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Parthenogenesis and activation of mammalian oocytes for *in vitro* embryo production: A review

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ABSTRACT

Parthenogenesis is a form of asexual reproduction found in females, where growth and development of embryos occurs without fertilization by a male. Parthenogenesis occurs naturally in aphids, Daphnia, rotifers, nematodes and some other invertebrates but can also be induced efficiently in mammalian oocytes by providing appropriate stimuli *in-vitro*. Recently, parthenogenesis has attracted wide attention because of the role of activated oocytes in the field of research that have been described such as intra cytoplasmic sperm injection, cloning by nuclear transfer, somatic cell cloning, investigating culture conditions etc. & potential for deriving pluripotent stem cell lines and their differentiation into various cell lines that can be utilized for various tissue engineering applications. The parthenogenetically activated oocytes possess maternal genome and can developed in to either haploid, diploid or polyploidy embryos with the help of it we can analyze the possible role of all the genes involved in imprinting processes as well as the role the paternal genome plays during early embryo development by comparing them with fertilized embryos. Several methods are able to induce parthenogenetic activation through the elevation of cytoplasmic free calcium in oocytes. But one common, universal method or activation agents has not been developed for all species because the process is highly specific for each species. Therefore, activation step for each species need to be optimized accordingly. This review describes the general method of activation of mammalian oocytes and their genomic imprinting analysis.

Keywords: Epigenetic Modification; Genomic Imprinting; *In-Vitro* Maturation; Oocytes Activation; Parthenogenesis

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1. INTRODUCTION

Parthenogenesis is a phenomenon of undoubted biological interest which leads to the production of living young in many types of animals, as well as in plants. Parthenogenesis may initiate early embryonic development in mammals, and its lack of success in this class poses some fundamental and as yet unresolved problems regarding the significance of fertilization in the physiology of reproduction and embryonic development. This is one of the reasons why parthenogenesis is once again an area of active research. An individual resulting from the development of an unfertilized egg is variously referred to as "parthenogenone", "parthenogen", or "parthenote". The last term is American, while "parthenogenone" is preferred in the British literature [1]. Parthenogenesis is a reproductive strategy typical of lower species where a female gives birth to offspring's without a paternal contribution. On the contrary, parthenogenesis is not a form of natural reproduction in mammals even if mammalian oocytes, under appropriate stimuli, can undergo to parthenogenetic activation. Parthenotes can be efficiently obtained *in-vitro* with a variety of mechanical, chemical, and electrical stimuli using oocytes of several species at different stages along oocyte meiosis resulting in parthenotes with different chromosome complements [2].

Induced parthenogenesis; the experimental induction of parthenogenesis in mammals began with the pioneering studies of Pincus and his collaborators in the rabbit. Pincus and Enzman [3] showed that the extrusion of polar bodies could be induced *in-vitro* not only by contact with sperm suspension, but also by heat treatment or exposure to butyric acid and hypertonic solutions. Subsequently Pincus and Shapiro [4]; described the effect of cold treatment on unfertilized tubal eggs *in-vitro* and claimed not only an increased incidence of cleavage but also the production of a living young. There has since been abundant confirmation of the possibility of inducing parthenogenetic development in mammals by experimental procedures but none of the embryos so formed has survived beyond the embryonic period.

Using gene targeting, Kono *et al*. [5] were able to manipulate two imprinted loci H19/IGF2 and DLK1/ MEG3 to produce bi-maternal mice at high frequency and subsequently showed that fatherless mice have enhanced longevity and in April 2004, they used parthenogenesis successfully to create a fatherless mouse at Tokyo University of Agriculture Japan.

The general procedure of parthenogenetic embryo development is almost similar to the *in-vitro* embryo development of fertilized oocytes except the step of parthenogenetic activation with different activation agents which could either be electrical, chemical or other types and thus includes collection of ovaries, recovery of oocytes, *in-vitro* maturation of oocytes (IVM), activation of oocytes with different activation agents and finally *in-vitro* development of parthenogenetic embryos.

2. *In-Vitro* MATURATION OF MAMMALIAN OOCYTES

Although recent efforts in *in-vitro* embryo production system have led to advances in many steps, the efficiency of *in-vitro* embryo production is still low with regards to obtaining the fully matured oocytes, as only 30% of oocytes develop into blastocysts, probably because the *in-vitro* environment cannot mimic *in-vivo* environments and results in embryos with altered morphology and gene expression. Therefore, it is needed to standardize the culture conditions that mimic *in-vivo* embryo development [6]. The improvement of caprine culture system is highly desirable in terms of the production of preimplantation stage embryos for both biotechnological studies and embryo transfer technique [7]. The improvement of *in-vitro* culture systems are important for production of embryos with high developmental competence that are used in agricultural and biomedical research, and animal biotechnology [8].

Oocytes can either be retrieved from slaughter house ovaries within stipulated time or from the live animals by different techniques. Superovulation (hormonal treatment) of donor is routinely done to increase number of ova released by the ovary. Laparoscopic ovum pick up is one of the best techniques because of less adhesion problems compared to laparotomy or surgical oocytes collection from live animals. The cost of oocyte retrieval from live animal is high due to unpredictable results and low oocytes quantity. Therefore, slaughter house ovaries are attractive alternative as source for oocyte retrieval as they are less expensive and most abundant source of immature oocytes for large scale production of caprine embryos [6]. Lesser time taken between the slaughter and transport of ovaries is always preferred. Time interval between collection of ovaries and harvesting of oocytes also vary from 1 hour [9] to 3 - 4 h [10-12] without any detrimental effects at appropriate temperature. Most of the researchers observed a temperature range of 30˚C - 37˚C during transport of ovary to be the optimum for IVMFC of mammalian oocytes [6].

The obvious advantage of oocyte recovery technique is speed of operation, quality of oocytes, and quantity of oocytes. Therefore, different techniques of oocyte collection are employed in order to obtain maximum good quality oocytes. Follicle puncture, Ovary Slicing and follicle aspiration are routinely used techniques for recovery of oocytes from slaughter house ovaries. However, different views come from different researchers. Goat ovaries are relatively smaller in size therefore aspiration of follicular oocytes is difficult [13]. Therefore, in case of goat ovary, slicing and puncture techniques are most common in terms of obtaining good quality and quantity of oocytes [14]. The efficiency of *in-vitro* embryo production profoundly influenced by the number and quality of oocytes which successfully complete maturation. The ability to identify good quality oocytes prior to *in-vitro* maturation is the important consideration for *in-vitro* embryo production system. The existence of a healthy population of somatic cells surrounding the oocytes is mandatory to facilitate the transport of nutriaents and signals into and out of the oocytes [15].

Selection criteria of cumulus oocyte complexes (COCs) are extremely important for successful *in-vitro* maturation. Morphology of the cytoplasm and cumulus cell investment surrounding oocytes is the primary criteria for the grading and selection of oocytes for IVM. Normal oocytes should have cumulus cell investment surrounding the zona pellucid (ZP), absence of cracked zona pellucida and absence of vesicle in ooplasm [16].

Presence of more and compact layers of cumulus cells is considered better [17]. Retrieved oocytes could be graded as excellent (A), good (B), fair (C) and poor (D) quality depending upon the cumulus investment and cytoplasmic distribution. Excellent (A); Oocytes with more than 4 layers of bunch of compact cumulus cells mass with evenly granulated cytoplasm. Good (B): oocytes with at least 2 - 4 layers of compact cumulus cell mass with evenly granulated cytoplasm. Fair (C): oocytes with at least 1 layer of compact cumulus cell mass with evenly granulated cytoplasm. Poor (D): oocytes with no cumulus cells or incomplete layer of cumulus cell or expanded cells and having dark or unevenly granulated cytoplasm [12]. It is desirable to select A and B quality oocytes for IVMFC.

Granulosa Cell monolayer supports cytoplasmic maturation of growing oocytes, which is evident by a better maturation rate, active fertilization, improved cleavage rate and subsequently a higher rate of morula formation [12,18]. Culture media supplemented with gonadotropins (LH and FSH) and estradiol-17ß are reported to improve maturation rates significantly [12,19-21].

In-vitro maturation can be achieved by the supplementation of 20% estrous goat serum. Supplementation of 20% EGS or 10% FBS + 3 mg/ml BSA in TCM-199 medium could also be used to achieve maturation followed by fertilization, embryo culture, and subsequent embryo transfer resulted in successful birth of caprine kid [10,16,22]. In our previous study we found slightly better *in-vitro* maturation rate in the presence of antioxidant ß-mercaptoethanol as compared to base medium (TCM-199 with NCS and 3 mg/ml BSA) but lesser than base medium containing hormones (LH-5µg/ml, FSH-5 µg/ml, Estradiol 1 µg/ml) [21]. A significant positive effect of epidermal growth factor (EGF) on IVM of oocytes was reported in cattle [23], sheep [24], pigs [25], buffalo [26] and in goats [21].

Further the time required for *in-vitro* maturation of various mammalian oocytes varies depending upon their own complexity. The time required for *in-vitro* maturation of mouse oocytes is 18 h [27]; Goat, 27 h [6], Rabbit, 14 - 15 h [28], Cat, 36 h [29], Camel, 24 - 48 h [30]. In that regard, bitches ovulate immature oocytes, and the oocytes require 2 - 5 days for meiotic maturation within the oviduct and remain surrounded by cumulus cell mass, unlike other mammals [31]. Good quality Buffalo oocytes were matured for 22 - 24 hrs. [32,33] whereas Procine oocytes, 22 - 24 hrs [34].

In compare to the majority of species, porcine oocyte maturation occurs over a longer period and this has resulted in the development of a two-stage maturation process. In the first step COCs are cultured in medium supplemented with hormone(s) such as eCG and hCG or HMG for 20 - 22 h in order to enhance nuclear maturation, in the second step, COCs are cultured in maturation medium without hormonal supplement(s) for 18 - 24 h. The removal of hormones is thought to slow nuclear maturation and enhance cytoplasmic maturation. North Carolina State University medium 23 (NCSU 23) supplemented with 10% porcine follicular fluid (pFF); NCSU 37 medium + 10% pFF; TCM 199 medium + 10% pFF and TCM 199 supplemented with 0.1% PVA are frequently used as a basic maturation medium for *in-vitro* maturation of procine oocytes [32]. However, Sow oocytes were significantly better than gilt oocytes when maturation rate, development to blastocyst and mean blastocyst cell number were compared.

Maturation can be judged directly by staining their nuclear and chromatin structure and/or by the ability of the oocytes to be fertilized or activated [6]. Cytoplasm of the oocyte may play a crucial role in assembling the correct metabolic environment for production of sufficient energy for cellular functions during maturation, cleavage and blastocyst formation [18]. However ooplasmic changes occur during oocytes maturation are still difficult to evaluate. Cytoplasmic maturation refers to the other maturation events that prepare the oocytes for fertilization and preimplantation development [35]. Degree of cumulus cells expansion can be used as a morphological indicator for maturation of oocytes. So, it can be said that expanded cumulus cells indicates mature and good quality oocytes while a compact cumulus cells characterizes immature oocytes [36]. When fully grown oocytes are released from their follicles to the culture medium they resume meiosis spontaneously in maturation medium. The reduced development of *in-vitro* derived zygote suggest that the conditions of IVM do not support cytoplasmic maturation, so it is very important that the improvement of the *in-vitro* maturation systems for oocytes aimed at defining *in-vitro* conditions that are more similar to the *in-vivo* environment [37]. However, development is still compromised compared to oocytes matured *in-vivo* and further research is required to optimize maturation in all species. Therefore development of appropriate IVM culture conditions that can mimic *in-vivo* culture condition for each type of mammalian oocytes is essential.

3. PARTHENOGENESIS

Meiotic maturation of oocytes starts during the fetal development of mammalian females, but is arrested at the late diplotene stage. The ability to resume meiosis and to continue in maturation beyond this stage is acquired gradually during the subsequent period of oocyte growth. In fully grown oocytes, meiosis continues after germinal vesicle breakdown (GVBD) through the stages of metaphase I, anaphase I and telophase I to the stage of metaphase II, when meiosis is again arrested. The ability to pass through all these stages is designated as full meiotic competence. During the growth period, oocytes pass through a stage in which meiotic competence is only partially developed [38]. These oocytes acquire the ability to undergo germinal vesicle break down (GVBD) and to enter metaphase I stage. However, they are unable to exit from metaphase I stage, to reach metaphase II stage, and to complete meiotic maturation [39-41]. Parthenogenetic activation of matured oocytes is a valid tool to assess their cytoplasmic maturation and quality. Furthermore, identification of an optimal protocol for oocyte activation is required for the production of genetically identical animals by somatic cell nuclear transfer.

Present methods for parthenogenetic embryo production *in-vitro* depend on the use of oocytes with full meiotic competence, which are present in the ovary in limited numbers. Numerous populations of follicles with growing oocytes that have partially developed meiotic competence cannot be used for these purposes. However, embryos produced from these oocytes could be used for breeding, production of cloned or transgenic animals, or for preservation of endangered breeds. To this end, cul-

ture systems for *in-vitro* growth and acquisition of full meiotic competence of mammalian oocytes have been developed [42,43]. However, the processes involved in the acquisition of full meiotic competence are not fully understood. Sedmíková *et al*. [44] demonstrated that drugs elevating intracellular calcium levels can overcome the meiotic block in oocytes with partially developed meiotic competence and can induce their maturation to the metaphase II stage. To achieve these results they used cyclopiazonic acid, the inhibitor of calcium-dependent ATPases, which elevates intracellular levels of free calcium ions through the mobilization of intracellular calcium deposits [45,46]. Successful activation has been achieved by a range of treatments including electrical stimulation, as well as chemicals such as strontium in mouse, ionomycin, calcium ionophore in cattle and sheep. In addition, there are many factors influencing efficient activation; concentration of chemical agents, duration between fusion and activation, activation media, strength of electric stimulation, post-treatments such as cytochalasin B or D (CB, CD), cycloheximide (CHX), or dimethylaminopurine (DMAP) etc. Several methods are able to induce parthenogenetic activation through the elevation of cytoplasmic free calcium in oocytes. One common, universal method or activation agents has not been developed for all species because the process is highly specific for each species therefore, combination of activation agents is also applied. Similarly concentration as well as incubation time of the activation agents are also species specific and need to be optimized.

Parthenogenesis in bovine oocytes can be induced with an electrical pulse [47,48], ethanol [49-51], calcium ionophore A23187 [52,53], cycloheximide (CHX) [54,55], 1,4,5-inositol triphosphate [56], ionomycin [57,58], or strontium [59]. When ethanol was used in combination with CHX, the success rate for parthenogenesis was further enhanced [51,54]. Similarly, better results were obtained after oocytes were first treated with ethanol or ionomycin prior to treatment with DMAP [60]. 9% ethanol (5 min) followed by 6-DMAP (4 h) promoted optimal parthenogenetic activation of bovine oocytes [61].

Activation response in bovine oocytes by several activation agents has been demonstrated to be oocyte age dependent also. Namely, the development to the pronuclear stage was investigated following activation treatment by ethanol [49,62] calcium ionophore [52,63] electric pulse [52,62,64,65] or cycloheximide [62-64]. While less than 40% of the young or aged (23 - 42 h) *in-vitro* matured oocytes were activated [49,64,66]. The relation between activation and aging of recipient oocytes is a factor which affects the development of activated embryos.

Aging of oocyte decreases the fertilization rate and the subsequent development [67,68]. Therefore, using young recipient oocytes is attractive and may increase the overall efficiency of cloning procedures if the oocytes can be activated adequately. Aoyagi *et al*. [69] demonstrated that, reconstituted embryos had a high developmental rate to the blastocyst stage when a combination treatment of Ca-ionophore, electric pulse and cycloheximide was used to activate young oocytes. Combined ethanol and cycloheximide treatment has been reported to effectively (over 90%) activate freshly matured bovine oocytes [51,54,62,70]. Therefore, the combined activation of young oocytes leads to a more efficient development of bovine embryos.

Among several activating artificial agents, some promote intracellular calcium increase, e.g. strontium [71, 72], ionomycin [73], electric pulse [74], and ethanol [73], and others inhibit protein synthesis e.g. cycloheximide [75] or protein phosphorylation e.g. 6-DMAP [73]. In the case of intracellular calcium releasing treatments, ionomycin induces a single increase and is frequently used in combination with protein phosphorylation inhibitors [73]. The great disadvantage of using the protein kinase inhibitors or the protein synthesis inhibitors is that these inhibitors do not specifically inhibit the activity of a particular kinase or the synthesis of a specific protein that control cell-cycle progression. However, they inhibit the activity of several kinases or the synthesis of several proteins that may be involved in other cell functions, whose inhibition may have a deleterious effect on the subsequent cellular events after oocyte activation [76]. Moreover, the calcium oscillations triggered by the sperm cells function not only in inducing resumption of meiosis but also in many other events [77]; for example, recruitment of specific maternal RNAs [78,79], which is essential for activation of zygotic genome [80] and may be extended to other unknown functions. Therefore, a new activation regimen without using either protein synthesis or protein phosphorylation inhibitors but with two trigger agents for single calcium increase effectively improved blastocyst yield.

Strontium (divalent cation), has also shown to promote multiple free calcium oscillations (similar to fertilization) in mouse oocytes [81]. Therefore it can also be used to activate oocytes of various animals including cattle. Meo *et al*. [82] successfully applied strontium efficiently for bovine oocyte activation at 20 mM in Ca^{2+}- and Mg^{2+}-free TALP medium for 6 h. Hosseini *et al*. [83]; studied fusion pulses along with a chemical activation protocol and sequential use of Ca^{2+} immobilizing agents, which may benefit the activation outcome in bovine oocytes. A combination of single, double or triple compounds of EP (electrical pulse), sequential combinations of calcium ionophore (CI), ethanol (ET), strontium (SR) along with 6-DMAP on *in-vitro* matured bovine oocytes showed the best cleavage rates with double (SR-CI, 84.4%), triple

(CI-SR-ET, 79.4%) and single (CI, 73.7%) compounds, respectively, which were not significantly different with each other and with *in-vitro* fertilized (85.5%) oocytes. The highest blastocyst rates were gained with ET-SR (24.5%), SR-CI-ET (20.4%) and CI (24.5%) accordingly which were not significantly different with each other but significantly lower than IVF (47%). Embryo cell counting further confirmed reasonably better quality of blastocysts produced using double, triple and single compounds.

In buffalo, ethanol has been employed as an agent [84], which activates oocytes by promoting the formation of inositol 1,4,5-triphosphate (IP3) at the plasma membrane and the influx of extracellular Ca^{2+} [85], causing a large, single rise in intracellular Ca^{2+} concentration [72]. Inomycin is another popular activating agent currently used in buffalo nuclear transfer protocols [86], which induces repetitive transient rises of Ca^{2+} lasting for several hours, probably by displacing bound Ca^{2+} in the oocytes. Electrical stimulation has also been used for activation of *in-vitro* matured oocytes in buffalo [87,88], whereas ethanol, ionomycin and calcium ionophore were used as chemical activators in buffalo [89,90]. Within the ethanol and ionomycin activation groups, ethanol supported the highest development in terms of cleavage (71:4 ± 7:8 versus 59:4 ± 10:7) and morulae-blastocysts rate (32:6 ± 6:5 versus 25:7 ± 8:3). Similarly, ethanol activation gave better results than the IVF control group, with higher cleavage rate (71:4 ± 7:8 versus 55:8 ± 5:8, respectively) and a higher proportion of oocytes developing into morulae-blastocysts (32:6 ± 6:5 versus 22:9 ± 7:5, respectively). It was also observed that aging negatively affects post-parthenogenetic and post-fertilization development [89]. Interestingly, despite the similar maturation rate of buffalo (87%) and cattle (94%) oocytes, the cleavage rate in buffalo oocytes is poor (64% versus 84%) or very poor (9% - 45%) and may be attributed to poor activation by sperm at the time of fertilization [91,92]. To ascertain possible reasons for low cleavage following IVF and to identify the role of sperm in the process of fertilization and cleavage, Mishra *et al*. [33] compared chemical activation protocols on *in-vitro* matured oocytes with IVF (natural activation) and observed that cleavage rate was significantly higher following ET + DMAP, ET + CHX and ET + CHX + DMAP activation (52.5%, 52.5% and 44.4%, respectively) compared to IVF (36.5%, 23.4% and 26.8%, respectively). Blastocyst development (30.9% versus 15.2%) was also significantly higher following ET + CHX + DMAP activation than IVF. Thus, buffalo oocytes had better inherent developmental competence and that the poor cleavage and embryo development following IVF may be due partly to the poor quality of frozen/thawed sperm, improper sperm capacitation and/or fertilization.

The literature on activation protocols for goat oocytes is limited therefore; effective activation protocols need to be developed. Ongeri *et al*. [93] compared the development of IVF goat embryos with those of non-fertilized parthogenetically developing oocytes activated by treatment with either ionomycin or ethanol, both followed by immediate exposure to 6-diethylaminopurine (6-DMAP). In both shipped and non shipped oocytes, parthenotes developing from ionomycin and ethanol activated oocytes had significantly greater blastocyst development compared to IVF embryos. Guo *et al.* [94] evaluated the ionomycin, strontium and electrical pulse for the effecttive activation and parthenogenetic development of goat oocytes. The activation of oocytes by ionomycin combined with 6-dimethylaminopurine, strontium plus cytochalasin B and electrical pulses combined with cytochalasin B revealed 79.3% - 81.6%, 2.2% - 78.8% and 65.5% of the oocytes cleaved and 16.2% - 24.8%, 0% - 15.6% and 11.1% of the cleaved embryos developed into blastocysts, respectively. In our lab, we tried to optimize the protocols for caprine oocytes activation through comparing the effectiveness of different concentration of ethanol treatment on the activation and subsequent development of oocytes. In the Experiment, matured oocytes were treated with single activation agent *i.e.* Ethanol with concentration ranging from 1%, 3%, 5%, 7% & 9% for 5 min. The cleavage rates were gradually higher with higher concentration of ethanol treatment. Development of the embryo up to morula stage were also follow the same trend upto 7% ethanol treatment and decreased at higher concentration *i.e.* at 9% ethanol. Blastomeres were also shows less compaction in all other treatments including 9% ethanol concentration. These results suggested that ethanol treatment (7% for 5 min) is most favorable for parthenogenesis of caprine oocytes and its further development *in-vitro* [95]. The use of Ca ionophore (5 μM) and 6-DMAP (2 mM), activation of caprine oocytes for the production of zona and zona-free parthenogenetic embryos in three different culture media revealed that zona parthenogenetic hatched blastocysts were highest in RVCL (6.8% ± 0.9%) as compare to mSOF (1.2% ± 0.7%) and EDM (5.5% ± 0.7%) ($P < 0.05$) media, respectively. Similarly, zona-free parthenogenetic blastocyst, formation was greater in the RVCL (8.8% ± 0.9%) as compare to mSOF (5.6% ± 0.5%) and EDM (5.1% ± 0.8%) ($P < 0.05$) media, respectively [96].

Several protocols have been used to successfully create parthenogenetic sheep embryos [73,97-99]. Grazul-Bilska *et al*. [100] validated and optimized the methodologies necessary to create parthenogenetic sheep embryos for future studies of placental development in normal and compromised pregnancies. The oocytes were activated using ionomycin (a calcium ionophore) and 6-dimethylaminopurine (DMAP; a protein kinase inhibi-

tor). Activation of oocytes in serum-free medium resulted in minimal cleavage rates. However, replacement of ionomycin with ethanol treatment resulted in decreased blastocyst formation (from 58% to 19%, respecttively) but not cleavage rates (83% and 81%, respectively [73]. It is known that ionomycin induces smaller and thus less cytotoxic rise of intracellular calcium-whereas ethanol induces extracellular as well as intracellular release of calcium. In another study, the rate of blastocyst formation was 25% after oocyte stimulation with direct current pulses and treatment with cycloheximide plus cytochalasin B [101]. Thus, the activation protocol may have a profound effect on success of the oocyte activation to obtain parthenogenetic embryos. Shirazi *et al.* [102] compare the effect of time of parthenogenetic activation (22 hr versus 27 hr after *In Vitro* Maturation-IVM) on in vitro development of ovine oocytes using either single (Ionomycin 5 μM for 5 min or Ethanol 7% for 7 min) or combined (ionomycin and ethanol with 6-DMAP 2 mM for 3 hr) activation treatments. The cleavage and blastocyst rates in single-treated groups were positively influenced by the extension of duration of IVM (27 hr). A trend of decreased numbers of total cells and ICM was observed in slightly aged oocytes. Moreover, developmental potential of ovine parthenotes, especially in young oocytes, was improved by the addition of 6-DMAP to the activation regimen.

In addition to the above mentioned oocyte activation factors, several other factors can activate oocytes to induce parthenogenetic development, including chilling or warming, exposure to colchicine, exposure to electric pulses in the presence of Gluta MAX-I, pricking, certain anesthetics, and factors disturbing the balance between free calcium and the state of the cycloskeletal system [103,104].

4. GENOMIC IMPRINTING ANALYSIS OF PARTHENOGENETICALLY ACTIVATED EMBRYOS

When the mammalian oocyte is fertilized with sperm, it receives the paternal genetic materials. The paternal alleles, like the oocyte alleles, have been subjected to epigenetic modifications during gametogenesis that cause a subset of mammalian genes to be expressed from one of the two parental chromosomes in the embryo. This regulatory mechanism is termed genomic imprinting [105, 106]. Additional epigenetic processes also occur during early development after fertilization [73]. Thus, the maternal and paternal genomes are not functionally equivalent, which is why both a maternal and a paternal genome are required for normal mammalian development. Mammalian parthenotes are able to undergo several cycles of cell division after oocyte activation, but never proceed to term, arresting at different stages of development, depending on the species [50,73,105,106].

Success rates and viability of parthenogenetic embryos appear to be organism dependent. Mouse parthenotes are capable of developing beyond the postimplantation stage *in-vivo* [107,108]; porcine parthenotes have developed up to post-activation day 29 (limb bud stage, past the early heart beating stage); rabbit parthenotes until day 10 - 11 [109] and primates (Callithrix jacchus) have only been shown to implant stage [110]. The reason for this arrested development is believed to be due to genetic imprinting. Since all genetic material in parthenotes is of maternal origin, there is no paternal imprinting component and this prevents proper development of extraembryonic tissues whose expression is regulated by the male genome [111].

Uniparental embryos, such as parthenotes or androgenotes, have been used to study imprinting processes as well as the role the paternal genome plays during early embryo development [112]. Since diploid parthenotes (DPs) and fertilized embryos show similar development, at least to the blastocyst stage, their gene transcription patterns during early developmental processes may not differ markedly. However, there may be some more subtle differences in that fertilized embryos may express Y-chromosome-linked genes and imprinting genes during early development, unlike the DPs. Comparison of the gene expression patterns of the fertilized embryo and the DP parthenote may thus illuminate the role(s) paternal genes play in later embryonic development. Compared to DPs, fewer haploid parthenotes (HPs) cultured *in-vitro* reach the blastocyst stage and those that does have lower cell numbers [28,113]. The reasons for this limited developmental potential of mammalian HPs are not clear. One possibility is that the lack of genetic component(s) in HPs may increase the duration of the cell cycle and consequently slow their development [28]. This explanation is supported by the observation that mouse HPs develop *in-vitro* more slowly than DPs during the preimplantation period [114]. Another possible explanation is that the low DNA content in HPs may not be sufficient to control the gene expression network, which could result in apoptosis [28,115,116] or the failure of developmental processes during preimplantation development.

To gain insights into the roles the paternal genome and chromosome number play in pre-implantation development, cultured fertilized embryos and diploid and haploid parthenotes (DPs and HPs, respectively), and compared their development and gene expression patterns. The DPs and fertilized embryos did not differ in developmental ability but HPs development was slower and characterized by impaired compaction and blastocoel formation. These results are consistent with previous reports that indicated HPs are developmentally retarded

and show slow development in mice [28,114]. While it remains unclear why HPs show more limited and slower development, it may be speculated that at least part of the reason may involve the difficulties HPs have in compacting. Compaction during embryonic development involves the formation of tight junctions between outer cells, which permits selective ion transport and facilitates blastocoel formation [117]. Thus, the incomplete compaction of HPs may be responsible, at least in part, for their impaired development to the blastocyst stage. Microarray analysis revealed that fertilized blastocysts expressed several genes at higher levels than DP blastocysts; these included the Y-chromosome-specific gene eukaryotic translation initiation factor 2, subunit 3, structural gene Y-linked (Eif2s3y) and the imprinting gene U2 small nuclear ribo nucleoprotein auxiliary factor 1, related sequence 1 (U2af1-rs1). It is found that when DPs and HPs were both harvested at 44 and 58 h of culture, they differed in the expression of 38 and 665 genes, respectively. When differentially expressed genes in the HPs as compared to the DPs at 58 h after activation were analyzed with regard to their putative molecular function. 176 highly expressed and 158 lower expressed genes were unclassified. Of the remainder, it was found that 12 highly expressed and 9 lower expressed genes were related to cell adhesion/cell junction/cytoskeletal-functions.

Moreover, compared to the 58 h DPs, the 58 h HPs showed lower expression of more nucleic acid-binding proteins, oxidoreductase, transcription factors, selected regulatory molecules, and transferase, and highly expression of more receptors, transcription factors, nucleic acid-binding proteins, kinases, and selected regulatory molecules.

However, when DPs and HPs were harvested at the midpoints of 4-cell stage (44 and 49 h, respectively), no differences in expression was observed. Similarly, when the DPs and HPs were harvested when they became blastocysts (102 and 138 h, respectively), only 15 genes showed disparate expression. These results suggest that while transcripts needed for early development are delayed in HPs, it does progress sufficiently for the generation of the various developmental stages despite the lack of genetic components.

Genomic imprinting, a specific genetic mechanism in mammals, plays important roles in the regulation of fetal growth, development, placental function, and postnatal behavior [118-120]. It endows some genes with different "imprints", which lead to their differential expression in fetuses and/or placenta and regulate the transfer of nutrients to fetus and placenta from the mother [121].

The establishment of genomic imprinting is controlled by DNA methylation, histone modifications, noncoding RNA, and specialized chromatin structure; DNA methylation is thought to be a major factor [122-124]. Specific DNA methylation in the differentially methylated regions (DMRs) of parental origin allows the discrimination between the maternal and the paternal alleles and leads to monoallelic expression of imprinted genes [125]. Uniparental fetuses, including parthenotes and androgenotes, show disrupted expression of several imprinted genes, such as Snrpn, Peg3, H19, and Gtl2 [126,127]. Studies in mouse uniparental embryos have revealed that the paternal genome is more important for the development of the extraembryonic tissues, while the maternal genome is more essential for fetal development. These distinctive differences are the result of genomic imprinting [128]. Parthenogenetic fetuses die by day 10 of gestation [129]. Likely, the cloned animal fetuses exhibit a high rate of developmental abnormalities due to inefficient epigenetic reprogramming of the donor nucleus within enucleated oocytes [130-132]. The aberrant epigenetic modifications caused by inefficient reprogramming everely undermine the developmental potency of cloned embryos [132-134]. But to date, our knowledge about the molecular mechanism of epigenetic reprogramming is still very limited [135]. Compared to the laborious manipulation of somatic cell nuclear transfer, the mouse parthenogenetic embryo is a most suitable alternative to study the events of methylation imprints. Similar to mouse parthenogenetic embryos, the aborted cloned bovine fetus also exhibits disrupted expression of imprinted genes and aberrant methylation imprints [136, 137]. To obtain further insight into the dynamics of methylation imprints during development of diploid parthenogenetic mouse embryos, [138] determined the methylation status of DMRs of three maternally imprinted genes and two paternally imprinted genes using bisulfite mutagenesis sequencing methods. They showed that the maternally imprinted genes Snrpn and Peg1/Mest were nearly unmethylated or heavily methylated, respectively, in their differentially methylated regions (DMRs) at the two-cell stage in parthenogenetic embryos. However, both genes were gradually de novo methylated, with almost complete methylation of all CpG sites by the morula stage in parthenogenetic embryos. Unexpectedly, another maternally imprinted gene, Peg3, showed distinct dynamics of methylation during preimplantation development of diploid parthenogenetic embryos. Peg3 showed seemingly normal methylation patterns at the two-cell and morula stages, but was also strongly de novo methylated in parthenogenetic blastocysts. In contrast, the paternally imprinted genes H19 and Rasgrf1 showed complete unmethylation of their DMRs at the morula stage in parthenogenetic embryos. These results indicate that diploid parthenogenetic embryos adopt a maternal-type methylation pattern on both sets of maternal chromosomes and that the aberrantly homogeneous

status of methylation imprints may partially account for developmental failure.

5. CONCLUSION

A variety of activation stimuli and activation protocols have resulted in the production of viable embryos for parthenogenetic as well as somatic cell cloning research in a range of species. However, both the stimuli and the protocol used must be optimized for use in each species and there is a need for understanding the mechanism and effects by various activation methods. Since the birth of Fatherless mouse (Kaguya) the first viable parthenogenetic mammal in 2004 in Japan, significant advances have been made in the field of parthenogenetic research in order to understand the molecular processes involved during genomic imprinting process which is the main (perhaps the only) barrier to parthenogenetic development in mammals, in which the individual contains no paternal genetic material. Development of Parthenogenetic embryos is a multifactorial process and advances in all areas will contribute to simplifying and improving the efficiency of the technique.

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