Transcriptional Drug Repositioning Improves Development Of Somatic Cell Nuclear Transfer Embryos Via Inhibition Of CDK4/6 And Cyclin D1 During First Cleavage of Bovine Pseudo-Zygotes

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Abstract
Since the birth of the first cloned sheep, ‘Dolly’, Somatic Cell Nuclear Transfer (SCNT) has become a powerful method in the fields of agricultural and biomedical research. However, due to the accumulation of errors during nuclear reprogramming, the efficiency of this technique remained low. In this study, we applied a transcriptional drug repositioning approach for selecting small molecules to improve SCNT efficiency. This involved identifying a blueprint signature of differentially expressed genes in SCNT embryos compared to IVF embryos and finding compounds that target those genes effectively. As a result of the analysis, two highly ranked compounds, namely the breast cancer treatment Palbociclib and the retinoid derivative Fenretinide, were selected which are selective inhibitor of the cyclin-dependent kinases 4/6 (CDK4/6) and inhibitor of Cyclin D1, respectively. We hypothesized that treatment of SCNT reconstructed oocytes during the first cleavage with these two small molecules may prolong the first cleavage in SCNT embryos, which can have a favorable impact on reprogramming by prolonging the exposure of the fibroblast cell to reprogramming factors in the oocyte. We found that the optimal concentration and time of treatment with either Palbociclib (100 nM for 12 h) and Fenretinide (16 µM for 6 h) prolonged the time of first cleavage in treated SCNT embryos, increased the cleavage rate after 72h and improved developmental competence of SCNT derived embryos similar to IVF embryos in vitro. Hence, a transcriptional drug repositioning approach was in this work for the first time applied successfully for improving bovine SCNT efficiency.

Introduction
The somatic cell nuclear transfer (SCNT) technique involves implanting a donor nucleus from a somatic (body) cell into enucleated oocyte and it requires extensive nuclear reprogramming. This reprogramming consists of erasing the former epigenetic memory in the somatic cell nucleus and adding "de novo" epigenetic information, which leads to conferring the totipotent state to the newly forming embryos. As a practical application, the first SCNT-derived calves were generated in 1998 by Cibelli and his colleagues. Although bovine species have relatively higher reproductive cloning efficiency compared to other mammals, cloning efficiency is still less than 10%3–5, which is far less than full-term developmental efficiency of in vivo and in vitro fertilized embryos6–8 This problem is caused by incomplete nuclear reprogramming of SCNT embryos, and hence methods are currently being sought to improve this situation.

One of the most important factors responsible for the insufficiency of early and late embryonic development following SCNT is abnormal transcriptional reprogramming.9–11 This phenomenon is mainly due to different biological nature of transferred somatic cells compared to specialized gametes, the sperm and the oocyte during fertilization.12–16

In this regard, using various epigenetic drugs such as DNA methyltransferase inhibitors (DNMTis)17–19, histone deacetylase inhibitors (HDACis)20–24 and histone methyltransferase inhibitors (HMTis)25,26 have
become increasingly common to improve the efficiency of transcriptional reprogramming during SCNT by modifying the epigenetic status of donor cells and/or reconstructed oocytes. Various DNMTis and HDACis have been extensively used to improve epigenetic reprogramming in SCNT derived embryos in different species. Several studies have shown that this approach can significantly increase the efficiency of early and/or full-term development in different species. In one of our previous studies, we assessed the effect of assisted epigenetic modifications in fibroblast donor cells using trichostatin A (TSA) on the transcriptome of bovine SCNT blastocysts with a microarray chip. In that study, it was found that TSA markedly changed epigenetic reprogramming, reconstituted oocytes (5mC, 5hmC) and blastocysts, but some canonical pathways (including WNT and FGF) were similarly affected in both control and SCNT treated groups. Even though there are known small molecules which are able to improve the success rate of SCNT embryo development to some extent, there is still a need to identify new classes of small molecules with different mechanisms of action, which more efficiently improve the efficiency of SCNT in both in vitro and in vivo conditions.

In practice, due to financial and labor cost of the experiment, it is not possible to test a large number of small molecules on in vitro produced embryos. It is challenging to identify which small molecules to pick for this purpose, and this is precisely what the current work addresses: In this study, transcriptional drug repositioning has for the first time been successfully applied for improving SCNT outcome. Transcriptional drug repositioning has recently attracted increasing attention as it enables to study gene expression profiles of different diseases, identify key genes and search for small molecules that can target those genes effectively among large scale database of compound treatments. Its successful application to provide therapeutic starting points in oncology and neurodegenerative diseases has been established before. Some of the current authors have also used this approach in novel areas of improving stem cell differentiation to cardiomyocytes and suggesting novel anti-malarial drugs, and hence its utility has been shown across many application areas before.

In this particular work, for improving SCNT outcome, gene expression data of SCNT embryos was compared to gene expression of in vitro fertilized (IVF) embryos and genes that were differentially expressed were identified. Given that IVF has generally higher success rates than SCNT, this selection provided genes that are differentially expressed in SCNT embryos, and which on the one hand provide a possible explanation for lower success rates, and on the other hand potentially need to be transcriptionally adjusted to improve SCNT success rates (see Fig. 1 for a schematic representation of this process). The Library of Integrated Network-Based Cellular Signatures (LINCS) was used as a initial dataset for selecting such small molecules. The LINCS database provides data for 20,413 compounds applied on 77 cancer cell lines (after certain time points with various concentrations) leading to 201,776 gene signatures of compound treatments which the algorithm was applied in order to rank-order compounds for the given purpose. The selected compounds were then experimentally evaluated further, as described in this work, to both validate the algorithm employed, as well as to provide practically useful novel small molecules to improve SCNT success rates.


**Results**

**mRNA expression of CDK4 and 6 and CCND1, 2 and 3 in MII oocytes, sperm, bovine fetal fibroblast cells, and embryos derived from IVF and SCNT**

Given that Palbociclib and Fenretinide are selective chemical inhibitor of CDK4/6 and inhibitor of CCND1, first we investigated mRNA expression levels of CDK4/6 and CCND1/2/3 in MII oocyte, sperm, fibroblasts, and 6 and 12 hpi/hpa IVF and SCNT embryos under untreated conditions.

Total RNA was successfully isolated from MII oocytes, sperm, and bovine fetal fibroblast cells (BFFs) using different methods. In the first step, the mRNA expression of $\text{CDK4/6}$ and $\text{CCND1}$ in MII oocytes, sperm and BFFs was evaluated and were normalized to the geomean of two previously validated endogenous reference genes, $\text{GAPDH}$ and $\beta\text{-ACTIN}$ (Fig. 2, A-E). While the mRNA expression of $\text{CDK4/6}$ was remarkably low in sperm, the expression levels were significantly higher in MII oocytes and BFFs ($P<0.05$, Fig. 2). The mRNA expressions of CCND1/2/3 were significantly lower in sperm compared to BFFs but just lower compared to MII oocytes (Fig. 2). In addition, the expression of these genes was also significantly higher in BFFs compared to MII oocytes. Furthermore, the mRNA expression of aforementioned genes was characterized in IVF derived embryos 6 and 12 hpi and also in SCNT derived embryos 6 and 12 hpa. As shown in the result (Fig. 2), the expression of $\text{CDK4/6}$ and $\text{CCND1/2/3}$ has a similar pattern, of being significantly higher in SCNT derived embryos compared to IVF derived embryos. In addition, the level of expression of these genes in SCNT embryos was similar to MII oocytes.

We can conclude that mRNA expression of CDK4/6 and CCND1 is significantly higher in SCNT embryos compared to IVF embryos, and hence SCNT embryos may benefit from treatment with Palbociclib and/or Fenretinide through inhibition of CDK4/6 and CCND1 and slowing down the first cleavage.

**Determination of the optimal concentration of Palbociclib and Fenretinide in parthenogenesis and parthenogenetic bovine embryos**

In order to obtain the optimum concentration of Palbociclib and Fenretinide, the parthenogenetically produced embryos were treated with selected concentrations of Palbociclib (50, 100, 200, 400 and 800 nM) and Fenretinide (4, 8 and 16 µM). These concentrations were selected because the concentrations of instances of Palbociclib and Fenretinide in the LINCS database were 400nM and 4µM, respectively (See Supplementary Table 1). Given that the G1 phase in parthenogenetic embryos is approximately 9 h, we limited the treatment duration to six hours.46

One of the main regulators in the G1 to S-phase transition during cell cycle is Cyclin D1 and its binding partners CDK4 and 6.47 Since the targets of Palbociclib and Fenretinide are CDK4/6 and Cyclin D1, respectively, we next examined the two-cell formation at 30 hpa. As shown in Fig. 3A, the addition of Palbociclib at concentrations of 50, 100, 200, 400 and 800 nM did not change the proportion of embryos ($P>0.05$) reaching two-cell stage at 30 hpa as compared to control embryos. In addition, no significant change ($P>0.05$) was also observed in cleavage rate at 72 hpa (Fig. 3B). However, addition of 100 nM
Palbociclib to culture medium for 6 hpa significantly ($P<0.05$) increased the proportion of oocytes that reached the blastocyst stage at day seven after activation (Fig. 3C). In a second set of experiments, bovine activated oocytes were also treated with 0 (control), 4, 8 and 16 µM of Fenretinide for 6 hpa. As shown in Fig. 4A and 4B, addition of Fenretinide at concentrations of 4, 8, and 16 µM neither changed the two-cell formation rate nor cleavage rate at 30 and 72 hpa, respectively ($P>0.05$). On the other hand, supplementation of culture medium with 4 µM Fenretinide for 6 hpa significantly ($P<0.05$) increased the blastocyst yield at day seven compared to other treatment groups (Fig. 4C). In conclusion, while both Palbociclib and Fenretinide did not change the two-cell formation rate and cleavage rate, they showed beneficial effects on blastocyst yield in parthenogenetic embryos.

**Determination of the optimal time for treatment of activated bovine oocytes with optimal Palbociclib concentrations in parthenogenetic embryos**

We next optimised the time of treatment of activated oocytes with the optimal concentration of Palbociclib and Fenretinide. Previously, it was shown that the observed difference between the onset of the S-phase was related to the duration of the G1-phase$^{46}$, and in particular the S-phase started at 9.5 to 15.5 hpi or hpa in IVF or parthenogenetic embryos, respectively. Therefore, we extended the time of treatment with the optimal concentration of Palbociclib (100 nM, as obtained in the previous experiment) from 6 hpa to 9 and 12 hpa.

While extending the time of treatment of activated oocytes with 100 nM Palbociclib from 6 h to 12 h resulted in a significant reduction in two-cell formation rate ($P<0.05$, Fig. 5A), no change was observed ($P>0.05$) in cleavage rate at day three (Fig. 5B). In addition, the blastocyst rate of those activated oocytes which were treated with 100 nM Palbociclib for 6 hpa was significantly higher than other treatment groups ($P<0.05$, Fig. 5C). Given that we did not observe any improvement in blastocyst formation rate after 12 h treatment with Palbociclib, no experiment was performed for a 15 h time point.

**Optimal concentration and time of treatment of Palbociclib and Fenretinide improved blastocyst yields in parthenogenetic embryos to levels similar to those of IVF embryos**

Next, independent parallel groups including IVF embryos, untreated Parthenogenetic embryos, and Parthenogenetic embryos treated with either 100 nM Palbociclib for 6 hpa (PA-PALB100 6h) or with 4 µM Fenretinide for 6 hpa (PA-FEN4 6h) were carried out in triplicate (simultaneously), to assess the developmental competence of the derived embryos. As shown in Fig. 6A and 6D, the first cleavage rate (two-cell formation rate) evaluated 30 hpa or hpi was higher in Parthenogenetic embryos compared with IVF derived embryos ($P<0.05$). In addition, neither treatment with Palbociclib, nor Fenretinide, prolonged the first cleavage (evaluated at 30 hpa) compared to the IVF group (Fig. 6A and 6D, $P>0.05$).

Subsequently, the cleavage and blastocyst rates were evaluated from the same group of oocytes/embryos. The cleavage rates assessed on day three of development were similar among the treatment groups for both Palbociclib and Fenretinide (Fig. 6B and 6E, $P>0.05$). The blastocyst rate on day seven, however showed a higher percentage in 100 nM Palbociclib for 6 hpa and 4 µM Fenretinide for
6 hpa as compared to non-treated PA group ($P<0.05$) and it was similar to the IVF group (Fig. 6C and 6F $P>0.05$).

**Effect of Palbociclib and Fenretinide on developmental characteristic of SCNT derived embryos**

We next determined whether the optimal concentrations of Palbociclib (100 nM) and Fenretinide (4 µM) for 6 hpa (in agreement with the results in Parthenogenetic embryos) could also promote the developmental competence of SCNT derived embryos. For this purpose, we treated the SCNT-reconstructed embryos with 100 nM Palbociclib for 6 hpa, and found no improvement was observed in two-cell formation (Fig. 7A), cleavage (Fig. 7B) and blastocyst (Fig. 7C) rates ($P>0.05$). This observation may be related to the different nature of SCNT embryos in comparison to Parthenogenetic embryos. In SCNT embryos a fibroblast cell, which has higher expression of \textit{CDK4/6} (Fig. 2), is introduced into an enucleated oocyte, while on the other hand fibroblast cells are absent in Parthenogenetic embryos. So, it is conceivable that longer periods of time are needed to inhibit the function of CDK4/6 in SCNT embryos for better reprogramming. For this purpose, we extended the time of treatment post activation and treated the reconstructed embryos for 9, 12 and 15 h with the optimal concentration of Palbociclib. As shown in Fig. 7A, the two-cell formation rate was decreased significantly ($P<0.05$) following the treatment of reconstructed embryos with 100 nM Palbociclib for 9 and 12 h in compare to other groups. In addition, the cleavage rate in the SCNT-PALB100 12h group was significantly higher than control group (Fig. 7B, $P<0.05$). Furthermore, blastocyst formation was also significantly higher in the SCNT-PALB100 12h group compared to the other groups (Fig. 7C, $P<0.05$). We can hence conclude that parthenogenetic and SCNT embryos have different requirements with respect to Palbociclib treatment conditions, which we assume to be due to different expression of \textit{CDK4/6} in both cases.

The optimal concentration and time of treatment of Fenretinide in Parthenogenetic embryos were next also used in SCNT embryos. No significant change ($P>0.05$) was observed in two-cell formation (Fig. 8A), cleavage (Fig. 8B) and blastocyst (Fig. 8C) rates. Similar to the data which were observed for Palbociclib, this observation also may be related to the different nature of SCNT and Parthenogenetic embryos. In particular, we investigated the hypothesis that higher concentration or longer period of time might be required for the inhibition of Cyclin D1 and improving the developmental competence of SCNT embryos. Hence firstly, increased concentrations of Fenretinide (8, 16 and 32 µM) in addition to the previous concentrations of 0 and 4 µM were used for treating SCNT embryos. As shown in Fig. 8A, increasing the concentration of Fenretinide did not change the two-cell formation rate at 30 hpa (Fig. 8A, $P>0.05$). On the other hand, the cleavage rates were significantly higher with concentrations of 16 and 32 µM Fenretinide, compared to other treatment groups (Fig. 8B, $P<0.05$). Furthermore, the highest blastocyst yield was observed with 16 µM Fenretinide in comparison to other treatment groups (Fig. 8C, $P<0.05$). Finally, when SCNT derived embryos were treated with different concentration of Fenretinide (0, 4, 8, 16 and 32 µM) for 9 hpa no change was observed ($P>0.05$) in two-cell formation (Supplementary Fig. 1A), cleavage (Supplementary Fig. 1B) and blastocyst rates (Supplementary Fig. 1C). Therefore, we conclude that parthenogenetic and SCNT embryos have different most effective treatment conditions for both Palbociclib and Fenretinide. This is likely to be due to the different nature of these two types of embryos (and associated higher expression of \textit{CDK4/6} and \textit{CCND1}), which is related to the introduction
of fibroblasts cell into enucleated oocytes in SCNT embryos, while fibroblast cells are absent in parthenogenetic embryos.

**Optimal concentration and time of treatment of Palbociclib and Fenretinide improved developmental competence of SCNT derived embryos similar to those of IVF embryos**

Independent, simultaneous parallel groups including IVF embryos, untreated SCNT embryos, and SCNT embryos treated either with 100 nM Palbociclib for 12 hpa (SCNT-PALB100 12h), or treated with 16 µM Fenretinide for 6 hpa (SCNT-FEN16 6h), were carried out in triplicate to next assess the developmental competence of the derived embryos. As shown in Fig. 9A, the two-cell formation rate (which was evaluated at 30 hpa or hpi) was higher in SCNT embryos, compared with IVF-derived embryos ($P < 0.05$). Following treatment of SCNT-reconstructed embryos with 100 nM Palbociclib for 12 hpa the first cleavage rate (evaluated 30 hpa) was lower compared to the SCNT group (Fig. 9A). However, despite the reduction of first cleavage rate in the SCNT-treated group compared to the non-treated SCNT group, the first cleavage rate remained significantly higher in the SCNT treated group than the IVF group ($P < 0.05$). The cleavage rate assessed on day three of development was significantly ($P < 0.05$) higher in SCNT-PALB100, 12h compared to IVF and SCNT groups (Fig. 9B). The blastocyst rate on day seven showed a higher percentage in the SCNT-PALB100 12h group compared to the non-treated SCNT group, and it was in particular similar to the IVF group ($P > 0.05$), as shown in Fig. 9C. As shown in Fig. 9D, treating SCNT embryos with 16 µM Fenretinide for 6 hpa did not change the percentage of successful first cleavage after 30 hpa in comparison with the non-treated SCNT group. In addition, the two-cell formation rate in the non-treated and treated SCNT groups was similar to each other, and significantly higher than in the IVF group. The cleavage rate at day three was significantly higher in the SCNT-FEN16 6h group, compared to the non-treated SCNT and IVF group (Fig. 9E). Finally, treating SCNT-reconstructed embryos with 16 µM Fenretinide for 6 hpa increased the blastocyst formation rate at day seven compared to that of the non-treated SCNT group, and to values similar to the IVF group (Fig. 9F). Overall, we can conclude that treating SCNT embryos with both Palbociclib and Fenretinide slows down the first cleavage rate at 30 hpa. In addition, SCNT embryos benefit from treatment with Palbociclib or Fenretinide through inhibition of CDK4/6 and CCND1 in terms of cleavage and blastocyst rate, which was improved to values similar to the IVF group.

**Expression of core pluripotency and trophectoderm markers in SCNT-derived blastocysts from the Palbociclib and Fenretinide treated groups**

We finally investigated the gene expression of two core pluripotency genes, namely $POU5F1^{48-50}$ and $NANOG^{51,52}$, and also $TEAD4^{53,54}$ as a critical regulator of trophectoderm development, on day seven in blastocysts using real-time RT-PCR analysis. The results (Fig. 10) showed that the blastocysts derived from both the SCNT-PALB100 12h and SCNT-FEN16 6h groups express a higher level of $NANOG$, $POU5F1$ and $TEAD4$ mRNA compared to non-treated SCNT embryos. In addition, the mRNA expression of those genes was also significantly higher in SCNT-treated embryos in comparison with IVF embryos. Hence,
treated the SCNT embryos with both Palbociclib and Fenretinide promote the quality of derived blastocysts in terms of expression of core pluripotency and trophectoderm markers.

Discussion

Despite many successes in the field of SCNT and cellular reprogramming, efficiency of this technique remains a challenge for its practical application in reproduction. Aberrant epigenetic reprogramming of somatic nuclei after SCNT is still considered as the main hurdle of SCNT procedure, and therefore, many studies have tried to overcome this obstacle via epigenetic modifications (induced DNA/histone hypomethylation or histone hyper-acetylation) of somatic donor cells and/or reconstructed oocytes.

In this work, a novel computational approach for selecting small molecules for improving cloning efficiency has been presented. To this end, large scale gene expression profiles of compound treatments were utilized, which were then matched with differentially expressed genes between SCNT and IVF-derived embryos (whose gene expression data was published earlier). Two highly ranked small molecules, Palbociclib and Fenretinide, were selected and used in this study for experimental validation. In this study, we treated the reconstructed oocytes immediately after activation using 100 nM Palbociclib and 16 µM Fenretinide for 12 and 6 h (after optimizing conditions), respectively. Overall, we found that treating SCNT embryos in this way improved developmental competence of SCNT bovine embryos.

Palbociclib and Fenretinide are selective inhibitor of the cyclin-dependent kinases 4/6 (CDK4/6) and inhibitor of Cyclin D1, respectively (see Supplementary Tables 3 and 4 for all available pharmacology data obtainable from the ChEMBL database for the concentrations bellow what we used in this study) and are widely used for cancer therapy. Both drugs act by inhibiting the formation of the CDK4/6-Cyclin D1 complex, and preventing the phosphorylation of Retinoblastoma protein (Rb) and thereby G1/S cell cycle transition.

While the oocyte is a main determinant factor of regulating cleavage, spermatozoa also plays an important role in the timing of early cleavage. In this regard, zygotes derived from low fertility sperm showed a delayed first cleavage compared with those derived from high fertility sperm. To the contrary, in another study comparing high and low fertility sperm, early cleavage was more prominent in the low fertility group. In addition, it has been shown before that fast-cleaved embryos, unlike slow-cleaved embryos, had increased loss of methylation at \textit{H19} and \textit{Snrpn} imprinted genes and aberrant \textit{H19} expression compared with in vivo counterparts. This demonstrates that fast cleaved-embryos had some abnormalities in epigenetic modification of imprinting genes which may hamper the proper embryonic development.

There are various important developmental events that occur prior to the first cleavage, including syngamy, early embryonic genome activation, and epigenetic reprogramming, which crucially affect SCNT outcomes. In previous studies in bovine SCNT, it has been observed that the first cleavage time is shortened in SCNT embryos, compared to IVF embryos. In this study, it was hypothesized that the
faster cleavage in SCNT is mainly due to the deficiency of sperm-borne regulatory factors, mainly miRNAs.\textsuperscript{70,71} Numerous studies have confirmed that sperm-borne miRNAs are important during the early embryonic development.\textsuperscript{62,72,73} It is known that, paternal miR-34c is important for the first cleavage\textsuperscript{62} and sperm-borne miRNA-449b increases the time of first cleavage and improves the epigenetic reprogramming in SCNT embryos comparable to that of IVF embryos.\textsuperscript{70} In addition, supplementation of SCNT embryos with sperm-borne small RNA prolonged the average cleavage time of SCNT embryos, which was longer than that of the control group, and similar to IVF embryos.\textsuperscript{71} Furthermore, in this previous study the authors found that sperm-borne small RNA can affect abnormal pronuclear-like structures, and improve developmental competence of bovine SCNT embryos during both pre- and post-implantation development.

Previous studies have shown there are six miRNAs which are exclusively expressed in spermatozoa and they are absent in oocytes,\textsuperscript{72} namely miR-34b, miR-34c, miR-99a, miR-214, miR-451, and miR-449, which commonly target genes of the CDK6 and CCND family (Supplementary References 1–16). Thus, we hypothesized that the small molecule inhibitors of CDK4/6 and CCND1 Palbociclib and Fenretinide prolong the first cleavage in SCNT embryos in a similar manner, and that postponing the first cleavage in SCNT embryos acts in favor of reprogramming of fibroblast cells, and thereby improves SCNT efficiency.

To verify this hypothesis, first we assessed the mRNA expression of CDK4/6 and CCND1/2/3 in MII oocyte, sperm, fibroblasts, and 6 and 12 hpi/hpa IVF and SCNT embryos. In this study, we observed that expression of these genes are higher in fibroblast than sperm. Furthermore, the relative expression of these genes are higher in SCNT embryos, compared with IVF embryos. These results are in accordance with a previous study\textsuperscript{74} which have demonstrated a higher expression of CDK4/6 in mouse fibroblasts than oocytes and 6 hpi IVF mouse embryos.\textsuperscript{74} In the current study, we treated the SCNT embryos with Palbociclib or Fenretinide post activation and investigated their effects on two cell formation after 30 hpa and embryonic development. The results show that the optimized concentration and time of treatment of both small molecules (100 nM Palbociclib for 12 hpa and 16 µM Fenretinide for 6 hpa) have an effect on reduction of two-cell formation rate, which is a consequence of prolonged first cleavage. Mechanistically, this effect can be explained by an inhibition of the formation of the CDK4/6-Cyclin D1 complex, preventing the phosphorylation of Retinoblastoma (Rb). The consequence is the prevention of pseudo-zygotes from exiting the G1 phase and entering the S phase of the cell cycle and thus delayed the first cleavage compared to that in the control SCNT group. Meanwhile, the cleavage ratio at 72 h in the experimental groups was higher than that in the control SCNT group. Furthermore, our results showed that later first cleavage has a beneficial effect on developmental competence of SCNT embryos in terms of blastocyst yield. To further investigate the effect of Palbociclib and Fenretinide on the quality of resultant embryos, we assessed the relative mRNA expression of three developmentally important genes including \textit{NANOG}, \textit{POU5F1} and \textit{TEAD4}. From this we could see that while the expression of these genes in SCNT-treated blastocysts was higher than in the SCNT-control blastocyst, and also the IVF blastocyst. Whether higher mRNA expression of these genes lead to higher post-implantation development requires further investigation.
This study has hence overall demonstrated the first application of transcriptional drug repositioning to identify two small molecules that were able to (i) prolong the time of first cleavage in treated SCNT embryos, (ii) to increase the cleavage rate after 72 h, and (iii) benefit the development of SCNT embryo \textit{in vitro} via inhibition of CDK4/6 and CCND1 during first cleavage. This represents a novel application of transcriptional drug repurposing strategies in this field, and it is expected that it also leads to increased SCNT efficiency in the future.

**Online Methods**

**Media and reagents**

All reagents and media were obtained from Sigma Chemical Co. (St. Louis, MO) and Gibco (Grand Island, NY, USA), respectively, unless otherwise specified.

The Institutional Review Board and Institutional Ethical Committee of the Royan Institute approved all animal care protocols and the proposal. In addition, all methods were performed in accordance with the Institutional Review Board and Institutional Ethical Committee of the Royan Institute guidelines and regulations.

The bovine ovaries used in the study were obtained from cows at the local slaughterhouse (Fasaran, Isfahan), with the permission of the manager of the slaughterhouse and the agreement of veterinary organization.

**Somatic donor cell preparation**

Bovine fetal fibroblast cells (BFFs) were prepared similar to the protocol which has been used in our previous studies.\textsuperscript{75,76} Briefly, the tissue from 2-month-old female fetuses was finely minced until it became possible to pipette. Subsequently, the minced tissue was disassociated by trypsinization [0.25\% trypsin/EDTA (Gibco, Invitrogen)]. Next, cell suspension was centrifuged and cultured in Dulbecco's modified Eagle medium F-12 (DMEM/F-12) containing 10\% FBS and 1\% penicillin-streptomycin at 37.5°C and 5\% CO\textsubscript{2} in a humidified atmosphere. After reaching confluency, BFFs were frozen in -196 °C liquid nitrogen. Frozen cells were thawed and used for various assessments.

**Bovine oocyte preparation**

The ovaries were transferred to the laboratory from slaughterhouse as soon as possible (about in 2 hours) in physiological saline, containing 100 IU/ml penicillin and 100 mg/ml streptomycin at 15–17°C. Upon arrival, the ovaries were washed, trimmed and then kept at 15–17 °C until the time of harvesting of the oocytes according to the previously described protocol.\textsuperscript{77} All the 2–8 mm follicles were aspirated with an 18 gauge needle attached to a vacuum pump for obtaining cumulus-oocyte complexes (COCs). Only oocytes containing homogenous cytoplasm and at least three layers of compact cumulus cells were selected for \textit{in vitro} maturation (IVM).
Preparation of Palbociclib and Fenretinide solution

Commercially available Palbociclib (PD0332991 isethionate; Sigma, PZ0199; ≥98%) and Fenretinide (Retinoic acid p-hydroxyanilide; Sigma, H7779; >98%) were dissolved in water and DMSO, respectively and then formulated to 0.4 and 2.4 mM stock solutions, respectively and stored at -20°C. Serum-free BO-IVF media (Cat. IVC1703N) was used to dilute the stock solutions to obtain the desired compound concentrations later.

IVM of bovine oocytes

At first, selected COCs were washed in HEPES tissue culture medium 199 (H-TCM) and subsequently in TCM medium mixed with sodium pyruvate, 10% FBS, 10 µg/ml follicle-stimulating hormone (FSH; Sigma F8174), 10 µg/ml luteinizing hormone (LH; Sigma L5269), 100mM 17β-estradiol (E2; Sigma E4389), 0.1 mM cysteamine (Sigma M9768), 10 ng/ml epidermal growth factor (EGF; Sigma E4127), and 100 ng/ml insulin-like growth factor 1 (IGF1; R&D 291-G1) (maturation medium: MM). Then the COCs were transferred and cultured in groups of 10 into 50µl droplets of MM and incubated for 24 h at 38.5 ºC in a humidified 5% CO₂ atmosphere under mineral oil.

Embryo production by parthenogenesis

To obtain the optimum concentration of Palbociclib and Fenretinide, the parthenogenetically produced embryos were treated with selected concentrations of Palbociclib (50, 100, 200, 400 and 800 nM) and Fenretinide (4, 8 and 16 µM). These concentrations were selected because the concentrations of instances of Palbociclib and Fenretinide in LINCS database were 400nM and 4µM, respectively (See Supplementary Table 1). We next determined a safe and non-toxic concentration of these two drugs for embryos with those concentrations as a starting point and using developmental competence of parthenogenetic (PA) embryos as an endpoint. Hence, parthenogenetic embryos were separately treated with 0, 50, 100, 200, 400 and 800 nM of Palbociclib or 0, 4, 8 and 16 µM of Fenretinide for 6 hours post activation (hpa).

The method which was used to produce parthenogenesis embryos was described previously. In brief, matured oocytes were denuded with 300 IU/ml hyaluronidase for three minutes. Then, the denuded oocytes were activated by 5 µl Ca-inophore for five minutes. The reconstructed oocytes were washed and transferred to 6-dimethylaminopurine (6-DMAP) which was supplemented with different concentrations of Palbociclib or Fenretinide. After 4 h incubation in 6-DMAP, the activated oocytes were washed and transferred to serum-free BO-IVF media (Cat. IVC1703N) containing the specified concentration of Palbociclib for 2, 5 and 8 h (6, 9 and 15 h in total or Fenretinide) or Fenretinide for 2 h (6 h in total). These time courses were selected based on a previous study which demonstrated that duration of G1 phase in parthenogenetic bovine embryos is about 9 h.

Six embryos were cultured in 20 µl serum-free BO-IVF media without Palbociclib or Fenretinide for seven days at 38.5 ºC, 5% CO2, 5% O2 in humidified air under mineral oil. Tow cell formation rate was reported.
30 h after activation. In addition, cleavage and blastocyst rates were reported at day three and seven of embryonic development.

**Embryo production by SCNT**

The optimum concentration and duration of treatment, which were derived for Palbociclib and Fenretinide in the previous section in parthenogenetically activated embryos, were used in SCNT-derived embryos. The method for production of SCNT embryos were largely based on those described before. In brief, denuded oocytes were exposed to 5 mg/ml pronase for 20–30 seconds, followed by deactivation with H-TCM + 20% FBS for 15 minutes for the removal of zona pellucida. Oocyte enucleation was performed manually using a fine pulled Pasteur pipette. Thereafter, zona-free oocytes were incubated in TCM supplemented with 4 µg/ml demecolcine for 1h at 38.5°C. Subsequently, the cytoplasmic protrusion, which contains MII spindle, was separated by a hand-held manual oocyte enucleation pipette. In order to transfer the somatic nucleus to the enucleated oocyte, a fibroblast cell was attached to the membrane of the enucleated oocyte in H-TCM medium supplemented with 10 mg/ml phytohemagglutinin. Then, the couplet of enucleated oocyte and fibroblast cell was electrofused using sinusoidal electric current (7 V/cm) for 10 sec, followed by two direct currents (1.75 kV/cm for 30 µsec and 1 sec delay). The procedure of activation and culture of embryos were as described in the previous section (Embryo production by parthenogenesis) for the production of parthenogenetic embryos. Tow cell formation rate was reported 30 h after activation. In addition, cleavage and blastocyst rates were reported at day three and seven of embryonic development.

**Embryo production by IVF**

In this study, the IVF procedure was performed as described previously and used as gold standard. Frozen semen straws were thawed (30 sec in air and then 45 sec in 37°C water bath). Afterward, the semen was centrifuged at 300 g for five minutes at room temperature (RT) to remove the cryoprotectant. The semen pellet was layered on a discontinuous Pure Sperm® gradient (40 % over 80 % solution prepared in H-TCM + 10% FBS), and motile sperms were collected after centrifugation at 100 g for 15 minutes at RT. The prepared samples were washed in HEPES buffered Tyrode’s albumin lactate pyruvate (TALP) medium. 1 × 10⁶/ml sperms were co-incubated with 10 mature COCs in 50 µl IVF-TALP (TALP supplemented with 6 mg/ml BSA and 20 µg/ml heparin) for 18 h at 38.5°C and 5 % CO₂ in the humidified atmosphere. After 18 h, the presumptive zygotes were denuded of cumulus cells and cultured in serum-free BO-IVF media for seven days at 38.5 °C, 5% CO₂, 5% O₂ in humidified air under mineral oil. Six and twelve hours post insemination (hpi) the zygotes were denuded and used for RNA extraction and reverse transcription.

**RNA extraction and reverse transcription**

Two methods were used to extract the RNA. RNA extraction of sperm pellet was performed using the standard TRIzol method and for the rest of samples, the RNeasy Micro Kit (QIAGEN, cat.No.74004) was used. This technique was carried out as previously described. Briefly, fibroblast cells, matured oocytes
(MII), sperm, 6 and 12 hours after insemination of IVF embryos (5 hours were given for sperm penetration) and 6 and 12 hours after activation of SCNT embryos were used to investigate the expression level of CDK4 and 6 and CCND1, CCND2 and CCND3 genes. Expression of pluripotency (POU5F1 and NANOG) and trophectoderm (TEAD4) markers were assessed to evaluate the quality of blastocysts derived from IVF, SCNT (SCNT-CRL) and treated SCNT embryos (SCNT-TRT).

Reverse transcription was immediately performed using a QuantiTect Reverse Transcription Kit (QIAGEN, cat.no.205311). The cDNA was frozen at -70°C in 200 µl micro-tube (each vial containing 30 µl of cDNA). PCR reactions were prepared using SYBR Green Master (Roche, cat.No.04673514001). Each reaction consisted of 1 µl cDNA, 7.5 µl SYBR Green PCR master mix, 0.9 µl of each primer pair, and 5.1 µl ultrapure water. The reactions were repeated at least three times.

Geometric means from B-ACTIN and GAPDH were used as the reference genes. Real time PCR amplification program steps were as follows: pre-denaturation at 95°C for 4 min, denaturation at 94°C for 10 sec, annealing temperature (different for each primer) for 30 sec and 72°C extension for 30 sec in 45 amplification cycles. Three technical replicates were done in each of the Real time PCR reactions, which were repeated three times in total. The ΔΔCT method was used to assess fold changes between the genes of interest (2^ΔΔCT) following RT-qPCR. The value comparative threshold cycle (CT) denotes the threshold cycle, and ΔCT was calculated as CT (of the target gene) - CT (of the geometric mean). The fold change in the gene expression was calculated using 2^−ΔΔCT, where ΔΔCT was calculated as ΔCT. The sequences of the primers for quantitative RT-PCR are presented in Supplementary Table 2.

Computational approach for identification of small molecules

201,776 small molecule treatments on 77 cancer cell lines were used and most 50 up/down regulated genes of each compound signature constituted a gene signature describing each compound treatment. Drug repositioning approach described earlier (See introduction section) was used to rank order compound treatments in LINCS database and target genes in the SCNT Vs IVF signature. The approach used Gene Set Enrichment Analysis to query full profile of differentially expressed genes in the SCNT with gene signatures in LINCS database and identify compounds that target the most differentially expressed genes in the SCNT profile in a reverse way. The approach identifies genes that are differentially expressed in SCNT compared to IVF which are hypothetically associated to the lower success rate of SCNT embryos compared to IVF embryos and then identifies small molecules to target those genes and reverse the SCNT gene expression profile to IVF profile. Such compounds are expected to increase success rate of SCNT. For shortlisting compounds, we first did this process once for all SCNT embryos Vs all three IVF embryos and once for all SCNT vs one IVF embryo. (The full rank ordered compound list is provided in Supplementary Table 1) The ranks of Palbociclib and Fenretinide were three and 18 respectively among 201,776 compound signatures in LINCS database when considering eight replicates of two IVF embryos and 150 and five respectively when taking into account four replicates of one of IVF
embryos. Moreover, the known targets of the compounds were considered for selection as discussed in the results.

**Statistical analysis**

Each experiment was performed at least in three replicates. The data are presented as the mean ± standard error of mean (S. E. M). Statistical analysis was performed by SPSS (version 25.0) using one-way analysis of variance (ANOVA). ANOVA was followed by Tukey post-hoc test to explore differences between multiple groups. P-values < 0.05 were considered statistically significant. All diagrams were designed by PRISM version 8.0 software.

**Declarations**

**Authors’ contributions**

PB & SH performed the experiments. FJ wrote the manuscript and supervised the performance of the experiments. MH, MR and RM performed the experiments and analyzed the data. SMH contributed in the experimental part and providing the data. YK developed the computational drug repositioning approach that led to shortlisting compounds and contributed to writing the manuscript. AB supervised the computational part of the work and MHN conducted the research and wrote the manuscript.

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**Conflict of interest**

Authors declare no conflict of interest in this study.

**Ethics approval and consent to participate.**

The Institutional Review Board and Institutional Ethical Committee of the Royan Institute approved all animal care protocols and the proposal. In addition, all methods were performed in accordance with the Institutional Review Board and Institutional Ethical Committee of the Royan Institute guidelines and regulations.

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**Figures**

Figure 1
Workflow of computational approach and experimental validation. (A) The gene expression profile of SCNT and IVF bovine embryos were compared, genes were rank ordered based on differential gene expression profile. LINCS database which comprises 201,777 compound treatments on cell lines after different time periods and concentrations was used. Gene Set Enrichment Analysis were carried out to rank order all the compounds in LINCS database to target the most differentially expressed genes in profile of SCNT Vs IVF effectively. Palbociclib and Fenretinide were shortlisted as they were highly ranked based on the bioinformatics scoring system. (B) Protein targets of the shortlisted compounds were looked up from ChEMBL database as well as literature and literature evidence was checked for selecting ideal drug candidates. Palbociclib and Fenretinide were shortlisted as they were highly ranked based on the bioinformatics scoring system and there was supporting literature for CDK4,6 targets of Palbociclib and CCND1 target of Fenretinide. (C) In the experimental validation phase, optimal concentrations and treatment duration were identified. Next the effect of adding Palbociclib and Fenretinide to SCNT protocol was compared to standard SCNT protocol based on three determining factors of Two cell formation rate, cleavage rate and blastocyst formation rate.

Figure 2

Real-time reverse-transcriptase PCR gene expression analysis of (A) CDK4, (B) CDK6 and the CCND family including (C) CCND1, (D) CCND2 and (E) CCND3 in MII oocyte, sperm, bovine ear fibroblast (BEF), IVF embryos at 6 and 12 hpi, and SCNT embryos at 6 and 12 hpa. Fold-change values were calculated from three biological replicates and are shown relative to MII oocyte following normalization to ACTB. Values are means ± standard errors. Different letters indicate significant differences among the groups (P<0.05).
Figure 3

Effect of administration of different concentrations of Palbociclib for 6 hpa on in vitro development of parthenogenetic bovine embryos. (A) Two cell formation rate, (B) cleavage rate and (C) blastocyst formation rate of bovine parthenogenetic embryos cultured in the presence of different concentrations of Palbociclib (in nM) at 6 hpa. Data are expressed as the mean ± SEM. The experiments were replicated at least three times. Different letters indicate significant differences among the groups (P<0.05).

Figure 4

Effect of administration of different concentrations of Fenretinide at 6 hpa on in vitro development of parthenogenetic bovine embryos. (A) Two cell formation rate, (B) cleavage rate and (C) blastocyst formation rate of bovine parthenogenetic embryos cultured in the presence of different concentrations of Fenretinide (µM) during 6 hour post activation (6 hpa). Data are expressed as the mean ± SEM. The experiments were replicated at least three times. Different letters indicate significant differences among the groups (P<0.05).

Figure 5

Effect of treatment time (hours post activation) with 100 nM Palbociclib on in vitro development of parthenogenetic bovine embryos. (A) Two cell formation rate, (B) cleavage rate and (C) blastocyst
formation rate of bovine parthenogenetic embryos cultured in the presence of 100 nM Palbociclib at 6, 9 and 12 hpa. Data are expressed as the mean ± SEM. The experiments were replicated at least three times. Different letters indicate significant differences among the groups (P<0.05).

Figure 6

Optimal concentration and time of treatment with Palbociclib and Fenretinide. Optimal concentration and time of treatment with Palbociclib (A-C) and Fenretinide (D-F) improved developmental competence of bovine parthenogenetic embryos to levels similar to those of bovine IVF embryos. (A) Two cell formation rate, (B) cleavage rate and (C) blastocyst formation rate of bovine parthenogenetic embryos cultured in the presence of 100 nM Palbociclib for 6 hpa compared to bovine IVF and parthenogenetic embryos. (D) Two cell formation rate, (E) cleavage rate and (F) blastocyst formation rate of bovine parthenogenetic embryos cultured in the presence of 4 µM Fenretinide for 6 hpa compared to bovine IVF and parthenogenetic embryos. Data are expressed as the mean ± SEM. The experiments were replicated at least three times. Different letters indicate significant differences among the groups (P<0.05).

A

B

C

D

E

F

Figure 7

Effect of treatment time (hours post activation) with 100 nM Palbociclib on in vitro development of bovine SCNT derived embryos. (A) Two cell formation rate, (B) cleavage rate and (C) blastocyst formation rate of bovine SCNT derived embryos cultured in the presence of 100 nM Palbociclib for 6, 9, 12 and 15
hpa. Data are expressed as the mean ± SEM. The experiments were replicated at least three times. Different letters indicate significant differences among the groups (P<0.05).

Figure 8

Effect of administration of different concentrations of Fenretinide for 6 hpa on in vitro development of bovine SCNT derived embryos. (A) Two cell formation rate, (B) cleavage rate and (C) blastocyst formation rate of bovine SCNT derived embryos cultured in the presence of different concentrations of Fenretinide (µM) during 6 hpa. Data are expressed as the mean ± SEM. The experiments were replicated at least three times. Different letters indicate significant differences among the groups (P<0.05).

Figure 9

Optimal concentration and time of treatment of Palbociclib (A-C) and Fenretinide (D-F) improved developmental competence of bovine SCNT derived embryos to levels similar of bovine IVF embryos. (A) Two cell formation rate, (B) cleavage rate and (C) blastocyst formation rate of bovine parthenogenetic embryos cultured in the presence of 100 nM Palbociclib for 12 hpa compared to bovine IVF and SCNT derived embryos. (D) Two cell formation rate, (E) cleavage rate and (F) blastocyst formation rate of bovine SCNT derived embryos cultured in the presence of 4 µM Fenretinide for 6 hpa compared to bovine IVF and SCNT derived embryos. Data are expressed as the mean ± SEM. The experiments were replicated at least three times. Different letters indicate significant differences among the groups (P<0.05).
Figure 10

Real-time reverse-transcriptase PCR gene expression analysis of pluripotency-related (OCT4 and NANOG) and trophectoderm-related (TEAD4) genes in blastocysts produced from bovine SCNT derived embryos treated with 100 nM Palbociclib for 12 hpa or 16 µM Fenretinide for 6 hpa. Fold-change values were calculated from three biological replicates and are shown relative to IVF derived blastocysts following normalization to ACTB. Values are means ± standard errors. Different letters indicate significant differences among the groups (P<0.05).

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