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Analysis of Permissive and Repressive Chromatin Markers in *In Vitro* Fertilized Bovine Embryos Just After Embryonic Genome Activation

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1. Introduction

Preimplantation development in mammalian species is a challenging stage of embryogenesis, which makes *in vitro* fertilization and embryo culture sophisticated biotechniques. One of the main tasks is to provide cultured embryos an adequate environment that allows embryonic-maternal transition, and consequent achievement of an independent gene expression program.

In this respect, mammalian models have been used to further elucidate the mechanisms of embryonic genome activation. Within the main players in this context we can include chromatin compactor/relaxing agents, and among them histone modifying enzymes and their consequent posttranslational modifications.

In this chapter, we aim to discuss some histone modification marks observed during embryonic genome activation, and how their monitoring can provide useful information about early embryo development.

1.1 *In Vitro* Production (IVP) of mammalian embryos: How other species can contribute to human IVP improvement

In vitro early embryo development was first established in human species in 1978 and represented an important achievement for Reproductive Medicine. However, despite several decades of research and clinical experience, *in vitro* development of blastocysts and pregnancy following embryo transfer has not been yet fully dominated. Although improvements of medium, atmosphere, hormone stimulation, oocyte recovery and embryo transfer protocols were achieved during the last 30 years, rates of *in vitro* developed blastocysts are still around 40% in most systems (Bannister & Kouzarides, 2011; Bolton et al., 1991; Hardy et al., 1989; Zhang et al., 2010). Furthermore, the identification of developmentally competent embryos is still a difficult task.

Among several critical steps of IVP, one that has the most stochastic consequence is the activation of embryonic genome. At this time point, a phenomenon observed in many

species is the developmental block of embryos due to the inability to activate zygotic genes and continue cleavage. As the usage of human embryos for research involves major ethical issues, most researches are conducted in other mammalian models. In this context, despite several advantages of mouse physiology as a model for human development, bovine early embryo development presents more similarities to human at some aspects.

Mouse embryos develop to the blastocyst stage in 3.5 days and the rate of blastocyst production in most labs is about 90%. Furthermore, developmental block during embryonic genome activation (EGA) is not a common phenomenon for most mouse embryos, occurring normally to only some inbred mice strains (Shire & Whitten, 1980). On the contrary, one of the main causes of embryonic arrest for bovine embryos appears to be the inability to overcome chromatin repression before maternal RNAs stock has been depleted, which occurs approximately by the 8cell stage (Meirelles et al., 2004). Failure to activate zygotic genome on time is also an issue for human embryos, and most arresting embryos stop development at this time point (Artley et al., 1992). The timing of EGA is also closer between bovine (8-16cell) (Badr et al., 2007) and human (4-8 cell) (Braude et al., 1988) embryos, which corresponds to day 3 of embryonic development in both bovine (Oliveira et al., 2010) and human (Sepulveda et al., 2011) embryos.

The reason why embryonic block does not affect most of mouse embryos might be related to the fact that EGA initiates in this species just a few hours after fertilization (Hamatani et al., 2004), while for bovine and human embryos it takes up to 3 days. In fact, the lower rates of blastocysts observed for bovine and human comparing to mouse embryos might be as well a result of extended IVC, since culture environment can affect epigenetic modifications pattern (Enright et al., 2003). This aspect may contribute to the different gene transcription profiles observed between bovine embryos produced in vitro and in vivo (Wrenzycki et al., 2004). In IVF embryos, some genes are expressed at a low level, such as transcripts associated with compaction/cavitation (Wrenzycki et al., 1996), stress adaptation (Rizos et al., 2002), embryonic metabolism (Bertolini et al., 2002), and X chromosome inactivation (Wrenzycki et al., 2002).

Therefore, bovine embryogenesis seems to be an appropriate model for studying histone modifications in human pre implantation development, including in vitro embryonic genome activation. We believe that epigenetic marks can reflect developmental competence, and also that the manipulation of those epigenetic states might be useful to elucidate their roles during mammalian early embryo development.

1.2 Maternal-zygotic transition: Achievement of an independent gene expression program

1.2.1 Degradation of maternal mRNAs transcripts

The oocyte is the largest mammalian cell, and has an extensive amount of cytoplasm comprising an abundant reserve of RNAs. After oocyte maturation, nuclei is arrested in metaphase, when transcription stops and translation is reduced. The huge amount of RNA accumulated during oocyte growth is then kept and utilized during the first embryonic divisions as a maternal stock (Lonergan et al., 2003). Those maternal RNAs are essential for embryonic development until the event of EGA occurs, as they provide most translational elements used for protein synthesis during initial divisions. Therefore, adequate availability

of maternal transcripts should be present until that moment, which also explain why oocyte quality is so important for species which EGA occurs 2 or more cell cycles after fertilization, as in bovine embryos (Meirelles et al., 2004).

The first few days of oocyte maturation and embryo development in human are characterized by a significant decrease in transcript levels, including pro apoptotic genes, suggesting that decay of those maternal RNAs are essential for embryo development (Dobson et al., 2004). Such a staged down regulation has not been reported for any organism during early development, and it is not clear whether it is required for genome activation to occur, or whether it is an independent event in early embryo development. Vassena et al. (2011) reported this maternal mRNA turnover to occur in two waves, tiled during early development. The first wave, termed 'early maternal', probably takes place between the Metaphase II (MII) and 2-cell stage, and during this time, maternal mRNAs are loaded onto polysomes, translocated and degraded. A second wave of maternal mRNA degradation, termed 'late maternal', included transcripts that decrease gradually over time.

1.2.2 Activation of embryonic genome

Embryonic genome activation timing differs between mammalian species. It occurs at the 2-cell stage in mouse (Bensaude et al., 1983; Moore, 1975), at the 4- to 8-cell stage in horse (Brinsko et al., 1995) and human (Braude et al., 1988), and at the 8- to 16-cell stage in cow (De Sousa et al., 1998), sheep (Crosby et al., 1988), rabbit (Brunet-Simon et al., 2001) and monkey (Schramm & Bavister, 1999). Maternal-zygotic transition is essential for the activation of a large number of genes, thus achieving a gene expression pattern compatible with embryonic development and differentiation (Schultz, 2002).

Most studies involving EGA are carried out in mouse embryos. In this species, the first signs of zygotic transcriptional activity occur few hours after fertilization, before first cleavage, mainly in the male pronucleus (Aoki et al., 1997; Nothias et al., 1996). This wave is named Minor Zygotic Activation (Minor ZGA), and it is responsible for a small set of peptides that are transiently increased by the 2cell stage. Then, during transition from 2 to 4 cell stage, a dramatic increase in transcriptional activity is described, entitled Major Zygotic Activation (Major ZGA), and embryonic gene expression pattern is established. Embryos that fail to activate their own genome are arrested (Rambhatla & Latham, 1995). At this moment, genes that encode basic cellular machinery are activated (Davis & Schultz, 1997). Nonetheless, embryonic genome must suffer a third wave of transcription activation at the 4cell stage. This wave represents the second major transition of gene expression profile, and is named mid pre-implantational gene activation (Hamatani et al., 2006). At this point, genes associated with critical function in early embryos, such as key regulators for epiblast (EPI) and trophoctoderm (TE) specification, are activated.

Human EGA was first described during the transition from 4- to 8- cell stages (Tesarík et al., 1988). However, Vassena et al. (2011) recently identified transcriptional activity in the human embryo at the 2-cell stage. The authors also identified three waves of transcriptional activation in the human embryo: at 2-cell stage, 4-cell stage, and between the 6- and the 8- to 10-cell stages. The major wave, which the authors interpreted as similar to Major ZGA in mouse, occurred between the 8-10 cell stages. In bovine, Minor ZGA is described from 1- to

4-cell stage, but major transition, which is responsible for embryonic developmental competence, occurs during the transition from the 8-16cell stage (Badr et al., 2007).

In conclusion, we can observe that embryonic genome activity is dramatically transformed in just a few days, or even hours. Those transcriptional profiles reflect chromatin architecture and compaction, which is built in part by chromatin modifications. In the next topic, we will address how histone modifications can play a role in embryonic genome activation.

1.3 Epigenetic mechanisms during early embryo development

Epigenetic refers to the control of gene function and expression without changing genomic sequence. This regulation allows the existence of tissue-specific gene expression patterns within one organism (Li, 2002). Epigenetic modifications can control gene expression by a range of processes which include DNA methylation, post-translational histone modifications and non-coding RNAs. Covalent modifications of histones occurs on amino residues, primarily on the amino-terminal tail (Bannister & Kouzarides, 2011), and have fundamental functions on chromatin condensation, DNA replication, DNA repair, and gene regulation. Therefore, based on biochemical interaction, histone modifications can induce chromatin decondensation, allowing transcription factors to bind, or maintain nucleosomes tightly wound.

Examples of some modifications that are commonly associated with active sites of transcription are acetylation of histone H3 and H4, as well as di- and trimethylation of lysine 4 on histone H3 (Kouzarides, 2007). On the other hand, constitutively silenced DNA regions such as telomere, centromeres, and heterochromatin are hypoacetylated and highly methylated on particular amino acid residues (lysine 9 and lysine 27 of histone H3).

Among histone modifications, acetylation and methylation have been so far the most described. Unlike histone acetylation, that is always associated with transcriptional activation, histone methylation can induce different outcomes depending on which residue is modified (Martin & Zhang, 2005). Both modifications are conducted by enzymes. For acetylation, histone acetyltransferase (HAT; adds acetyl groups) and histone deacetylase (HDAC; removes acetyls groups) are involved on lysine regulation (Wolffe & Guschin, 2000). Several of these enzymes (HDACs 1, 2, 3, 7, and HAT1) have been detected in bovine embryos (McGraw et al., 2003), and as more research is conducted, it becomes apparent that crosstalk between enzymes is a common feature.

Therefore, a wide array of epigenetic modifications participates in early embryo development, including DNA methylation and histone modifications, and they are crucial for genomic imprinting and X-chromosome inactivation in female embryos (Dean et al., 2003). Time course studies demonstrate that those modifications are switched between embryonic stages and are well orchestrated to warrant normal development. For instance, levels of histone methylation are higher and histone acetylation levels are lower in male gametes compared to female gametes (Adenot et al., 1997; Kim et al., 2003; Sarmiento et al., 2004; Spinaci et al., 2004), resulting in minimal gene expression at this stage. Then, demethylation occurs soon after fertilization (Sanz et al., 2010). Histone methylation levels are reduced in 2 to 4 cell embryos, and start to increase at the 8- to 16-cell stage, concurrently with zygotic genome activation (Santos et al., 2003). Histone acetylation levels peak at the

time of zygotic genome activation, corresponding to a dramatic increase in gene expression levels, and then diminishes during the morula stage (Maalouf et al., 2008).

At the blastocyst stage, DNA and histone methylation are elevated in the ICM, whereas DNA and histones are hypomethylated in the trophectoderm, which clearly reflects a difference between the two cell lineages (Reik et al., 2003). However, studies suggest that this epigenetic status segregation can occur even before. It has been demonstrated that chromatin modifications are differentially distributed between blastomeres as early as at 4-cell stage. Torres-Padilla et al. (2007) described CARM1 enzyme, a histone H3 arginine methyltransferase, as being involved in pluripotency achievement. Furthermore, methylation of arginine histone H3 residues is increased in 4-cell blastomeres that will contribute to ICM formation. Thus, histone modifications are involved in lineage specification in early embryos, and seems to occur earlier than most events related to embryo differentiation.

In addition, studies have demonstrated that histone modifications are disrupted by embryo manipulation and in vitro culture. Santos et al. (2003) demonstrated that bovine embryos produced by somatic cell nuclear transfer (SCNT) presented hypermethylation of histone H3-K9. A study also reported that acetylation of lysine 5 on histone H4 (H4-K5ac) appears to change dramatically during early embryo development of IVF produced embryos, but remains consistently elevated in SCNT produced bovine embryos (Kang et al., 2002).

Compared to in vitro fertilized embryos, SCNT bovine embryos have elevated heterochromatic histone methylation (H3K9me2) and H3K9-acetylation in the trophectoderm layer (Santos et al., 2003). These and other modifications could explain the altered expression of vital developmental genes later in development. Bovine cultured cells also present a disrupted pattern of epigenetic modifications (Enright et al., 2003). In this respect, we described that it is possible to artificially increase histone acetylation during in vitro culture. Trichostatin A (TSA), a histone deacetylase inhibitor, can be added to culture medium at low concentrations without causing detrimental effects to embryonic development (Oliveira et al., 2011).

Embryos cultured in TSA supplemented medium present higher acetylation levels, and develop normally. Furthermore, female and male embryos respond to TSA treatment in a different way (Oliveira et al., 2010), and this might be related to X chromosome inactivation event (unpublished results).

In conclusion, epigenetic modifications are essential during early embryo development, and they have a predictable and well orchestrated pattern of expression which correlates with developmental potential, and seems to influence lineage specification. It has been demonstrated that manipulation of embryos leads to disruption in histone modifications pattern, as we summarized in this topic. Therefore, monitoring of histone post-translational modifications during preimplantation development is an important tool for assessing culture environment. In addition, a better understanding of how those modifications can be manipulated might be even more interesting aiming the achievement of improved phenotype blastocysts and ES cells derivation, in terms of pluripotent capacity of inner cell mass. In this respect, we are studying concomitantly two well described histone modifications, H3k9ac (permissive chromatin) and H3k27me3 (repressive chromatin), in bovine embryos. At this stage, we aimed to describe how they correlate and are distributed during EGA, comparing blastomeres within one embryo.

2. In Vitro Production (IVP) of bovine embryos

Development of bovine embryos in vitro for research purposes is mainly carried out using oocytes obtained from slaughterhouse ovaries. Blastocyst production includes oocyte maturation, in vitro fertilization and embryo culture, and can be accomplished in 7-8 days, with rates of approximately 40%. Here we briefly describe all steps of this process.

2.1 Supplements

Reagents and culture media were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise stated.

2.2 Preparation and selection of oocytes

Bovine ovaries were collected at a local slaughterhouse and processed within 2 h after slaughter. The ovaries were washed in saline (37°C) and follicles measuring 3 to 8 mm in diameter were aspirated with an 18-gauge needle coupled to a 20-mL syringe. Follicle liquid was placed in a 50 mL conic tube for 20 min sedimentation at 37°C, and then 10 mL of the sediment was collected and transferred to 100 mm Petri dishes. Cumulus-oocyte complexes (COCs) presenting at least three layers of cumulus cells and homogenous cytoplasm were selected under a stereomicroscope. The COCs were washed in HEPES-buffered TCM-199 (Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal calf serum (FCS; Cripion Biotecnologia, Andradina, SP, Brazil), 16 µg/mL sodium pyruvate and 83.4 µg/mL amikacin (Instituto Biochimico, Rio de Janeiro, RJ, Brazil).

2.3 In vitro maturation (IVM)

Groups of 15 COCs were transferred to 100-µL drops of medium containing sodium bicarbonate-buffered TCM-199 supplemented with 10% FCS, 1.0 µg/mL FSH (Folltropin™, Bioniche Animal Health, Belleville, ON, Canada), 50 µg/mL hCG (Profasi™, Serono, Sao Paulo, SP, Brazil), 1.0 µg/mL estradiol, 16 µg/mL sodium pyruvate and 83.4 µg/mL amikacin, covered with sterile mineral oil (Dow Corning Co., Midland, MI, USA) and incubated for 24 h at 38.5°C in an atmosphere of 5% CO₂ in air under saturated humidity.

2.4 In vitro fertilization (IVF)

After in vitro maturation (IVM) the cumulus cells were partially removed from the oocytes by vigorous pipetting. Groups of 20 oocytes were washed twice and transferred to 80-µL drops of TALP-IVF medium supplemented with 0.6% BSA, 10 µg/mL heparin, 18 µM penicillamine, 10 µM hypotaurine and 1.8 µM epinephrine, and covered with sterile mineral oil. A frozen straw of semen was thawed at 35.5°C and centrifuged on a discontinuous 45/90 Percoll gradient for 7 min at 3600 × g. The pellet was collected (100 µL) and resuspended in 700 µL TALP-IVF medium and again centrifuged for 5 min at 520 × g. After centrifugation, 30 µL of the medium containing the pellet was collected from the bottom of the tube and homogenized in a conical tube. The suspension was adjusted for a final concentration of approximately 104 mobile spermatozoa for each oocyte. The plates were incubated at 38.5°C for 20 h in an atmosphere of 5% CO₂ in air under saturated humidity. Semen from the same bull and the same batch was used for all replicates.

2.5 In vitro culture (IVC)

After IVF, presumptive zygotes were denuded of cumulus cells by vigorous pipetting. Embryos were washed three times and transferred in groups of 15 to 20 to be cultured in 100- μ L drops of SOF medium supplemented with 5 mg/mL BSA and 2.5% FCS. The dishes were then incubated in an atmosphere of 5% O₂ in air under saturated humidity for 5 days at 38° C. The cleavage rate, blastocyst development, and blastocyst hatching were evaluated 48 h, 7 days and 9 days after IVF, respectively.

2.6 Immunocytochemistry of H3K9ac and H3k27me3

For this experiment, we used day 5 embryos, 70h after IVF. At this timepoint, approximately 30-50% of embryos should be at the 5th cell cycle. Embryos were fixed in 4% paraformaldehyde for 30 min at 37°C and stored at 4°C in PBS supplemented with 3% BSA and 0.5% Triton X-100 for up to 1 week. Fixed embryos were incubated in blocking solution (3% BSA and 0.2% Tween-20 in PBS) for 1 h at room temperature. Next, the embryos were incubated with the primary antibodies (mouse anti-H3K9ac monoclonal antibody, 1:200, and rabbit anti-H3k27me3 monoclonal antibody (1:200; Upstate Biotechnology, Lake Placid, NY, USA) for 12 h at 4°C. The embryos were then washed three times in PBS for 10 min and incubated with the secondary antibody (chicken anti-mouse-alexa 488; 1:200; Invitrogen Molecular Probes, Eugene, OR, EUA), and goat anti-rabbit-alexa 555 (1:200; Invitrogen Molecular Probes, Eugene, OR, EUA) for 1 h. Nuclei were counterstained with 10 μ L/mL Hoechst 33342 for 20 min. The embryos were washed three times for 10 min in PBS and examined under a fluorescence microscope. Reactions in which the primary antibody was omitted served as negative control. Images of each structure were captured with an AxioCam camera and stored using the AxioVision 4.7.1 software (Carl Zeiss, Jena, Germany).

Images were measured for fluorescence intensity on each blastomere (day 5 embryos) using Adobe Photoshop CS3 (Adobe Systems Inc., Beaverton, OR, USA). First, the three images from each embryo (HOECHST, Alexa 488 and Alexa 555) were placed together in a new file, in different layers. Nuclei were selected with the magic wand tool in HOECHST layer for each blastomere. Sections were measured using the histogram function through the red (H3k27me3) and green (H3k9ac) channels. Photoshop assigns intensity values between 0 and 255 to each pixel in the selected area and then averages these intensities, giving the mean intensity of the selected region. For each embryo, the mean intensity of blastomeres was normalized to the lowest level. After, levels were classified into 7 categories.

2.7 Statistical analysis

Mean frequency of each category of normalized blastomeres was analyzed by one-way ANOVA and means were compared by the Tukey test. Statistical analysis was performed using the SAS 9.1 software (SAS Institute Inc., Cary, NC, USA).

3. Results

We evaluated two replicates and 12 embryos during the transition from 8- to 16-cell stages, totaling 169 blastomeres. The pattern detected for each embryo can be seen in Figure 1. As we can observe, levels of H3k27me3 varied accordingly to levels of H3k9ac. In others words, blastomeres that presented higher H3k27me3 tended to present higher H3k9ac, which

means that, for those embryos, global increases on repressive marks (H3k27me3) leads to increases in permissive marks (H3k9ac) as well.

Then we normalized the fluorescence level of each blastomere to the lowest level obtained, within each embryo. In this analysis, we observed that some embryos displayed a high individual variation between blastomeres, as demonstrated in Figure 2.

Therefore, we divided the embryos into two classes: A, for embryos that presented similar H3k9ac and H3k27me3 between blastomeres (8 embryos, 66%), and B, for embryos that exhibited variations between blastomeres (at least 2 blastomeres presenting 2fold increase in H3k9ac compared to the lowest blastomere) (4 embryos, 33%). Within each class, we classified the blastomeres accordingly to their intensity level: 1 to 1.5 (I), 1.5 to 2 (II), 2 to 2.5 (III), 2.5 to 3 (IV), 3 to 3.5 (V), 3.5 to 4 (VI), 4 to 4.5 (VII).

Fig. 1. Pattern of H3k9ac and H3k27me3 for 12 different embryos. Rows represent fluorescence levels of individual blastomeres. A) Embryos that presented similar levels of H3k9ac and H3k27me3 between blastomeres. B) Embryos that presented at least 2 blastomeres presenting 2-fold increases on H3k9ac levels, comparing to lowest level.

In class A embryos, we detected for H3k9ac a higher ($P < 0.05$) frequency of level I blastomeres, corresponding to 80%. The other blastomeres were classified in level II (18.3%) and III (1.6%). In this class of embryos, we did not detect any blastomeres in levels IV-VII, which means that most blastomeres presented similar lower levels of H3k9ac. The same pattern was observed in class I embryos for repressive mark H3k27me3. These results indicate that H3k9ac and H3k27me3 levels are constant between blastomeres in class I embryos, and their variation to the lowest blastomere level is only 2.5 fold maximum, in a small percentage of cells. Also, H3k9ac and H3k27me3 exhibited the same pattern of expression.

Fig. 2. Levels of H3k9ac and H3k27me3 in individual blastomeres from 12- to 16-cell embryos. I) Immunocytochemistry reaction for H3k9ac (Alexa 488, green) and H3k27me3 (Alexa 555, red) in class A (a,b) and class B (d,e) embryos. Nuclei were counterstained with HOECHST 33342 (c,f). II) Percentage (mean $\pm$ S.E.) of blastomeres from class A (a,b) and B (c,d) in H3k9ac and H3k27me3 level categories. In each embryo, blastomeres were normalized to the lowest H3k9ac level. ^{ABC}Means with different letters within the same group are not equal (ANOVA one way and Tukey post test, $P < 0.05$).

On the other hand, when we assessed class B embryos, we observed that only 36% blastomeres were classified as level I. This percentage was superior ($P < 0.05$) when compared to the percentage of embryos classified as levels V (9.2%), VI (1.9%) and VII (1.9%), but was similar to the frequency observed for levels II (14.8%), III (23.2%) and IV (12.4%). Thus, it can be suggested that a higher variation among H3k9ac levels is present in class B embryos. Additionally, for H3k27me3, the same pattern was observed, although level I frequency was higher (48.4%) and similar to level III (18.5%), and both levels I and III were superior ($P < 0.05$) to levels II (11.7%), IV (5.0%), V (6.6%), VI (3.8%) and VII (5.8%). Based on these results, it can be inferred that, for class B embryos, levels of H3k9ac and H3k27me3 displayed a remarkable variation between blastomeres, up to 4.5 fold higher than the lowest blastomere level.

We also wanted, we wanted to confirm if the variations observed for H3k9ac and H3k27me3 were occurring in the same intense for both marks. Therefore, each blastomere level was tested for Person's correlation analysis. It was observed an extremely high correlation coefficient ($r = 0.913$) and a significant P value ($P < 0.0001$), confirming our hypothesis that H3k9ac and H3k27me3 are highly correlated (Figure 3). In other words, we observed that in 12- to 16-cell embryos repressive and permissive marks vary in the same direction and intensity.

Fig. 3. Correlation between H3k9ac and H3k27me3 in individual blastomeres from 12- to 16-cell embryos.

4. Conclusions and future directions

Our results describe the presence of two distinguishable populations of bovine embryos during the 4th cell cycle, considering their epigenetic status. One population presented similar levels of repressive and permissive marks in all blastomeres, while the second one displayed a remarkable variation among their blastomeres.

Those changes were reported 2 cell cycles earlier than lineage specification in blastocysts. In mice, it has been demonstrated that differences in histone modifications between blastomeres as early as at the 4-cell stage reflect pluripotency, 2 cycles before trophectoderm differentiation (Torres-Padilla et al., 2007). Furthermore, only a specific population of embryos, based on their cleavage pattern, presented this difference in histone modification levels between blastomeres. Therefore, it is possible that the same phenomena happens in bovine embryos, and that might explain our findings.

In other words, those variable histone modification levels within class B embryos might show differences in pluripotent competence between blastomeres in the same embryo. In this case, cellular differentiation, which can be clearly seen after blastocyst formation, might have already been initiated in the 16-cell bovine embryo. However, this preliminary data should be further investigated. Time lapse studies, following the cell fate decisions of those blastomeres and better characterization of how they were derived are needed to elucidate this question.

In addition, here we demonstrated that global levels of permissive and repressive marks are correlated at this time point. These results suggest that, in 4th cell cycle embryos, no global switch between repressive and permissive marks is detected; and that level of those marks still goes along together.

In conclusion, embryonic genome activation is a crucial step across early embryo development, and it is accompanied by a dramatic change in epigenetic profile of blastomeres. Monitoring of histone modifications related to euchromatin and heterochromatin is an important tool to assess developmental competence in a sense that those marks are altered when manipulation and environmental stress conditions are applied. In this chapter, we described the pattern of IVC embryos in the cycle after embryonic genome activation, considering a repressive (H3k27me3) and a permissive (H3k9ac) histone modification mark. Those experiments would also be useful in order to compare culture conditions of IVF embryos, and how they would respond after environmental challenges. Interestingly, we found two distinguishable populations of embryos, one presenting similar profiles between blastomeres and other presenting remarkable changes between blastomeres. This observation should be further studied, as it might be reflecting distinct cleavage pattern embryos and pluripotency competence.

5. Acknowledgment

The authors are grateful to FAPESP for the financial support provided.

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In Vitro Fertilization - Innovative Clinical and Laboratory Aspects

Edited by Prof. Shevach Friedler

ISBN 978-953-51-0503-9

Hard cover, 156 pages

Publisher InTech

Published online 11, April, 2012

Published in print edition April, 2012

The field of In Vitro Fertilization is a relatively new field in medicine, constantly on the move. This field is an exquisite example of the vast power in the complementary use of basic research with clinical practice and opened a new route of great basic and clinical research possibilities. The knowledge base that allowed the accomplishment of the idea of in vitro fertilization and embryo transfer has much developed since. The vast body of research pertaining to this field allowed deepening our understanding in the processes related to reproduction. In this book on in vitro fertilization we present new and interesting updated information in various aspects of this field. This work is a result of collaborative work of an international group of professionals dedicated to contribute to the advancement of our knowledge.

How to reference

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Clara Slade Oliveira, Naiara Zoccal Saraiva, Leticia Zoccolaro Oliveira and Joaquim Mansano Garcia (2012). Analysis of Permissive and Repressive Chromatin Markers in In Vitro Fertilized Bovine Embryos Just After Embryonic Genome Activation, In Vitro Fertilization - Innovative Clinical and Laboratory Aspects, Prof. Shevach Friedler (Ed.), ISBN: 978-953-51-0503-9, InTech, Available from: <http://www.intechopen.com/books/in-vitro-fertilization-innovative-clinical-and-laboratory-aspects/analysis-of-permissive-and-repressive-chromatin-markers-in-in-vitro-fertilized-bovine-embryos-just-a>

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