Optimization of culture conditions for artificial blastocysts

Maria Inês Baptista Leite [1,2]

[1]Instituto Superior Técnico, Universidade de Lisboa, Lisboa, Portugal
[2]MIRA Institute, University of Twente, Enschede, Netherlands

The main goal of this thesis was to optimize the method to develop artificial blastocysts (also called blastoids). In parallel different studies were developed with the aim of ensuring that the compounds tested are not harmful to the blastoids and reveal some details of biological mechanisms that take place during blastocyst formation.
The stem cells were seeded on a platform and their seeding cell densities were optimized. Dose responses of blastoids to several compounds of the blastoids medium were performed to address the optimum concentration of each one.
With the different studies was possible to conclude that one specific pathway was important during the preimplantation stage of the mouse blastocyst and a screening revealed 15 potential hits that promote cell differentiation.

Key words: mouse blastocyst, stem cells

Introduction

How a single fertilized egg gives rise to a complete embryo is a complex and intriguing process for biologists to resolve in detail, already since Aristotles' times. For *in vitro* study of preimplantation development, the mouse embryos are flushed from the uterus or oviduct of a pregnant mouse. Presently, pre-implantation development can be recreated in vitro in a chemically-defined culture medium, using stem cells under a controlled environment without significantly impairing the developmental potential of embryos [1]. However, animals are normally sacrificed in the process of harvesting embryos and natural embryos can only be obtained in relatively small numbers compared to large-scale generation of artificial blastocysts. Hence, one way to overcome previous limitations is to develop artificial blastocysts. In this thesis, a method is described that allows the formation of a cellular model that resembles the development of a natural blastocyst, including its stereotypical morphogenetic processes.

To accomplish that is necessary to understand some early events that take place between egg fertilization and the pre-implantation blastocyst, with special interest in the three distinct tissue lineages: the epiblast (EPI), the primitive endoderm (PE), and the trophectoderm (TE) (see [2] for detailed review).

The TE is a single epithelial layer that surrounds a fluid filled cavity and the inner cell mass (ICM). The PE and EPI are two lineages derived from the ICM. The PE appears as a layer of cells on the surface of the ICM lining the cavity, with deeper cells comprising the pluripotent EPI [2-4].

Several transcription factors are required for the proper segregation of each lineage. The principal transcription factors of TE are Cdx2, Tead4, Eomes and Sox2 [1, 2, 5]. One ICM-specific transcription factor is Oct4 [2]. Gata6 and Pdgfrα

are PE markers and Nanog is one EPI marker [3, 6, 7].

Several pathways such as Hippo, FGF/MAP and Wnt/β-catenin signaling pathways have crucial importance in cell differentiation during blastocyst development [4, 8-12]. An interesting feature of the first three lineages of the mouse is that stem cells can be derived from each one of them. Embryonic stem (ES) cells are likely derived from and represent the EPI, the trophoblast stem (TS) cells from the TE and extraembryonic endoderm (XEN) cells from the PE [2, 13, 14].

The main aim of this master thesis was to optimize the method to develop artificial blastocysts and, in paralel, to perform some studies to better understand some of the biological mechanisms that govern the blastocyst development. For that purpose, it is envisaged the *in vitro* recreation of blastocyst in a chemically-defined culture medium, using stem cells without significantly impairing the developmental of embryos. The blastoids obtained in the end mimick the *in vivo* blastocyst.

Results and Discussion

Method's Optimization

- Study of the influence of cell number in blastoids development

To study the effect of cell number on blastoids developmental potential, the cell number was modulated by seeding different concentrations of cells per well. One hour after seeding pictures of the platform were acquired and cells were counted (using the ImageJ software) showing a good correlation with the seeded amount (data not shown).

In addition, the effect of a specific compound (A) in the blastoids development was also examined in this experiment. A previous work had shown that this compound promotes cavitation in blastoids and its concentration was previously optimized. In order to use it in future screenings it was important to test its effect on blastoids development, considering the ICM and the cavitated blastoids sizes.

Consistent with the earlier observations, in this experiment the yield of cavitation was higher when the blastoids were cultured in the presence of the tested compound.

As expected, the yield of cavitation (formation of the blastocyst's cavity), when cultured with A, increased continuously with cell number, reached a plateau and decreased from this point on. Probably this is due in part to the fact that at a high cell density the blastoid forms a big ICM which impaired the blastocoel formation.

The results show that ICM size increased with cell number. While in the cavitated blastoids the ICM increased, with and without A. Finally, the size of cavitated blastoids increased with cell number.

From these observations is possible to conclude that there is an optimum cell density for culturing blastoids, when cultured with A.

- Study of the influence of different medium compounds in blastoids development

The addition of compounds that will stimulate a good blastoid development is mandatory. In this context, it was decided to study the influence of adding two different compounds to the blastoid culture medium, B and C, that are crucial during the preimplantation stage of the mouse blastocyst. Individual dose responses were performed to determine the optimal concentration of each mentioned compound.

The effect of combining A with B was assessed at a range of concentrations. Gradual and progressive increase of yield of cavitation with the increase of B concentration was observed with and without A.

Once again, higher yields were obtained when the blastoids were cultured with A. The difference between the conditions with or without A is easily noted in higher concentrations of B. Furthermore, the difference between concentrations of B is more significant in the samples with A. In simple terms, together they promoted cavitation more efficiently than either did separately. Consequently it might be true that A and B act together to promote blastocoel formation.

The B both ICM's and cavitated blastoids' sizes increased gradually. Interestingly, with higher concentrations of B they both decreased. The sizes reduction might indicate cell toxicity at a combination of A with high concentrations of B.

To examine the influence of C in the blastoids development the blastoids were cultured with increasing concentrations of C.

Surprisingly, the yield of cavitation did not increase in a dose dependent manner. On the other hand a gradual increase in ICM size of both cavitated and non cavitated blastoids was observed with increasing doses of C, which is essential for a good blastoid formation.

At this point a more complex experiment was performed with a combination of different concentrations of A, B and C. The data was not as accurate and consistent with the previous studies as it was hoped.

Among all the tested compounds, the yield of cavitation and the size of cavitated blastoids are more influenced by B. In general, higher concentrations of A or B corresponded to the highest yields of concentration and the biggest cavitated blastoids, with few exceptions. Besides that, statistical analysis revealed that the results were not significantly different. Consequently, to firmly establish the dose response of blastoids to these compounds it is recommended to repeat this experiment with a control condition and more samples per condition.

- Optimization of staining of blastoids

As it was mentioned before, the three germ layers can be easily identified by its transcriptional factors. Therefore, to be able to study their expression along the blastoids development, the Cdx2 (expressed in TE [15]) and Nanog (expressed in epiblast [15]) staining was optimized. The available protocols were optimized for the staining of 2D monolayers being necessary its adaptation to 3D cultures.

Nanog is a EPI-specific factor that maintains the pluripotency by inhibiting PE formation [13]. It starts being expressed in all cells at the 8-cell stage, and at around 32-cell stage, it is co-expressed in all cells along with the Gata6. Nanog persists within the ICM, and becomes exclusive in EPI cells until the late blastocyst stage when it starts to decline and later becomes re-established in the germline [3, 10].

In the case of the Nanog, stainings in the 2D and 3D cultures were performed separately, starting by investigating the presence of this protein in the 2D cultures. Two different controls were used to verify the accuracy of the antibody staining: a non-specific primary antibody instead of the specific one and a secondary antibody without adding a primary. As expected, the Nanog expression was undetectable in both cases (data not shown).

Likewise, the difference between the positive and negative control is clear. In the positive there was an intense Nanog expression while in the negative control the levels of expression were significantly low (data not shown).

It is known that the non-ionic detergents can reduce non-specific hydrophobic interactions. These give large enough pores for antibodies to enter the cells without dissolving plasma membrane [62]. Therefore, 0,05% Tween 20 in PBS or ice cold methanol were used to improve permeabilization and, consequently, optimize the staining. However, the results were not improved.

In what concerns to the blastoids staining (3D cultures), the results were not as good. The DAPI was very diffused probably because of the short time of incubation (data not shown). This step

should thus be repeated with a longer time of incubation.

Studies about blastocyst development

As previously referred, several studies were performed to better understand some biological mechanisms during blastocyst development. In order to avoid a high complex and through thesis, it will only be explained and discussed the three of them.

- Z signaling in A treatment

The aim of the next experiment was to address the role of Z signaling in the blastocyst development.

In this experiment the Z signaling was promoted through the addition of D. In the same way, Z signaling was inhibited with E. At last, the Z secretion was inhibited with F. Once again the blastoids were cultured with and without A and kept for 4 days.

Among the tested conditions two were associated with high levels of yield of cavitation – addition of D and addition of D+A. Actually, the A effect was only detected when this was combined with A. There were no statistical differences between A and control through all conditions.

The results tend to show a synergy between D and A in the promotion of an efficient cavitation. Besides, as D promotes Z signaling, this supports the importance of this pathway in blastocoel formation, being a crucial step.

On the other hand, a clearly yield reduction was detected when the blastoids were cultured with E. Consistent with the previous hypothesis, these results suggest that Z signaling is essential during cavitation. It is worth to notice that the fact that yield in control being similar to the yield with D may indicate that the last one can only promote cavitation when the Z signaling is activated. It was also noticed that the size of cavitated blastoids was significantly small, which may indicate a deficient blastoid development. Finally, the yield of cavitation with F was still lower in comparison with the absence of both E and F but not as low as with E.

To sum up, these results showed that Z pathway is also important in the early stages of the blastocyst and it Is crucial during cavitation.

- Interaction of A with different growth factors

As the concentration of A increases the yield of cavitation, it was intended to use this compound in the future to improve screening read-out. Therefore it was tested the impact of the combination of A with growth factors on blastoids development. The blastoids were cultured with a low, medium or high concentration of each growth factor.

In general, the growth factors promoted a yield of cavitation similar to the control condition. These may indicate that these tested growth factors do not influence the blastoids cavitation. Moreover, when comparing the results obtained between low, medium and high concentrations no statistically significant differences were observed. This can have two interpretations. On one hand it could mean that the concentration does not influence the cavitation of the blastoids. On the other hand it could also mean that the tested concentrations were not sufficiently different from each other to obtain significantly different results. As it is believed that in this case the concentrations are already significantly different from each other, the second hypothesis probably does not apply. But, to be firmly sure it is recommended to repeat the experiment with even more disparate concentrations.

Surprisingly, the ICM size of cavitated blastoids was identical upon addition of each one of the growth factors. Once again, the results obtained with different concentrations did not diverge from each other. At last, the size of cavitated blastoids was maintained among all other conditions.

To conclude, A can be used with these growth factors because in general it did not impair the blastoids development.

Conclusions and future perspectives

How a single fertilized egg gives rise to a complete embryo is still an intriguing process but, more than thirty years since the first models of lineage restriction were proposed, we have begun to understand the molecular foundations of lineage restriction and fate commitment in the early mouse embryo. This advance was possible due to technological improvements and pioneering studies with stem cell lines from the first three lineages of the mouse, among others. Actually, lessons learned through these studies extend beyond blastocyst formation, and have contributed to advancement in our knowledge in different ranges, such as body patterning and stem cell therapeutics.

The protocol to develop artificial blastocysts here described is included in these studies. The stem cells are cultured in a chemically defined culture medium under a controlled environment that promotes the blastocyst formation with a potential to reduce the amount of blastocysts being harvested for future studies.

The main goal of this thesis was to optimize the culture conditions to produce artificial blastocysts and good results were obtained. In parallel, different assays were developed with the aim of ensure that the compounds tested are not harmful to the artificial blastocysts and reveal some details of biological mechanisms that take place during blastocyst formation.

The Z signaling study suggested that this pathway has influence in the early blastoids formation because when it was inhibited the cavitation yield was significantly low. It was also observed that A in combination with the tested growth factors do not impair the blastoids cavitation.

Despite the good results achieved by this method there are still several improvements that can be implemented, beside the ones already mentioned. One drawback of this protocol is the variability introduced by serum and feeder cells. For better reproducibility of results and less risk of contamination is crucial to develop a standardized media.

The seeding process of the cells and the pipetting steps can also be optimized. The use of robotic systems in the protocol and in other high-throughput screens would lead to more accurate and reliable results by reducing the variability inside the wells, between the wells and between different plates.

Finally, to validate the method, the blastoids must be compared with naive blastocysts using different approaches. Cytogenetic analysis, immunoprecipitation of methylated DNA, cell cycle distribution by propidium iodide staining and flow cytometry, quantitative RT-PCR (qRT-PCR) to analyze the expression of specific transcription factors, immunofluorescent staining and immunoblotting against the same transcription factors are some of them.

Materials and Methods

Mouse fibroblast feeder layer (mEF) culture

Both embryonic and trophoblast stem cells were seeded on a mouse embryonic fibroblast (mEF) feeder layer. Before seeding the mEF, the well plates (Nunclon Delta Surface by Thermo Scientific Nunc) were coated with a gelatin solution (0,1%) by incubating for at least 15 minutes at 37 ºCelsius. After removing the remaining gelatin, the fibroblasts were seeded in mEF medium (Dulbeco's Modified Eagle Medium (DMEM, Gibco by Life Technologies) supplemented with 1 % L-glutamine (200mM in stock); 0,5 % Penicillin Streptomycin (Pen/Strep, Gibco by Life Technologies); 10 % fetal bovine serum (FBS, Hubrecht) and 1 % Hepes Buffer Solution (Gilbco by Life Technologies)) and incubated at 37 ºC and 5% CO_2. All the used

fibroblasts were till passage 4 (maximum 5) and seeded at an approximate confluent density of $1x10^4$ cells/cm^2.

Cell culture

The cells were previously isolated from preimplantation blastocysts from pregnant mice, separated from the others and seeded on well plates on the top of a mEF feeder layer. These culture plates were incubated at 37 ºC and 5% CO_2.The cells were usually seeded at a density of 10 000 cells/cm^2.

Immunostaining

Blastoids were fixed in 4% paraformaldehyde in PBS for 30 minutes or overnight (depending on the cells sensibility) at 4ºC. The used primary antibodies were rabbit Cdx2 (EPR-2764y, rabbit anti-human/mouse) and rabbit Nanog (abcam ab84447 Lot GR53934). Secondary antibodies were goat anti rabbit, labelled with Alexa fluorophores (Invitrogen, Paisley, UK) diluted 1:500 in PBS+0,1% TritonX-100. Sometimes for control it was also used a non-specific primary antibody, Mouse IgG isotype.

Image acquisition and processing

The plates with the blastoids were imaged with BD pathway 435, Biosciences. The images obtained were processed and analyzed using the Cell Profiler Software. With the obtained information was possible to distinguish the cavitated blastoids from the others because the periphery radial intensity profile (Radial Distribution MeanFrac) of the first ones is different than the other ones. The used threshold was 0,9. The ICM and the blastoid size correspond to the sum of the pixel intensities within the ICM cells and the area of the blastoid, respectively. The conversion in μm^2 was 6,0 μm^2/pixel.

Statistical analysis

Statistical analysis was performed using the GraphPad Prism software. *P-value*, which was determined by ANOVA with Bonferroni post hoc tests, is shown wherever the difference between compared groups was significant (***p<0,001, **p<0,01, and *p<0,05)). The values plotted in bar graphs represent the mean results obtained per condition ± SD.

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