance of a cavity as a measure of TE formation is not strong. Other markers of the cellular behavior of the TE could be added.
3. L 45. Suggests that in mouse, developmental potential may be restricted earlier than previously thought. The term “restricted potential” should be used with caution. Appearance of lineage bias does not necessarily mean that there is concomitant lineage restriction. Biased blastomeres may still show full potential when removed from their position in the intact embryo. Indeed, that is exactly what the authors observe here and discuss on l234.
4. As well as between blastomere variation, they also report variation among embryos of the same stage. Why is this?
5. Is the cell division rate higher in large vs small blastomeres?

Reviewer #2

(Remarks to the Author)
This manuscripts describes transcript abundance in individual cleavage stage bovine blastomeres from several embryos. In a novel approach, the authors also correlated expression patterns and with blastomere size and blastomere size with developmental potential. The authors compare inter and intra embryonic blastomere variation as a way of demonstrating that the intraembryonic variation they see from 4-cell stage to 8-cell stage is not an artefact of the amplification process of scRNA seq, but there is no independent data (RNAscope, digital PCR) shown to verify these findings. Using intraembryonic enrichment as a criterium, the authors detect MAPK as one of the pathways showing differential distribution and then compare member genes to CDX2 which has already previously been shown to be differentially expressed at the 8 and 16 cell stage in cattle by the Wolf and Blum groups (Lavagi et al., 2018). References to this paper are missing in this manuscript as are a comparison of the results and conclusions. In the comparisons of high and low CDX2 blastomeres, another signaling pathway, namely WNT signaling is shown in Figure 3 but not analysed further even though of equal P value and higher GAGE statistic than MAPK signaling. The further analysis of large and small 8-cell stage blastomeres is an interesting approach though the lack of developmental potential of small blastomeres may simply reflect the presence of too little cytoplasm, which can be deleterious to further development (see “The development capacity of blastomeres from 4- and 8-cell sheep embryos” by S. M. Willadsen in J Embryol Exp Morphol 1981 Vol. 65 Pages 165-72) and the author’s interpretations are thus fraught with problems.
Here some more detailed comments, in order of reading the manuscript.
Line 76 add: this complements prior work on cattle 8 and 16 cell stage embryos (Lavagi, I., Krebs, S., Simmet, K., Beck, A., Zakhartchenko, V., Wolf, E., Blum, H., 2018. Single-cell RNA sequencing reveals developmental heterogeneity of blastomeres during major genome activation in bovine embryos. Sci Rep 8, 4071.). This paper needs also be related to in the discussion.
Fig. 1B Requires the use of more distinctive symbols (as for 2-cell) for the individual embryos of 4 cell and 8-cell stages.
Figs. 2 and 3 Font sizes too small to read and markers too faint.
Line 123, Do the authors mean most as opposed to several?
Line 146 CDX2-expressing cells
Lines 147 to 150 has mistakes in terms of which panel of Fig 3 is referred to. No legend for Fig. 3 panels D and E. Panel E should show R-squared not r (and as mentioned before, all too small to see). Comparison should probably be done using logs of TPM to avoid skewing by the few very large values.
Gene names inconsistently written in italics.
Lines “168 These results suggest that large blastomeres are transcriptionally biased
169 toward the activation of TE-associated pathways, such as the MAPK pathway, indicating
170 the potential unequal inheritance of maternal transcripts” should be removed as no evidence is shown that MAPK bias is correlated with TE nor that any bias is due to unequal inheritance, could e.g. also be due to unequal mRNA degradation etc.
Supplementary Table 1 (erroneously labelled Table 3) is at odds with Fig 4: see P-values of MAPK14. Clearly the MAPK pathway differences are minor (2^2) in comparison to some other genes which show a 2^5 change. The change needs to be normalised to all genes sequenced as it may be that most genes are overexpressed in these datasets and thus the fold change of the MAPK pathway is irrelevant.
The conclusion in lines 185-187 “185 maintaining their ability to contribute to both the TE and ICM lineages. Collectively, these results suggest that blastomere size is linked to both the transcriptional and functional properties relevant to early lineage bias” is unwarranted as no embryos derived from the smallest blastomere were examined by marker expression.

Lines 240 onward in discussion needs to deal with Lavagi 2018 model and with lineage bifurcation model proposed in Hu, B., Jin, H., Shi, Y., Yu, H., Wu, X., Wang, S., Zhang, K., 2024. Single-cell RNA-Seq reveals the earliest lineage specification and X chromosome dosage compensation in bovine preimplantation embryos. FASEB J 38, e23492.

Reviewer #3

(Remarks to the Author)
This manuscript enriches our understanding of embryonic blastomere diversification (or symmetry breaking) in a mammalian species other than mouse or human. Briefly, the study compared individual blastomeres from the same bovine embryo at successive cleavage stages (1-, 2-, 4-, and 8-cell), using single-cell RNA sequencing; moreover, at the 4- and 8-cell stage, the smallest and the largest blastomere from the same embryo were compared with each other too. As such the study makes a valuable contribution to the field.

I have the following remarks.

Major remarks

To my knowledge there is only one other study that examined the transcriptomes of 8-cell bovine embryos at the single cell level (PMID 29511234); although the comparison of all blastomeres from the same embryo is missing in PMID 29511234, I think Hinata and colleagues should mention PMID 29511234 and should compare their results with those of PMID 29511234 as far as possible.

Developmental rates to blastocyst don’t matter much as long as the focus is to compare for gene expression all sister blastomeres of the same embryo up to the 8-cell stage. But when the Authors compare small and large blastomeres for blastocyst formation, then the numbers and rates should be presented in a Table. In Fig.4c 168 hpi: some of the embryos look dead, not delayed, rather just dead. For this experiment in which the blastocyst formation of small and large

blastomeres is comparatively assessed, it is important to provide information about the actual blastocyst rates.

It needs to be better introduced that there are size differences between the sister blastomeres. The Authors go straight to the molecular analysis of this trait, but before doing so, they should present quantitative data documenting that there is a difference (small, large) in the first place (for example this could be done by showing the histograms of the statistical distributions of the diameters). I see the pictures of small and large blastomeres (Fig.4), and I trust that the differences are real, but still I think that they should be quantified.

In the mouse (yes I know, the study by Hinata and colleagues is about bovine) small and large blastomeres were found to not behave differently in terms of blastocyst formation and cell lineage composition (PMID 28811525). By contrast in the present study the blastomeres of different sizes seemed to have distinct developmental properties. Is this a biological difference of the two species, or is it more specific of bovine? Do differences at the mRNA level necessarily produce differences at the protein level? It is well known that mRNA-protein correlation is generally poor. Therefore, the obvious question I ask is whether the divergence of transcripts between sister blastomeres reflects in the proteins. At the end (Figure 4), the Authors show data for two proteins (CDX2, SOX2). It would be informative to compare the mRNA and protein data of Cdx2/CDX2 and Sox2/SOX2. I understand that this cannot be done within the same embryos, but still, it might be possible (and informative) to see if changing mRNA levels between blastocysts of small vs large blastomeres reflect in changing intensities of protein signal.

Minor remarks

Larger blastomeres cavitate earlier; but ultimately also the smaller blastomeres cavitate. How robust is this difference of timing? Larger blastomeres might simply have more ATP and/or more mitochondria, which make all processes run faster, rather than having a predisposition toward trophectoderm. Since Fig.4d shows a borderline p value of 0.048. I suggest to show the data points on top of the two histogram bars.

Fig 3 legend is incomplete (‘d’ and ‘e’ shown in the figure are not explained in the legend).

Fig 4b: connecting the points of small and large blastomere with a line is, in my opinion, misleading, as it suggests that intermediate-sized blastomeres have an intermediate behavior. While this is plausible, it is an assumption. What statistical test was used to assess statistical significance? Please mention it in the legend.

Fig 4e: please show scale bars

I hope the Authors find my comments useful.

The manuscript has several supplementary files, I apologize if some of the information I asked for is already included in the supplementary files. In this case, please make it more clear in the main text, that the information is available.

Regards,

Michele Boiani

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have responded well to all the comments of the reviewers and I am satisfied with their answers. The study is a useful contribution to our understanding of early embryo development

Reviewer #2

(Remarks to the Author)
I believe the manuscript has been significantly strengthened by the authors’ substantial revisions and additional experiments and thank them for their sincere efforts to validate their findings. Apart from one minor comment, which the authors may wish to address, I believe the manuscript is fine for publication.
The minor comment refers to lines 240-243 showing significant increases in total and CDX2+ cells whereas increases in SOX2+ cells were not significant. Might be good to look at the changes in proportions too (i.e. CDX2+ relative to total cells in large and small-derived embryos) as this better accommodates the structure of the experiment (that is, not losing the per embryo component of the data).

Reviewer #3

(Remarks to the Author)
I have read the revised manuscript. I appreciate the thoroughness of the revision and the new experiments that have been conducted, such as the aggregation of large and small blastomeres. I also appreciate the many other improvements/extensions (e.g. table 1). The Authors should be commended for their work. All of my previous criticisms have been addressed, and I hope the Authors agree with me that it was worth the effort. My only comment is whether the small blastomeres were all nucleated. That is, whether the Authors checked with e.g. Hoechst staining that the nucleus was present in the S blastomeres used for gene expression analysis and for aggregation. It is known that bovine zygotes often undergo abnormal divisions and produce (smaller) cells without nucleus (example: Destouni et al. https://pubmed.ncbi.nlm.nih.gov/27197242). Apart from this question, on which I would love to hear the Authors' answer, I approve the revision without hesitation.
Best regards,
Michele Boiani

Version 2:

Reviewer comments:

Reviewer #3

(Remarks to the Author)
The Authors' response to my comment about anucleated blastomeres is convincing. I thank the Authors for explaining, and I have no further comments.
Michele Boiani

Senior editors' comments:

We hope you will find the referees' comments useful as you decide how to proceed. Should further experimental data or analysis allow you to address these criticisms, we would be happy to look at a substantially revised manuscript. In particular, a thorough discussion of the related paper PMID: 29511234 should be provided. We also expect that you carry out additional validation of the main findings with independent data and additional analyses of alternative signalling pathways to strengthen your findings.

Response:

We thank the Senior Editors for their careful assessment of our manuscript and for outlining the key points required to strengthen the study. In response, we have substantially revised the manuscript and performed additional experiments and analyses to directly address the major concerns raised.

First, we now provide a thorough discussion of the related study (PMID: 29511234) in both the Background and Discussion sections. We explicitly position our findings in the context of prior work on transcriptional heterogeneity in bovine embryos, highlighting both shared observations and critical methodological differences. In particular, whereas previous studies analysed blastomeres without preserving complete embryo-of-origin information or were limited to a single developmental stage, our study examines complete sets of sister blastomeres from individually identified embryos across multiple cleavage stages. This approach allows us to assess how transcriptional asymmetries emerge and evolve within the same embryo and complements prior findings rather than contradicting them.

Second, to provide independent validation of our main transcriptomic findings, we added real-time PCR–based validation experiments using cDNA derived from individual blastomeres (Supplementary Fig. 4). These analyses confirm inter-blastomere variability and size-associated differences in the expression of key genes identified by single-cell RNA-seq, supporting the robustness of the sequencing-based observations.

Third, in response to the request for additional analyses of alternative signalling pathways, we expanded our analyses beyond the MAPK pathway. We now include examination of components of the Wnt signalling pathway, as well as analysis of YAP nuclear localization (Fig. 5A and B) as an independent functional readout of trophectoderm-associated signalling. The YAP analysis provides orthogonal, non-transcriptomic evidence supporting an early TE-biased state and strengthens the mechanistic interpretation of our findings.

In addition, we performed immunofluorescence analyses on cavitated embryos derived not only from single isolated blastomeres (Fig. 5) but also from reaggregated embryos reconstructed exclusively from small or large blastomeres (Fig. 6). This allowed us to assess lineage-associated marker expression under conditions where the reduced cell mass

inherent to single-blastomere–derived embryos was partially compensated by reaggregation. These analyses revealed that embryos derived from large blastomeres exhibit a greater total cell number and a preferential increase in $CDX2^{+}/SOX2^{-}$ trophectoderm cells, while still retaining $SOX2^{+}$ cells, indicating a biased but non-restricted developmental state.

Finally, we conducted additional re-analyses focusing on transcriptional variability among blastomeres, including refined normalization and variability metrics. These analyses further support the conclusion that inter-blastomere heterogeneity emerges progressively during cleavage-stage development and is not an artefact of amplification or sampling.

Together, these substantial additions—including independent molecular validation, pathway-level analyses (Wnt and YAP), immunofluorescence-based lineage assessment in both single-blastomere–derived and reaggregated embryos, and reanalysis of transcriptional variability—significantly strengthen the manuscript and directly address the concerns raised by the referees and Senior Editors.

We have carefully and systematically addressed all comments raised by the reviewers through substantial revisions, additional experiments, and complementary analyses, as detailed below.

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

In this ms, the authors explore transcriptional variation among blastomeres of preimplantation bovine embryos. They carefully selected embryos at the 1-, 2-, 4- and 8-cell stage, where they could collect a complete set of isolated blastomeres from the same embryo, and then performed single cell RNA-seq on all the isolated blastomeres. They found uniform expression between 2-cell blastomeres, but increasing levels of divergence between blastomeres at the 4- and particularly at the 8-cell stage. This divergence was most noted in genes associated with trophectoderm differentiation, such as Cdx2, and the MAPK signaling pathway. This bias towards TE-associated gene expression was most obvious in the larger blastomeres of 4- and 8-cell sets. To assess whether this variation in gene expression might correlate with later lineage bias, they cultured isolated size-classified blastomeres through to the blastocyst stage equivalent and observed more cavity formation in the cultures isolated from larger blastomeres. These structures showed Cdx2+ cells by immunofluorescence but also expressed Sox2+ ICM-like cells.

Overall, this is a carefully carried out set of experiments designed to see whether it is possible to observe early bias in gene expression in bovine embryos, as has been reported in mouse

and somewhat in human embryos (Junyent et al Cell. 2024 May 23;187(11):2838-2854.e17. doi: 10.1016/j.cell.2024.04.029). Although they see evidence of lineage bias in gene expression, they do not observe actual lineage restriction in the outcomes of the later development of individual blastomeres. Larger blastomeres were more likely to form cavities and to have enough cells to undertake antibody staining for Cdx2 and Sox2. But they did contain roughly the expected proportion of Cdx2 to Sox2 positive cells. Smaller blastomeres apparently failed to progress but whether this is related to lineage bias or lack of competency for any further cell division in isolated small blastomeres is not clear. An important experiment that might determine this would be to produce full-sized embryos by reaggregating all large or all small blastomeres or a mixture, so that the general growth insufficiency of individual small blastomeres would be compensated. In this situation, would there be a true bias towards TE vs ICM fate in 'all large' embryos versus 'all small' embryos?

Response:

We thank the reviewer for this insightful and constructive comment. We agree that an important question is whether the divergent developmental outcomes of large (L) and small (S) blastomeres reflect early lineage bias or are instead attributable to differences in general developmental competence. To address this issue, we performed a series of additional analyses and experiments.

First, we generated additional embryos derived from L or S blastomeres (n = 9 and n = 5 embryos, respectively), enabling quantitative analyses of lineage-associated markers. In these experiments, embryos derived from L blastomeres tended to contain a higher total number of cells than those derived from S blastomeres. However, no significant differences were detected in the proportions of $CDX2^{+}/SOX2^{-}$ (TE-like) or $CDX2^{-}/SOX2^{+}$ (ICM-like) cells between the two groups (Fig. 5F and G). In contrast, additional analysis of YAP localization revealed markedly stronger nuclear enrichment in embryos derived from L blastomeres compared with those derived from S blastomeres (Fig. 5A and B). Given that nuclear YAP activity promotes trophectoderm identity, this finding provides molecular evidence for an early TE-oriented bias in L blastomeres.

Second, we identified several functional differences between L and S blastomeres. Embryos derived from L blastomeres more readily initiated and maintained cavitation (Table 1), initiated compaction earlier (Fig. 5D), and underwent an additional round of cell division prior to compaction onset, resulting in a higher number of blastomeres at the onset of compaction (Fig. 5E). These observations indicate that L blastomeres exhibit coordinated differences in developmental dynamics compared with S blastomeres (Fig. 5H).

Third, in direct response to the reviewer's suggestion, we generated reaggregated embryos composed exclusively of large blastomeres ("all-large") or exclusively of small blastomeres

("all-small"). In these reaggregation experiments, embryos in both groups initiated cavitation; however, the extent of cavity expansion was consistently weaker in all-small aggregates compared with all-large aggregates (Fig. 6C). Quantitative analyses further revealed that embryos reconstructed from L blastomeres contained a higher total number of cells, with a particularly pronounced increase in $CDX2^{+}/SOX2^{-}$ (TE-like) cells. At the same time, embryos derived from L blastomeres also showed a tendency to contain more $CDX2^{-}/SOX2^{+}$ (ICM-like) cells than those derived from S blastomeres (Fig. 6D and E), indicating that cell fate was not irreversibly fixed.

Taken together, these additional results clarify the reviewer's concern. While differences in overall developmental competence may contribute to the observed phenotypes, they cannot fully account for the divergent outcomes between L and S blastomeres. Instead, our findings support the interpretation that size-associated differences among bovine blastomeres reflect an early, non-deterministic bias in developmental potential—characterized by enhanced proliferative capacity and TE-associated molecular features in large blastomeres—rather than fixed lineage restriction. We have incorporated these new data and interpretations into the revised manuscript.

Other comments:

1. There is a focus on MAPK signaling as likely involved in TE vs ICM fate. However, it is known that in mouse and human, the HIPPO signaling pathway is critical for this lineage decision. Assessment of the localization of nuclear YAP in embryos derived from isolated embryos should be performed. This is particularly important because YAP localization is highly dependent on physical signals, which may underly effect of the blastomere size differential here.

Response:

Thank you for this important suggestion. Following the reviewer's recommendation, we quantified YAP nuclear localization in blastomeres of different sizes. Our analysis revealed that large blastomeres exhibit markedly stronger nuclear enrichment of YAP, whereas small blastomeres show weak or minimal nuclear localization. Because nuclear YAP promotes trophectoderm identity, these findings provide direct evidence that large blastomeres possess an early TE-oriented bias. We have added these results and their interpretation to the revised manuscript (Fig. 5A and B).

2. Use of the appearance of a cavity as a measure of TE formation is not strong. Other markers of the cellular behavior of the TE could be added.

Revised Response:

We agree that cavitation alone is not a definitive measure of trophectoderm (TE) formation. Therefore, we performed additional analyses to assess TE-associated properties using markers and developmental behaviors beyond cavitation.

First, as an alternative lineage-related readout, we cultured individual embryos reconstructed from small (S) or large (L) blastomeres until cavitation and compared the numbers of $CDX2^+$ and $SOX2^+$ cells by immunostaining (Fig. 5F). Although embryos derived from L blastomeres tended to contain a greater total number of cells, no significant differences were detected in the numbers of $CDX2^+/SOX2^-$ (TE-like) or $CDX2^-/SOX2^+$ (ICM-like) cells between embryos derived from S and L blastomeres (Fig. 5G).

Second, following the reviewer's suggestion, we examined YAP nuclear localization as an additional TE-associated molecular feature. Quantitative analysis revealed markedly stronger nuclear enrichment of YAP in embryos derived from L blastomeres compared with those derived from S blastomeres. Given that nuclear YAP activity is a key regulator of TE specification, this result provides independent molecular evidence supporting an early TE-oriented bias in L blastomeres (Fig. 5A and B).

In addition to molecular markers, we identified several behavioral differences between S- and L-derived embryos. Embryos reconstructed from L blastomeres initiated compaction earlier (Fig. 5D) and exhibited a shorter cell-cycle duration at the 8-cell stage, resulting in a higher number of blastomeres at the onset of compaction (Fig. 5E). Notably, in mouse embryos, outer cells that preferentially contribute to the TE lineage have been reported to exhibit shorter cell cycles than inner cells that give rise to the inner cell mass (McDole et al., 2011). The shorter cell-cycle duration observed in L blastomeres may therefore represent a conserved feature associated with cells biased toward TE contribution.

Together, these additional analyses demonstrate that while cavitation alone is insufficient to define TE identity, large blastomeres exhibit a constellation of molecular (YAP nuclear localization) and behavioral (shorter cell cycles) features consistent with an early, non-deterministic bias toward trophectoderm development.

3. L 45. Suggests that in mouse, developmental potential may be restricted earlier than previously thought. The term "restricted potential" should be used with caution. Appearance of lineage bias does not necessarily mean that there is concomitant lineage restriction. Biased blastomeres may still show full potential when removed from their position in the intact embryo. Indeed, that is exactly what the authors observe here and discuss on l234.

Response:

We thank the reviewer for this clarification. We fully agree that early lineage bias does not

equate to irreversible lineage restriction. Consistent with this interpretation, embryos derived from large blastomeres still contained $SOX2^{+}$ cells, indicating preserved bipotency despite a bias toward trophectoderm-related features. Moreover, embryos derived from small blastomeres were also able to generate both ICM- and TE-like cells when cavitation could be maintained. Accordingly, we have revised the manuscript to avoid the term “restricted potential” and now explicitly emphasize that the observed size-associated differences reflect an early developmental bias rather than lineage commitment.

4. As well as between blastomere variation, they also report variation among embryos of the same stage. Why is this?

Repose:

We appreciate this question. The variation observed among embryos of the same nominal developmental stage reflects the well-known developmental asynchrony characteristic of bovine embryos, which largely arises from natural differences in oocyte quality. Unlike experimental model organisms such as mice, which are maintained under highly controlled laboratory conditions, bovine oocytes are collected from ovaries obtained from multiple animals managed at different farms and under diverse husbandry conditions. In addition, the physiological status of the donor animals can vary substantially. As a consequence, variability in oocyte quality is unavoidable in bovine studies and contributes to embryo-to-embryo differences in developmental competence, even when embryos are classified at the same morphological stage. This intrinsic heterogeneity should therefore be considered a biological feature of bovine embryogenesis rather than experimental noise.

5. Is the cell division rate higher in large vs small blastomeres?

Response:

We thank the reviewer for this important question. Time-lapse analyses revealed that embryos derived from large (L) blastomeres initiated compaction earlier than those derived from small (S) blastomeres. In contrast, no significant difference was observed in the timing of cavitation between the two groups (Fig. 5D).

Further analysis showed that the duration of the 8-cell stage was shorter in embryos derived from L blastomeres, resulting in an increased number of progeny cells originating from L blastomeres at the onset of compaction (Fig. 5E). These findings indicate that L blastomeres undergo cell divisions more rapidly during early cleavage stages.

Together, these results suggest a link between cell-cycle dynamics and early lineage bias, consistent with the idea that accelerated cell-cycle progression may contribute to the preferential contribution of large blastomeres to subsequent developmental processes.

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Reviewer #2 (Remarks to the Author):

This manuscripts describes transcript abundance in individual cleavage stage bovine blastomeres from several embryos. In a novel approach, the authors also correlated expression patterns and with blastomere size and blastomere size with developmental potential. The authors compare inter and intra embryonic blastomere variation as a way of demonstrating that the intraembryonic variation they see from 4-cell stage to 8-cell stage is not an artefact of the amplification process of scRNA seq, but there is no independent data (RNAscope, digital PCR) shown to verify these findings.

Response:

We agree that independent validation of single-cell RNA-seq data is important, particularly when assessing intra-embryonic transcriptional heterogeneity. In the revised manuscript, we have therefore added real-time PCR–based validation experiments performed on cDNA synthesized from individual blastomeres.

Quantitative real-time PCR is widely used as a standard and robust approach for validating single-cell RNA-seq results, especially when the same single-cell–derived cDNA is analyzed across platforms. We employed a cDNA synthesis system with demonstrated reliability for single-blastomere inputs, allowing direct comparison between sequencing-based and amplification-based measurements.

Using 8-cell-stage embryos—where transcriptional divergence was most pronounced—we validated two representative genes, *CDX2* and *MAPK14*. The real-time PCR analyses demonstrated that

(i) both genes exhibit significant variability between sister blastomeres,

(ii) their expression levels are positively correlated, and

(iii) large blastomeres express significantly higher levels of both genes than small blastomeres.

Importantly, these results recapitulate the intra-embryonic expression patterns observed by scRNA-seq, supporting the conclusion that the transcriptional divergence detected at the 4- to 8-cell stages is biological rather than an artefact of amplification.

While we acknowledge the reviewer’s suggestion to use RNAscope or digital PCR, these approaches are not currently optimized for sequential isolation and analysis of individual bovine blastomeres. Given these technical constraints, real-time PCR provided the most appropriate and reliable independent validation strategy for the specific experimental design used in this study. The validation data are now presented in the revised Supplementary Fig.

4.

Using intraembryonic enrichment as a criterium, the authors detect MAPK as one of the pathways showing differential distribution and then compare member genes to CDX2 which has already previously been shown to be differentially expressed at the 8 and 16 cell stage in cattle by the Wolf and Blum groups (Lavagi et al., 2018). References to this paper are missing in this manuscript as are a comparison of the results and conclusions.

Response:

We thank the reviewer for this valuable suggestion. In the revised Discussion, we now cite and discuss the work of Lavagi et al. (2018). We have incorporated the following sentence into the Discussion: In this context, previous studies provide important context for interpreting these findings. Lavagi et al. (2018) reported blastomere-to-blastomere variability in lineage-associated genes, including *CDX2*, at the 8-cell stage, although they did not interpret this heterogeneity as the onset of lineage bifurcation18. More recently, Hu et al. (2024) demonstrated that the first unequivocal transcriptional separation between the ICM and TE lineages emerges at the early blastocyst stage33. Together, these studies indicate that bovine lineage segregation becomes transcriptionally defined relatively late. Rather than contradicting these reports, our findings instead suggest that early transcriptional divergence functions as a priming phase that biases developmental trajectories prior to definitive lineage separation between the ICM and TE lineages. This pattern aligns with conceptual models of symmetry-breaking, in which subtle asymmetries—generated by stochastic partitioning during cleavage divisions and reinforced by transcriptional circuits—gradually bias cells toward distinct developmental trajectories before overt lineage segregation occurs 34.

In the comparisons of high and low CDX2 blastomeres, another signaling pathway, namely WNT signaling is shown in Fig. 3 but not analysed further even though of equal P value and higher GAGE statistic than MAPK signaling.

The further analysis of large and small 8-cell stage blastomeres is an interesting approach though the lack of developmental potential of small blastomeres may simply reflect the presence of too little cytoplasm, which can be deleterious to further development (see “The development capacity of blastomeres from 4- and 8-cell sheep embryos” by S. M. Willadsen in J Embryol Exp Morphol 1981 Vol. 65 Pages 165-72) and the author’s interpretations are thus fraught with problems.

Response:

We sincerely thank the reviewer for raising this important point and for highlighting the

potential confounding effect of reduced cell mass or cytoplasmic volume on blastomere developmental capacity. This concern was critical for the interpretation of our results and directly motivated additional experiments.

To address this issue, and in line with the reviewer's suggestion, we performed new reaggregation experiments using reconstructed 8-cell–stage embryos composed exclusively of either smallest blastomeres (all-small) or largest blastomeres (all-large). In these reconstructed embryos, cavitation was observed in all cases; however, embryos derived from small blastomeres exhibited markedly fragile cavity expansion and were unable to maintain stable cavitation (Fig.. 6B and C). In contrast, embryos reconstructed from large blastomeres showed robust cavity expansion and contained a greater total number of cells, with a particularly pronounced increase in trophectoderm (TE) cells (Fig. 6D and E).

These results indicate that while smallest blastomeres retain the intrinsic ability to initiate cavitation, largest blastomeres preferentially contribute to embryonic growth and TE cell expansion. Thus, although reduced cell mass can negatively affect developmental robustness, it cannot fully account for the size-associated differences observed, supporting our interpretation that these differences reflect an early, non-deterministic bias rather than a trivial consequence of cytoplasmic insufficiency.

Line 76 add: this complements prior work on cattle 8 and 16 cell stage embryos (Lavagi, I., Krebs, S., Simmet, K., Beck, A., Zakhartchenko, V., Wolf, E., Blum, H., 2018. Single-cell RNA sequencing reveals developmental heterogeneity of blastomeres during major genome activation in bovine embryos. Sci Rep 8, 4071.). This paper needs also be related to in the discussion.

Response:

Thank you for highlighting this important study. We agree that it is highly relevant to our work. We have now cited Lavagi et al. (2018) in both the Background and Discussion sections and explicitly discussed how our findings complement their analysis of transcriptional heterogeneity at the 8-cell stages in bovine embryos.

Fig. 1B Requires the use of more distinctive symbols (as for 2-cell) for the individual embryos of 4 cell and 8-cell stages.

Response:

We thank the reviewer for this helpful suggestion. We have revised Fig. 1B to use more distinctive symbols for individual embryos to improve visual clarity and facilitate embryo-level comparisons.

Figs. 2 and 3 Font sizes too small to read and markers too faint.

Response:

We thank the reviewer for pointing this out. The font sizes and marker visibility in Fig. 2 and 3 have been increased to improve readability in the revised manuscript. In addition, we have reviewed all other figures and made further adjustments where necessary to enhance overall visual clarity.

Line 123, Do the authors mean most as opposed to several?

Response:

We thank the reviewer for this comment. This section has been revised throughout.

Line 146 CDX2-expressing cells

Response:

Thank you for pointing this out. We have corrected the wording.

Lines 147 to 150 has mistakes in terms of which panel of Fig 3 is referred to. No legend for Fig. 3 panels D and E. Panel E should show R-squared not r (and as mentioned before, all too small to see). Comparison should probably be done using logs of TPM to avoid skewing by the few very large values.

Gene names inconsistently written in italics.

Response:

Thank you for these comments. We have generated a revised Fig. 3 and updated the figure legend accordingly, correcting panel references, adding missing legend descriptions, revising the correlation metric, and improving overall readability.

Lines “168 These results suggest that large blastomeres are transcriptionally biased
169 toward the activation of TE-associated pathways, such as the MAPK pathway, indicating
170 the potential unequal inheritance of maternal transcripts” should be removed as no evidence is shown that MAPK bias is correlated with TE nor that any bias is due to unequal inheritance, could e.g. also be due to unequal mRNA degradation etc.

Response:

We appreciate the reviewer’s comment. As suggested, we have removed the sentence stating that the MAPK-related transcriptional bias in large blastomeres “indicates the potential unequal inheritance of maternal transcripts.” Our data do not directly demonstrate the origin of this bias.

Supplementary Table 1 (erroneously labelled Table 3) is at odds with Fig 4: see P-values of MAPK14. Clearly the MAPK pathway differences are minor (2^2) in comparison to some other genes which show a 2^5 change. The change needs to be normalised to all genes sequenced as it may be that most genes are overexpressed in these datasets and thus the fold change of the MAPK pathway is irrelevant.

Response:

We thank the reviewer for this important and careful comment. We have addressed these points as follows.

First, we performed differential expression analysis using a fully normalized dataset. As the reviewer correctly anticipated, this analysis identified *RAC1* as a differentially expressed gene, whereas *MAPK14* was not detected as a DEG in the normalized dataset (Supplementary Data 8). Consistent with this, the paired Wilcoxon signed-rank test shown in Fig. 4 yielded a borderline p value for *MAPK14* ($p = 0.06$).

Second, despite not reaching significance in the DEG analysis, *MAPK14* expression showed a consistent directional trend: in 4 out of 5 embryos, *MAPK14* expression was higher in large (L) blastomeres than in small (S) blastomeres. Moreover, bootstrap analysis revealed that this difference reached statistical significance (Supplementary Table 2). These results indicate that *MAPK14* exhibits reproducible inter-blastomere bias, even though its effect size is smaller than that of some other genes.

Third, we would like to clarify that the genes examined in Fig. 4B were not intended to represent DEGs between S and L blastomeres. Instead, these genes were selected from the set of genes exhibiting high inter-blastomere variability, as shown in Fig. 2D were subsequently compared between S and L blastomeres. To avoid the misunderstanding that the genes shown in Fig. 4B represent S–L DEGs, we have revised the figure legend accordingly.

The conclusion in lines 185-187 “185 maintaining their ability to contribute to both the TE and ICM lineages. Collectively, these results suggest that blastomere size is linked to both the transcriptional and functional properties relevant to early lineage bias” is unwarranted as no embryos derived from the smallest blastomere were examined by marker expression.

Response:

Thank you for this insightful comment. Because S-derived embryos rarely progressed to stages permitting reliable immunostaining, we produced additional embryos to increase the number of S-derived structures available for analysis. Using these additional samples, we performed further assessments of YAP localization and lineage-marker expression in embryos derived from both large (L) and small (S) blastomeres. Specifically, we examined

nuclear YAP enrichment at the morula stage and evaluated CDX2 and SOX2 expression in single-blastomere–derived blastocyst-like structures as well as in reconstructed embryos composed exclusively of either L or S blastomeres. These analyses revealed that L-derived embryos contained a higher number of $CDX2^{+}/SOX2^{-}$ cells and displayed markedly stronger nuclear YAP localization compared with S-derived embryos. These findings provide functional evidence that large blastomeres have an increased propensity to generate TE-fated daughter cells relative to small blastomeres. Together, these results support the interpretation that blastomere size correlates with molecular and functional properties indicative of early TE-biased tendencies. All new data have been incorporated into the revised manuscript.

Lines 240 onward in discussion needs to deal with Lavagi 2018 model and with lineage bifurcation model proposed in Hu, B., Jin, H., Shi, Y., Yu, H., Wu, X., Wang, S., Zhang, K., 2024. Single-cell RNA-Seq reveals the earliest lineage specification and X chromosome dosage compensation in bovine preimplantation embryos. FASEB J 38, e23492.

Response:

We thank the reviewer for pointing out that the Discussion should address both the model proposed by *Lavagi et al.* (2018) and the lineage-bifurcation framework described by *Hu et al.* (2024). In the revised manuscript, we have now incorporated a dedicated paragraph that explicitly discusses these two studies and situates our findings within the broader conceptual framework of bovine lineage specification.

Specifically, we now note that *Lavagi et al.* reported blastomere-to-blastomere heterogeneity in lineage-associated transcripts such as *CDX2* at the 8-cell stage, although they did not interpret this variability as evidence of early lineage bifurcation. We further discuss the recent findings of *Hu et al.* demonstrating that the first unequivocal transcriptional separation between the ICM and TE lineages appears at the early blastocyst stage. By integrating these observations, the revised text clarifies that bovine lineage segregation becomes transcriptionally resolved relatively late.

To place our work in this context, we highlight that our results reveal transcriptional divergence between sister blastomeres emerging already at the 4-cell stage and increasing by the 8-cell stage. As described in the added paragraph, this earlier divergence is consistent with the symmetry-breaking and lineage-competition model proposed from mouse studies, in which small early asymmetries—arising from stochastic partitioning during cleavage and reinforced by transcriptional circuits—gradually bias cell trajectories before overt bifurcation. Together, this newly added discussion shows how our findings extend and unify the models proposed by *Lavagi et al.* and *Hu et al.*, demonstrating that bovine embryos exhibit an

upstream priming phase that precedes—and may bias—the later ICM–TE segregation.

Reviewer #3 (Remarks to the Author):

This manuscript enriches our understanding of embryonic blastomere diversification (or symmetry breaking) in a mammalian species other than mouse or human. Briefly, the study compared individual blastomeres from the same bovine embryo at successive cleavage stages (1-, 2-, 4-, and 8-cell), using single-cell RNA sequencing; moreover, at the 4- and 8-cell stage, the smallest and the largest blastomere from the same embryo were compared with each other too. As such the study makes a valuable contribution to the field.

I have the following remarks.

Major remarks

To my knowledge there is only one other study that examined the transcriptomes of 8-cell bovine embryos at the single cell level (PMID 29511234); although the comparison of all blastomeres from the same embryo is missing in PMID 29511234, I think Hinata and colleagues should mention PMID 29511234 and should compare their results with those of PMID 29511234 as far as possible.

Response:

We thank the reviewer for this important comment. We agree that, to our knowledge, PMID:29511234 represents the only previous study that analyzed single-cell transcriptomes of bovine 8-cell embryos, and we have now cited this study in the revised manuscript.

As noted by the reviewer, PMID:29511234 did not include a comparison of all blastomeres derived from the same embryo, which constitutes a key methodological difference from our approach. Nevertheless, where direct comparison was possible, we have compared our results with those reported in PMID:29511234 and discussed similarities and differences, particularly with respect to genes and pathways associated with early lineage bias.

Specifically, we have added a new paragraph to the Discussion that places our findings in the context of previous single-cell transcriptomic studies of bovine embryos. In this section, we discuss the blastomere-to-blastomere variability in lineage-associated genes, including *CDX2*, reported by Lavagi et al. (2018; PMID:29511234), and clarify how our sister-blastomere–based analysis extends these observations. We also compare our findings with recent evidence demonstrating that the first unequivocal transcriptional separation between the inner cell mass and trophectoderm lineages emerges at the early blastocyst stage (Hu et

al., 2024).

Together, these comparisons support the interpretation that early transcriptional divergence observed in our study does not represent definitive lineage segregation, but rather functions as a priming phase that biases developmental trajectories prior to overt lineage separation. This interpretation is consistent with conceptual models of symmetry breaking, in which subtle transcriptional asymmetries generated during cleavage divisions are progressively reinforced, ultimately biasing cells toward distinct developmental trajectories before clear lineage segregation becomes apparent.

Developmental rates to blastocyst don't matter much as long as the focus is to compare for gene expression all sister blastomeres of the same embryo up to the 8-cell stage. But when the Authors compare small and large blastomeres for blastocyst formation, then the numbers and rates should be presented in a Table. In Fig.4c 168 hpi: some of the embryos look dead, not delayed, rather just dead. For this experiment in which the blastocyst formation of small and large blastomeres is comparatively assessed, it is important to provide information about the actual blastocyst rates.

Response:

As suggested by the reviewer, we have prepared a table summarizing the cavitation rates of embryos reconstructed from small (S) and large (L) blastomeres, which is now included as Table 1. We intentionally avoided the term *blastocyst*, as it was not possible to verify the presence of both the inner cell mass and trophectoderm in all embryos.

Consistent with the reviewer's observation, embryos reconstructed from S blastomeres were less likely to undergo cavitation. Approximately half of these embryos arrested at the compacted morula stage without initiating cavitation, and even among those that did cavitate, only a small fraction were able to maintain the blastocoel. Therefore, as suggested by the reviewer, the embryos shown in the previous images are likely to represent developmental arrest rather than delayed development.

In the revised manuscript, we have therefore replaced these images with representative time-lapse sequences of embryos derived from blastomeres that successfully initiated cavitation (Fig. 5C). Furthermore, analysis of developmental timing revealed that embryos reconstructed from S blastomeres initiated compaction later than those derived from L blastomeres, whereas no clear difference was observed in the timing of cavitation. These results are now shown in Fig. 5D.

It needs to be better introduced that there are size differences between the sister blastomeres. The Authors go straight to the molecular analysis of this trait, but before doing so, they should

present quantitative data documenting that there is a difference (small, large) in the first place (for example this could be done by showing the histograms of the statistical distributions of the diameters). I see the pictures of small and large blastomeres (Fig.4), and I trust that the differences are real, but still I think that they should be quantified.

Response:

We thank the reviewer for this helpful suggestion. To better introduce the size differences between sister blastomeres, we have now quantitatively analyzed blastomere diameters at both the 4-cell and 8-cell stages. These data are presented as distributions of blastomere diameters and have been added as a new panel (Fig. 4A). This analysis confirms the presence of consistent size asymmetry among sister blastomeres within individual embryos and provides an objective basis for defining the smallest and largest blastomeres used in subsequent molecular and functional analyses. We have also revised the text to introduce these quantitative size data prior to the molecular analyses, thereby clarifying the rationale for examining size-associated transcriptional and functional differences.

In the mouse (yes I know, the study by Hinata and colleagues is about bovine) small and large blastomeres were found to not behave differently in terms of blastocyst formation and cell lineage composition (PMID 28811525). By contrast in the present study the blastomeres of different sizes seemed to have distinct developmental properties. Is this a biological difference of the two species, or is it more specific of bovine? Do differences at the mRNA level necessarily produce differences at the protein level? It is well known that mRNA-protein correlation is generally poor. Therefore, the obvious question I ask is whether the divergence of transcripts between sister blastomeres reflects in the proteins. At the end (Fig. 4), the Authors show data for two proteins (CDX2, SOX2). It would be informative to compare the mRNA and protein data of Cdx2/CDX2 and Sox2/SOX2. I understand that this cannot be done within the same embryos, but still, it might be possible (and informative) to see if changing mRNA levels between blastocysts of small vs large blastomeres reflect in changing intensities of protein signal.

Response:

We appreciate the reviewer's insightful comment regarding whether transcriptional differences among blastomeres are reflected at the protein level. We agree that this is a highly interesting and important issue. Indeed, recent studies have reported approaches to analyze protein expression at the single-blastomere level, which have advanced our understanding of lineage bias during early cleavage stages.

However, in bovine embryos, methods that allow simultaneous and quantitative assessment of mRNA and protein levels in individual blastomeres are not yet established, and substantial

technical challenges remain.

As an additional experiment to address this point within current technical constraints, we examined nuclear localization of YAP protein at the blastomere stage (Fig.5A and B). YAP is a mechanosensitive effector of the HIPPO pathway and a well-established regulator of trophectoderm-associated cell states. We found that nuclear YAP localization was enriched in the largest blastomeres, indicating that transcriptional differences between small and large blastomeres are accompanied by functional differences relevant to TE contribution.

Furthermore, as suggested by the reviewer, we analyzed both mRNA (Supplementary Fig.5) and protein expression of CDX2 and SOX2 (Fig. 5F and G) at the blastocyst-like stage in embryos derived from small and large blastomeres. In addition, we quantified mRNA expression of other pluripotency-associated markers, including *NANOG* and *OCT4*. In cavitated embryos, both small- and large-blastomere–derived embryos expressed CDX2 and SOX2 at both the mRNA and protein levels, and no significant differences were observed between groups in *NANOG* or *OCT4* mRNA expression.

These findings indicate that, regardless of blastomere size, embryos retain the potential to generate both TE- and ICM-associated cell populations at the mRNA and protein levels. At the same time, the enrichment of nuclear YAP, the increased propensity for cavitation, and the enhanced trophectoderm expansion observed in embryos derived from large blastomeres suggest that large blastomeres are functionally biased toward TE contribution.

Together, these results support the interpretation that transcriptional differences observed among blastomeres are linked not only to molecular heterogeneity but also to functional differences influencing developmental behavior, without implying irreversible lineage restriction.

Minor remarks

Larger blastomeres cavitate earlier; but ultimately also the smaller blastomeres cavitate. How robust is this difference of timing? Larger blastomeres might simply have more ATP and/or more mitochondria, which make all processes run faster, rather than having a predisposition toward trophectoderm. Since Fig.4d shows a borderline p value of 0.048. I suggest to show the data points on top of the two histogram bars.

Response:

In response to the reviewer's comment, we performed additional time-lapse analyses, which are now presented in Fig. 5D. These analyses demonstrate that there is no difference in the timing of cavitation between embryos reconstructed from small (S) and large (L) blastomeres. Importantly, the absence of cavitation at 168 h was not due to delayed cavitation, but rather

to a failure to maintain cavitation after it had been initiated.
As suggested by the reviewer, we have replaced the previous figure with a table summarizing these outcomes. To avoid the potential misinterpretation that the lack of cavitation at 168 h reflects delayed timing, we explicitly distinguish between “cavitation observed” and “cavitation maintained” in the table (Table 1).

Fig 3 legend is incomplete (‘d’ and ‘e’ shown in the figure are not explained in the legend).

Response:

We thank the reviewer for this comment. Fig. 3 has been revised accordingly and is now provided as the revised Fig. 3. In addition, we have ensured that all figure legends have been updated to include complete explanations for all panels.

Fig 4b: connecting the points of small and large blastomere with a line is, in my opinion, misleading, as it suggests that intermediate-sized blastomeres have an intermediate behavior. While this is plausible, it is an assumption. What statistical test was used to assess statistical significance? Please mention it in the legend.

Response:

We thank the reviewer for this comment. We agree that connecting the smallest and largest blastomeres with a line could be misleading, as it may imply intermediate behavior of medium-sized blastomeres. To avoid this potential misinterpretation, we have removed the connecting lines in Fig. 4B in the revised manuscript.
Regarding statistical analysis, statistical significance was assessed using a paired Wilcoxon signed-rank test, as the smallest and largest blastomeres were derived from the same embryo. This information has now been added to the figure legend.

Fig 4e: please show scale bars

Response:

We thank the reviewer for this comment. Scale bars have now been added to the revised figure, which is presented as Fig. 5F.

I hope the Authors find my comments useful.

The manuscript has several supplementary files, I apologize if some of the information I asked for is already included in the supplementary files. In this case, please make it more clear in the main text, that the information is available.

Response:

We thank the reviewer for these constructive comments. We have carefully reviewed the supplementary materials and, where relevant information was already included, we have now clarified this more explicitly in the main text by adding appropriate references to the corresponding supplementary figs and tables.

Response to the Editor and Reviewers

We sincerely thank the Editor and the Reviewers for their careful and constructive evaluation of our manuscript and for their positive consideration of our work for publication in *Communications Biology*. We have revised the manuscript accordingly, and all changes are highlighted in the revised version.

We have carefully reviewed the journal's editorial policies and ensured full compliance. Individual data points have been added where required, numerical source data are provided as Supplementary Data, and ARRIVE have been fulfilled. All figures comply with the Digital Image Integrity Guidelines.

Reviewer #1

Comment:

The authors have responded well to all the comments of the reviewers and I am satisfied with their answers. The study is a useful contribution to our understanding of early embryo development

Response:

We thank the reviewer for the positive evaluation and encouraging remarks. We appreciate the recognition that the revisions and additional experiments have strengthened the manuscript.

Reviewer #2

Comment:

Might be good to look at the changes in proportions too (i.e., CDX2+ relative to total cells in large and small-derived embryos) as this better accommodates the structure of the experiment.

Response:

We thank the reviewer for this helpful suggestion. We have now calculated the proportion of CDX2-positive cells relative to the total cell number on a per-embryo basis. The revised analysis is presented in revised Fig. 5F and Fig. 6E and described in the Results section.

Although embryos derived from the largest blastomeres exhibit higher CDX2-positive cell numbers, the proportion of CDX2-positive cells did not differ significantly between groups. This lack of difference in CDX2-positive ratio appears to be attributable, at least in part, to a concomitant tendency toward increased CDX2- / SOX2+ (ICM) cell numbers in rec-L embryos. Thus, both TE and ICM cell numbers increased in parallel, resulting in no significant change in TE proportion.

Reviewer #3

Comment:

Were the small blastomeres all nucleated? It is known that bovine zygotes often undergo abnormal divisions and produce smaller cells without nucleus…

Response:

We thank the reviewer for raising this important point. We agree that abnormal cleavage events can generate anucleate cytoplasmic fragments in bovine embryos (e.g., Destouni et al., 2016). In our previous studies, we have demonstrated that abnormal cleavage patterns, particularly direct cleavage, are frequently associated with aberrant nuclear status, including anucleate or otherwise abnormal blastomeres (Suzuki et al., 2023; Nagai et al., 2021).

In the present study, only 2-cell stage embryos at 28 hpi corresponding to normally cleaved embryos were selected for subsequent analyses. Embryos were assessed by time-lapse imaging, and only those exhibiting normal cleavage patterns were included. Embryos showing abnormal cleavage events or cytoplasmic fragmentation were excluded. Given the established association between abnormal cleavage and aberrant nuclear status, this selection strategy substantially reduced the likelihood of including anucleate or otherwise aberrant blastomeres.

Furthermore, many of the smallest blastomeres underwent subsequent cleavage divisions and progressed to the compaction stage (Table 1), demonstrating sustained mitotic activity. This developmental competence strongly indicates that the analyzed blastomeres were nucleated. We have clarified this selection strategy in the revised Blastomere isolation section of the Methods.