Major transcriptomic, epigenetic and metabolic changes underlie the pluripotency continuum in rabbit preimplantation embryos

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First decision letter

MS ID#: DEVELOP/2022/200538

MS TITLE: Major transcriptomic, epigenetic and metabolic changes underly the pluripotency continuum in rabbit preimplantation embryos

AUTHORS: Wilhelm Bouchereau, Luc Jouneau, Catherine Archilla, Irene AKSOY, Anais Moulin, Nathalie DANIEL, Nathalie Peynot, Sophie Calderari, Thierry JOLY, Murielle GODET, Yan JASZCZYSZYN, Marine Pratlong, Dany Severac, Pierre Savatier, Veronique Duranthon, Marielle AFANASSIEFF, and Nathalie Beaujean

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 significant criticisms and recommend a substantial 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, 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 major revision. If it would be helpful, you are welcome to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating your plans for addressing the referee’s comments, and we will look over this and provide further guidance.

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

In this study, Bouchereau et al performed transcriptome analysis of various stages of pre-implantation embryos in rabbits, a useful model to study the development of bilaminar disc embryos similar to human and non-human primates.
The complemental analysis using bulk and single-cell RNA-seq together with the validations by immunofluorescent staining (IF) revealed early lineage segregation of extra-embryonic lineages, the transition of pluripotent states and dynamics of their metabolic and epigenetic changes.
Finally, the comparative analysis including published mouse and money datasets identified commonly expressed gene sets observed in ICM of blastocyst across these species.
Collectively, this study would be informative to understand the conserved and divergent mechanism underlying the regulation of pluripotency, which may help to capture the pluripotent cells in culture. The experiments are well-designed and the quality of the datasets is high enough as a resource for the community.

Comments for the author

I have minor concerns and suggestions for improving the manuscript.
1. Line 71-72, 76-77, “Primed” and “Formative” pluripotency markers The authors describe primed and formative pluripotent states are associated with the expression of representative transcription factors. However, so far, we do not know unique markers which can distinguish these two states, except for some lineage markers modestly upregulated in primed pluripotent stem cells. Indeed the authors also describe “formative/primed pluripotency marker OTX2” in Line 249. Thus, I would suggest that the authors change the statement.
2. Line 123
“Most of the pluripotency…” should be “Most of the naive pluripotency…”.
3. Line 205, Fig.3B The image of SOX2 fluorescence is somehow too dim to see compared with its z-stack image underneath. The authors should replace it with a brighter image.
4. Line 213, Hopf et al. 2011 The report showed data using conventional in situ hybridization rather than FISH to detect the expression pattern of BMP2 and 4. It should be corrected.
5. Line 222-223 It would be informative to show the distribution of definitive endoderm (DE) by IF of some markers.
6. Line 225-229 Did the authors identify rabbit PGCs from their 10x dataset? If so, it would be informative to show their representative gene expressions and where they are in the UMAP.
7. Line 235-241 The genes listed in “Primed ID” seems to be early primitive streak makers rather than pluripotency markers. Related to comment 1, the “Formative ID”
should be “Formative/Primed ID”, and “Primed ID” should be alternatives such as the use of “Gast” as in the previous paper (Nakamura et al., 2016).
8. Line 252, Figs. 5D, S4A While the expression of ESSRB is detected both in ICM and TE in rabbit blastocyst, ESRRB is almost absent in mouse TE and human ICM (Blakeley et al.,
2015). I suggest discussing it in the manuscript.
In addition, the DPPA5 staining looks not specific in ICM. I am wondering whether the antibody specifically stains DPPA5? Did the authors test the same antibody for a later stage epiblast for the negative control?
9. Line 265-267, 5mC and 5hmC staining The dynamics of the signals, in particular 5mC, look subtle. Can the authors quantify the levels?
10. Line 276, Fig. 6B It would be nice to have single cell data for epigenetic modifiers as in Fig. 7A and B. As the authors suggest E5.0 is a transition state (Line 416) and that stage shows a unique pattern of 5hmC in IF, it would be good to include this stage using 10x dataset.

References
Blakeley, P., Fogarty, N. M., del Valle, I., Wamaitha, S. E., Hu, T. X., Elder K., Snell, P., Christie, L., Robson, P. and Niakan, K. K. (2015). Defining the three cell lineages of the human blastocyst by single-cell RNA-seq. Development 142, 3151-3165.
Nakamura, T., Okamoto, I., Sasaki, K., Yabuta, Y., Iwatani, C., Tsuchiya, H., Seita, Y., Nakamura, S., Yamamoto, T. and Saitou, M. (2016). A developmental coordinate of pluripotency among mice, monkeys and humans. Nature 537, 57-62.

Reviewer 2

Advance summary and potential significance to field

In their manuscript Bouchereau and colleagues present a comprehensive transcriptome analysis of rabbit pre-implantation embryos from E2.7 (morula stage) to E6.6 (early primitive streak stage) using bulk and single-cell RNA-sequencing. They also extend their analysis to studies of oxidative phosphorylation and glycolysis and analyse active and repressive epigenetic modifications during blastocyst formation and expansion.

Comments for the author

The work of Bouchereau and colleagues is of a high value for the whole scientific community working on the processes related to establishment and maintenance of naïve, transformative and primed pluripotency as well as for scientists working on the lineage formation in mammalian systems. The work presented here is of a high quality, the comparisons between different mammalian species are very useful and are presented in a very clear and easy to follow way. There are, however, a few problems that need to be addressed before publication of this beautiful work.

Major points.
1. Page 5 Line 128: “Both TE markers (GATA3 and TFAP2C) and ICM markers (such as DDPA5, SOX15, KLF4, STAT3, KLF17, ESRRB) were enriched in the E2.7 morula samples, suggesting early commitment of blastomeres either to ICM or TE cells.” It is an interesting observation; can the authors confirm it on the protein level? Importantly, as this result was not mirrored by the single cell data in the current manuscript, how certain are the authors of this claim?
2. The authors need to make citations more consistent. They provide citations for some of the marker genes, but not for others. Perhaps the authors should consider creating a comprehensive table with all necessary citations for the marker genes used/mentioned in the text.
3. It is not always clear from the current manuscript what exactly is already known about rabbit development and what are the new findings presented in the current manuscript. Although I appreciate that the authors list the main findings in the discussion, there is a plethora of additional information “hidden” in the text that is not highlighted in the discussion.
4. Confusingly, some of the “marker” genes are used as markers of several lineages at once (for example OTX2 is presented as primed pluripotency marker, as well as PE marker). Although in some instances this is justified, it needs a clear explanation in the main body of the text. For example, a reader not very familiar with the field may find the sentence on page 1 line 200-201: “At E4.0 and E5.0, expression of the PE-specific gene OTX2 gene was quite homogeneous among endodermal cells” and the sentence on page 5 line 123: “Most of the pluripotency genes had lower transcript levels in epiblast samples (EPI at E6.0, E6.3, and E6.6, thereafter called EPI_6.0, EPIant_6.3 and EPIant_6.6, respectively), which showed higher expression of late epiblast markers (e.g. OTX2)” confusing at the best, or worse - directly contradictory. If the authors aim to present this manuscript to the wider audience, they need to make it much more accessible.
5. Linked to that problem, it is not clear how the authors assessed the difference in OTX2 levels between PE and EPI cells in figure 3B. The term “strong” is quite vague and this reviewer is not fully convinced whether indeed one could use OTX2 as a marker to distinguish EPI and PE (see my previous point on OTX being a marker of both those lineages). Furthermore, in Figure 3B I cannot see anything on the first SOX2 panel (xy view).
6. In the discussion section (page 13) the authors wrote: “Novel markers of naive pluripotency were identified, including MKRN1 and OOEP”, I understand that the word “novel” relates to the rabbit embryos, as the authors admit that these genes were previously implicated in the establishment of pluripotency in other species. Also, such a strong statement would require additional confirmation via immunostaining.

Minor points:
1. Page 5 line 115: “The 51 samples –namely morula, trophectoderm (TE), inner cell mass (ICM), epiblast at E6.0 (EPI), anterior epiblast at E6.3/6.6 (EPIant), primitive endoderm (PE) resulting from these dissections separated according to developmental time and lineage when projected onto the first two components of principal component analysis (PCA) (Fig. 1B).” While

figure 1 is fairly easy to understand, the sentence in the results is confusing and not easily understandable. I would urge the authors to revise it.
2. Fig 1 B. It may be worth changing the colour code in this figure (or consider using different ways to mark different lineages), as for example it is very difficult to distinguish between 3.5 ICM and EPI 6.0 or 4.0 ICM and EPIant 6.6.

First revision

Author response to reviewers' comments

Reviewer 1 Advance Summary and Potential Significance to Field:

In this study, Bouchereau et al performed transcriptome analysis of various stages of pre-implantation embryos in rabbits, a useful model to study the development of bilaminar disc embryos similar to human and non-human primates. The complemental analysis using bulk and single-cell RNA-seq together with the validations by immunofluorescent staining (IF) revealed early lineage segregation of extra-embryonic lineages, the transition of pluripotent states, and dynamics of their metabolic and epigenetic changes. Finally, the comparative analysis including published mouse and money datasets identified commonly expressed gene sets observed in ICM of blastocyst across these species. Collectively, this study would be informative to understand the conserved and divergent mechanism underlying the regulation of pluripotency, which may help to capture the pluripotent cells in culture. The experiments are well-designed and the quality of the datasets is high enough as a resource for the community.

We thank the reviewer for his/her positive appreciation of our work

Reviewer 1 Comments for the Author:

I have minor concerns and suggestions for improving the manuscript.

1. Line 71-72, 76-77, “Primed” and “Formative” pluripotency markers
The authors describe primed and formative pluripotent states are associated with the expression of representative transcription factors. However, so far, we do not know unique markers which can distinguish these two states, except for some lineage markers modestly upregulated in primed pluripotent stem cells. Indeed, the authors also describe “formative/primed pluripotency marker OTX2” in Line 249. Thus, I would suggest that the authors change the statement.
We agree with the reviewer that few, if any, of these markers are strictly specific to naive, formative, and primed states. We have modified the text in several places (especially lines 71, 76, 182, 237/238, and 364 in red in the text) to indicate that the expression of these genes is enriched in the naive, formative, or primed state, as appropriate.

2. Line 123
“Most of the pluripotency…” should be “Most of the naive pluripotency…”.
This has been corrected in the revised manuscript.

3. Line 205, Fig.3B
The image of SOX2 fluorescence is somehow too dim to see compared with its z-stack image underneath. The authors should replace it with a brighter image.
We have modified the figure 3B to show: 1) a z-section of the z-stack with weak OTX2 labelling colocalizing with SOX2 labelling; 2) a z-section with stronger OTX2 labelling, which does not colocalize with that of SOX2. This new figure now better matches the text ("Strong OTX2 signal was only observed in SOX2-negative VisE cells underlying the SOX2/OTX2 double-positive EPI cell layer").

4. Line 213, Hopf et al. 2011
The report showed data using conventional in situ hybridization rather than FISH to detect the expression pattern of BMP2 and 4. It should be corrected.
This has been corrected in the revised manuscript (now line 214).

5. Line 222-223
It would be informative to show the distribution of definitive endoderm (DE) by IF of some markers.
Previous studies (Hassoun et al., 2009a; Hassoun et al., 2009b; Hopf et al., 2011; Viebahn et al., 2002) already reported the segregation between mesoderm and endoderm after E6.0 in rabbit embryos. Therefore, we chose not to focus on the distribution of definitive endodermal markers in our study.

6. Line 225-229
Did the authors identify rabbit PGCs from their 10x dataset? If so, it would be informative to show their representative gene expressions and where they are in the UMAP.
Kobayashi et al. 2021 previously analysed this issue in detail, showing the emergence of PGCs at E6.6. We looked at the co-expression of TFAP2C and NANOS3 to identify these cells in our dataset (see Figure 1 below). These cells are quite rare in our dataset and only confirm the fact that PGCs appear between E6.0 and E6.6 in rabbit embryos as shown previously. Due to space limitations, we do not feel it is useful to add this figure to the manuscript.

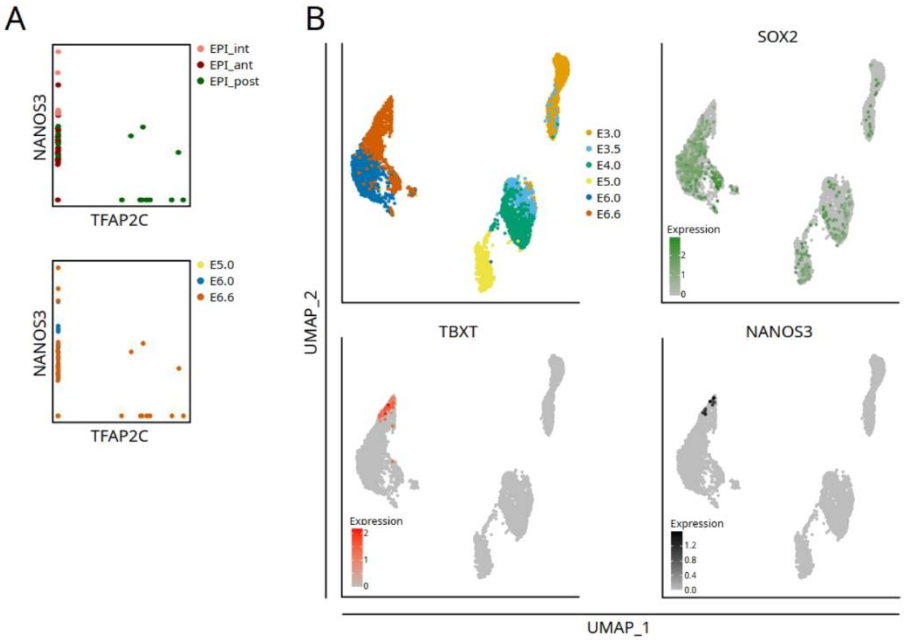

Figure 1: Identification of PGCs in the 10X dataset. (A) Scatter plot showing the expression of TFAP2C and NANOS3 in E5.0, E6.0 and E6.6 EPI cells. Cells are coloured according to their lineage or embryonic stage. (B) UMAP representation of the PLURI dataset. Colours correspond either to the embryonic stage or to the expression level of SOX2 / TBXT / NANOS3.

7. Line 235-241
The genes listed in "Primed ID" seems to be early primitive streak makers rather than pluripotency markers. Related to comment 1, the "Formative ID" should be "Formative/Primed ID", and "Primed ID" should be alternatives such as the use of "Gast" as in the previous paper (Nakamura et al., 2016).
We agree with the reviewer's comment. Figure 5 and text (now line 239-241) have been modified accordingly.

8. Line 252, Figs. 5D, S4A
While the expression of ESSRB is detected both in ICM and TE in rabbit blastocyst, ESRRB is almost absent in mouse TE and human ICM (Blakeley et al., 2015). I suggest discussing it in the manuscript.
This point is discussed in the revised manuscript as suggested (line 467-477).

In addition, the DPPA5 staining looks not specific in ICM. I am wondering whether the antibody specifically stains DPPA5? Did the authors test the same antibody for a later stage epiblast for the negative control?

We tested another antibody and performed comparative immunostaining at day 4.0 and day 6.0. The new images are now included in Figure 5 (panel D). The text has been changed accordingly (line 253-256).

9. Line 265-267, 5mC and 5hmC staining. The dynamics of the signals, in particular 5mC, look subtle. Can the authors quantify the levels?
We agree that the modifications are subtle. Quantification of the signal confirmed our findings (now in supplementary Figure S5). The text has been changed accordingly (line 303-308).

10. Line 276, Fig. 6B
It would be nice to have single cell data for epigenetic modifiers as in Fig. 7A and B. As the authors suggest E5.0 is a transition state (Line 416) and that stage shows a unique pattern of 5hmC in IF, it would be good to include this stage using 10x dataset.
We thank the reviewer for raising this point. We have added the data in the revised manuscript (see Fig. S5D, lines 278 and 295).

Reviewer 2 Advance Summary and Potential Significance to Field:

In their manuscript Bouchereau and colleagues present a comprehensive transcriptome analysis of rabbit pre- implantation embryos from E2.7 (morula stage) to E6.6 (early primitive streak stage) using bulk and single-cell RNA- sequencing. They also extend their analysis to studies of oxidative phosphorylation and glycolysis and analyse active and repressive epigenetic modifications during blastocyst formation and expansion.

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We thank the reviewer for his/her positive appreciation of our work

There are, however, a few problems that need to be addressed before publication of this beautiful work.

Major points.

1. Page 5 Line 128: “Both TE markers (GATA3 and TFAP2C) and ICM markers (such as DDPA5, SOX15, KLF4, STAT3, KLF17, ESRRB) were enriched in the E2.7 morula samples, suggesting early commitment of blastomeres either to ICM or TE cells.” It is an interesting observation; can the authors confirm it on the protein level? Importantly, as this result was not mirrored by the single cell data in the current manuscript, how certain are the authors of this claim?
We agree with the reviewer that this result was not mirrored by the single cell data. We analysed expression of TFAP2C and ESRRB in E2.7 morulae by immunodetection, which confirmed the single cell RNAseq data (Figure 2). Therefore, we decided not to raise this point and the corresponding sentences have been deleted in the revised manuscript.

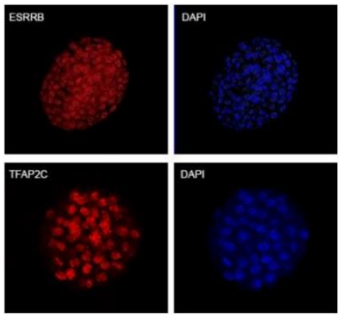

Figure 2: Immunofluorescence detection of TFAP2C and ESRRB in E2.7 morulae.

2. The authors need to make citations more consistent. They provide citations for some of the marker genes, but not for others. Perhaps the authors should consider creating a comprehensive table with all necessary citations for the marker genes used/mentioned in the text.
Thank you for raising this point. We now provide a Supplementary table (Table S1) with all the references for the markers mentioned in the text.

3. It is not always clear from the current manuscript what exactly is already known about rabbit development and what are the new findings presented in the current manuscript. Although I appreciate that the authors list the main findings in the discussion, there is a plethora of additional information "hidden" in the text that is not highlighted in the discussion.
We agree with the reviewer that there is a "plethora of additional information hidden in the text". We discussed the expression patterns of *ESRRB*, *SUSD2*, and *CCND2* in the revised manuscript (line 467-477).

4. Confusingly, some of the "marker" genes are used as markers of several lineages at once (for example OTX2 is presented as primed pluripotency marker, as well as PE marker). Although in some instances this is justified, it needs a clear explanation in the main body of the text. For example, a reader not very familiar with the field may find the sentence on page 1 line 200-201: "At E4.0 and E5.0, expression of the PE-specific gene OTX2 gene was quite homogeneous among endodermal cells" and the sentence on page 5 line 123: "Most of the pluripotency genes had lower transcript levels in epiblast samples (EPI at E6.0, E6.3, and E6.6, thereafter called EPI_6.0, EPIant_6.3 and EPIant_6.6, respectively), which showed higher expression of late epiblast markers (e.g. OTX2)" confusing at the best, or worse - directly contradictory. If the authors aim to present this manuscript to the wider audience, they need to make it much more accessible.
Thank you for raising this point. We have modified the manuscript as follows:
→ Line 198-207: now read: "Although OTX2 is primarily described as a formative/primed marker gene, it is re- expressed in the VisE in mice (Perea-Gomez et al., 2001). In primate embryos, it is expressed in the late EPI and primitive endoderm, and is extinguished in the ParE (Boroviak et al., 2018). The expression pattern of OTX2 can therefore be used to determine the timing of VisE/ParE segregation. At E4.0 and E5.0, expression of OTX2 gene was quite homogeneous among endodermal cells. However, at E6.0 and E6.6, endodermal cells formed two closely related clusters and OTX2 expression was restricted to one of these two subgroups (Fig. 3A). To characterize these OTX2-positive cells, we performed immunofluorescence analysis of E6.0 embryos. Strong OTX2 signal was only observed in SOX2-negative VisE cells underlying the layer of SOX2/OTX2 double-positive EPI cells and was not detected in the ParE (Fig. 3B)."

5. Linked to that problem, it is not clear how the authors assessed the difference in OTX2 levels between PE and EPI cells in figure 3B. The term "strong" is quite vague and this reviewer is not fully convinced whether indeed one could use OTX2 as a marker to distinguish EPI and PE (see my previous point on OTX being a marker of both those lineages). Furthermore, in Figure 3B I cannot see anything on the first SOX2 panel (xy view).
We have modified the figure to show: 1) a z-section of the z-stack with weak OTX2 labelling colocalizing with SOX2 labelling; 2) a z-section with stronger OTX2 labelling, which does not colocalize with that of SOX2. This new figure now better matches the text ("Strong OTX2 signal was only observed in SOX2-negative VisE cells underlying the SOX2/OTX2 double-positive EPI cell layer").

6. In the discussion section (page 13) the authors wrote: “Novel markers of naive pluripotency were identified, including MKRN1 and OOEP”, I understand that the word “novel” relates to the rabbit embryos, as the authors admit that these genes were previously implicated in the establishment of pluripotency in other species. Also, such a strong statement would require additional confirmation via immunostaining.
OOEP has been listed as a "naive" marker in other species, however, to our knowledge, has not been described more clearly as such. In contrast, MKRN1 is a new naive maker, in rabbit, mouse and primates. We have mentioned this point in the discussion (see line 437). We have also provided new results showing immunolabelling for OOEP, KDM5B and MKRN1, that confirms the stronger expression in EPI cells in E4.0 vs E6.0 embryos (see line 380-382; Figures 5 and 8).

Minor points:

1. Page 5 line 115: “The 51 samples –namely morula, trophectoderm (TE), inner cell mass (ICM), epiblast at E6.0 (EPI), anterior epiblast at E6.3/6.6 (EPIant), primitive endoderm (PE) resulting from these dissections separated according to developmental time and lineage when projected onto the first two components of principal component analysis (PCA) (Fig. 1B).” While figure 1 is fairly easy to understand, the sentence in the results is confusing and not easily understandable. I would urge the authors to revise it.
This sentence has been modified in the revised manuscript (see page, line 115-118).

2. Fig 1 B. It may be worth changing the colour code in this figure (or consider using different ways to mark different lineages), as for example it is very difficult to distinguish between 3.5 ICM and EPI 6.0 or 4.0 ICM and EPIant 6.6.
Thanks for the advice. We have changed the symbols used to mark the different lineages to make things clearer.

Second decision letter

MS ID#: DEVELOP/2022/200538

MS TITLE: Major transcriptomic, epigenetic and metabolic changes underly the pluripotency continuum in rabbit preimplantation embryos

AUTHORS: Wilhelm Bouchereau, Luc Jouneau, Catherine Archilla, Irene Aksoy, Anais Moulin, Nathalie Daniel, Nathalie Peynot, Sophie Calderari, Thierry Joly, Murielle Godet, Yan Jaszczyszyn, Marine Pratlong, Dany Severac, Pierre Savatier, Veronique Duranthon, Marielle Afanassieff, and Nathalie Beaujean
ARTICLE TYPE: Techniques and Resources Article

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

Reviewer 1

Advance summary and potential significance to field

The authors adequately addressed all my concerns in the revision. I believe this paper will be very informative for the community as a resource.

Comments for the author

I do not have further requests.

Reviewer 2

Advance summary and potential significance to field

Comments for the author

In their revised manuscript, Bouchereau and colleagues have done most of the changes suggested by this reviewer and I am satisfied with their response to my comments. It is my pleasure to recommend this interesting work to be presented in Development.