

—Mini Review—

Towards further optimization of preimplantation embryo culture media: from the viewpoint of omics and somatic cell nuclear transfer (SCNT) studies

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Abstract: Assisted reproductive technology (ART) has greatly benefited numerous infertile couples who would never have had their babies without this technique. However, *in vitro* culture is reported to cause epigenetic and transcriptomic changes on preimplantation embryos, leading to adverse effect on development, and little is known about the molecular mechanisms underlying these changes. Here, we first introduce key studies that designate the effectiveness of an omic strategy to explore the molecular mechanisms governing preimplantation development of *in vitro*-cultured embryos. Furthermore, we review how *in vitro* culture components facilitate genomic reprogramming and zygotic genome activation (ZGA) contributing to preimplantation development after somatic cell nuclear transfer (SCNT). From these different perspectives, we would search for a breakthrough to further optimize preimplantation embryo culture conditions and improve ART.

Key words: Preimplantation embryo, ZGA, Metabolomics, Octanoate, SCNT

Introduction

Since the first IVF baby birth in 1978, nearly 4 million babies have been born assisted by *in vitro*-fertilization (IVF) [1]. The number of assisted reproductive technol-

ogy (ART) treatment cycles in Japan has been steadily increasing from 37,455 in 1997 to 326,426 in 2012. Moreover, 44,951 babies in Japan in 2013, 67,996 babies in the USA in 2013 [2] and 134,106 babies in Europe in 2011 [3] were conceived with ART treatments. However, ART conceptions are reported to be associated with an increased incidence of congenital abnormalities, low birthweight and preterm birth when compared with spontaneous conceptions [4], and there are concerns that *in vitro* culture potentially causes transcriptomic changes [5], and epigenetic adverse effects on preimplantation embryo development (e.g. Beckwith-Wiedemann syndrome [6–9], Angelman syndrome [10–15], Prader-Willi syndrome [15], Silver-Russell syndrome [15], Retinoblastoma [16–18], and congenital abnormalities [4]), as well as having a significant effect on the birthweight of offspring [19, 20].

The culture medium is assumed to be an important factor affecting ART. A study using mice compared five culture media: KSOMaa [Millipore], nonsequential systems (Global Medium [LifeGlobal], Human Tubal Fluid (HTF) [LifeGlobal]), sequential systems (Preimplantation 1/Multiblast [Somagen Diagnostics Inc.], and G1v5PLUS/G2v-5PLUS [G1.5/G2.5; Vitrolife AB, Goteborg, Sweden]). All media had a varying but compromised ability to maintain the methylation levels of the imprinted genes H19, Peg3 and Snrpn in *in vivo*-derived mouse blastocysts [21]. Also in a human study, blastocysts cultured in G5 (Vitrolife) or HTF medium (Lonza, Verviers, Belgium) from day 1 (the pronuclear stage) until day 6 were compared and examined individually for genome-wide gene expression [5].

Table 1. Representative genes which involve fatty acid metabolism from microarray analysis (from [22]).

Gene symbol	Description
ACADM	Medium-chain specific acyl-CoA dehydrogenase, mitochondrial
ACADS	Short-chain specific acyl-CoA dehydrogenase, mitochondrial
ACADVL	Very long-chain specific acyl-CoA dehydrogenase, mitochondrial
ACADL	Long-chain specific acyl-CoA dehydrogenase, mitochondrial
Acad9	acyl-Coenzyme A dehydrogenase family, member 9
CPT1A	Carnitine O-palmitoyltransferase 1, liver isoform
CPT1B	Carnitine O-palmitoyltransferase 1, muscle isoform
CPT2	Carnitine O-palmitoyltransferase 2, mitochondrial
Crot	Carnitine O-octanoyltransferase
Dci	Dodecenoyl-Coenzyme A delta isomerase (3,2 trans-enoyl-Coenzyme A isomerase)
ECHS1/SCEH ('crotonase')	Enoyl-CoA hydratase, mitochondrial; enoyl Coenzyme A hydratase, short chain, 1, mitochondrial
Ehhadh	enoyl-Coenzyme A, hydratase/3-hydroxyacyl Coenzyme A dehydrogenase
Hadha/Lchad	hydroxyacyl-Coenzyme A dehydrogenase/3-ketoacyl-Coenzyme A thiolase/enoyl-Coenzyme A hydratase (trifunctional protein), alpha subunit; L-3-hydroxyacyl-Coenzyme A dehydrogenase, long chain
Hadhb	hydroxyacyl-Coenzyme A dehydrogenase/3-ketoacyl-Coenzyme A thiolase/enoyl-Coenzyme A hydratase (trifunctional protein), beta subunit
SLC25A20	Carnitine acyl-carnitine translocase; Mitochondrial carnitine/acylcarnitine carrier protein
Etfα	electron transferring flavoprotein, alpha polypeptide
Etfβ	electron transferring flavoprotein, beta polypeptide
Etfdh	electron-transferring-flavoprotein dehydrogenase
HADH/SCHAD	hydroxyacyl-Coenzyme A dehydrogenase; L-3-hydroxyacyl-Coenzyme A dehydrogenase, short chain

Eighteen pathways, involved in apoptosis, metabolism, protein processing and cell-cycle regulation, showed significant overrepresentation of differentially expressed genes. DNA replication, G1 to S cell-cycle control, and oxidative phosphorylation pathways were up-regulated in the G5 group, compared with the HTF group, which coincided with developmental abilities such as cleavage development and implantation rate [5]. These results suggest that culture media can influence methylation status, alter gene expression, and even affect subsequent development.

Therefore, optimization of culture media for preimplantation embryos is an urgent issue. Here we review the current knowledge about the formulation and influence of culture media on gene expression and methylation status of preimplantation embryos, from the viewpoint of omics and somatic cell nuclear transfer (SCNT) studies, to consider their implications for infertility treatment.

Latest Knowledge from Omic Studies of Preimplantation Embryo Culture Medium

(i) *Can a medium-chain fatty acid, octanoate, be utilized as an energy source in preimplantation embryos?*

Hamatani and colleagues performed global gene expression analysis of mouse preimplantation embryos.

They demonstrated that there are distinctive patterns of maternal RNA degradation and embryonic gene activation, including two major transient "waves of *de novo* transcription" (Zygotic Genome Activation (ZGA) and mid-preimplantation gene activation (MGA)) [22]. Their global gene analysis predicted that ZGA genes involving fatty acid metabolism (fatty acid beta-oxidation, carnitine transport, short-chain fatty acid catabolic process, long-chain fatty acid metabolic process and regulation of fatty acid oxidation) would be transcribed in preimplantation embryos (Table 1). In fact, significant expressions of acyl-CoA dehydrogenases, ACADM throughout the preimplantation stages and ACADL only at the blastocyst stage were confirmed by western blotting analysis [23]. ACADM is known to catalyze C4-C12 fatty acids, and ACADL catalyzes C8-C20 fatty acids [24]. Subsequently, hydroxyacyl-CoA, a product of the fatty acid β -oxidation cycle, is catalyzed by the mitochondrial trifunctional protein, which is a heterooctamer multienzyme complex consisting of four α subunits (hydroxyacyl-CoA dehydrogenase alpha, HADHA), whose expression was confirmed by western blotting analysis [23], and the β subunit (hydroxyacyl-CoA dehydrogenase beta, HADHB) [25–28]. It therefore seems that all stages of preimplantation embryos are well-equipped for mitochondrial β -oxidation systems to metabolize fatty acids including

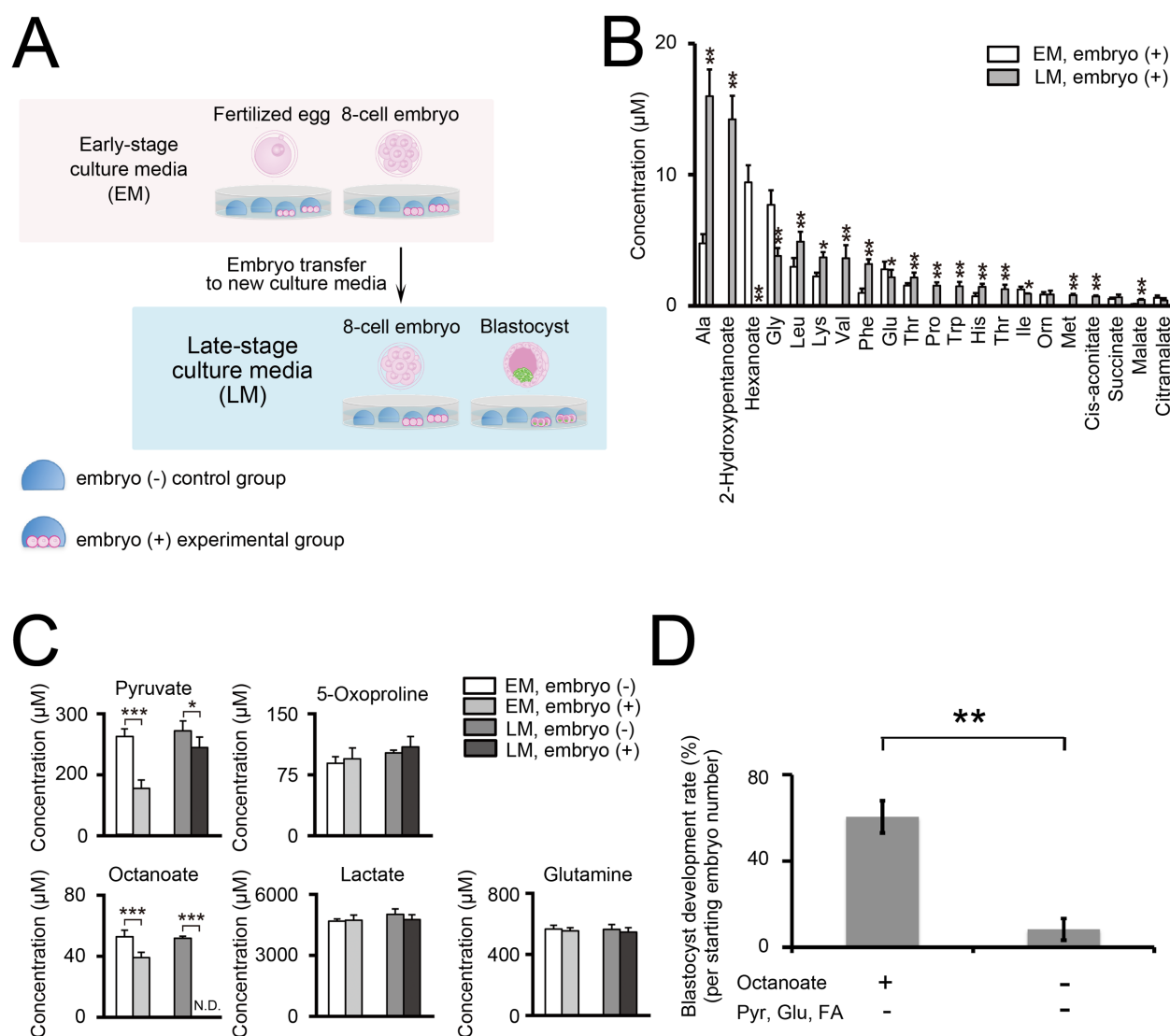


Fig. 1. CE-TOFMS analysis of metabolites in culture media during preimplantation development. a) Strategy of microdrop culture for synchronizing preimplantation embryos. b) Metabolites released into culture media during preimplantation development. c) Metabolites taken up from culture media during preimplantation development. d) Octanoate supplementation rescues preimplantation development in energy-depleted culture media (from [23]).

medium-chain fatty acids.

Accordingly, Yamada and colleagues analyzed the effects of low-molecular-weight metabolites in culture media on the growth of mouse preimplantation embryos, and focused on fatty acid metabolism in mouse preimplantation embryos using capillary electrophoresis time-of-flight mass spectrometry (CE-TOFMS) [23]. Culture media from *in vitro* cultures of embryos during the early and late preimplantation stages (the embryo group) or media incubated without any embryos (the control group) were analyzed (Fig. 1A). CE-TOFMS detected 28 me-

tabolites: 23 embryo-excreted metabolites including 16 amino acids and 5 media-derived metabolites (e.g. octanoate, a medium-chain fatty acid) (Figs. 1B, 1C).

Octanoate is known to act as a stabilizer when added to preparations of serum albumin prior to heat inactivation of viruses [29]. Compared to the negative control group, the embryo group media showed significantly lower concentrations of octanoate throughout the preimplantation stages. To further investigate the effect of octanoate on preimplantation embryo development under energy-depleted conditions, embryos were cultured

in vitro in media lacking glucose, pyruvate, and fatty acids. Few embryos ($9.8 \pm 2.9\%$) could reach the blastocyst stage. In contrast, $60.4 \pm 7.4\%$ of the embryos cultured in energy-depleted media supplemented with octanoate ($100 \mu\text{M}$) reached the 8-cell/morula stage ($P < 0.01$) (Fig. 1D). In addition, a ^{13}C -labeled octanoate tracer experiment revealed that octanoate was incorporated into the TCA cycle via β -oxidation and used as an alternative energy source in the mitochondria of preimplantation embryos.

Fatty acids including octanoate are known to be cytotoxic at high concentrations [30]. In fact, Fredrickson *et al.* compared the effect of different octanoate concentrations (400, 800, 1200 μM) on development, and showed that fetal and placental weights were increased with 800 μM octanoate relative to controls [31]. However, these octanoate concentrations ($>400 \mu\text{M}$) are much higher than the natural concentration in culture media (around 50 μM in mouse KSOM media) (Fig. 1C), making their comparison difficult. Our preliminary data show that the developmental rate of blastocysts increased in a dose-dependent manner at less than 150 μM of octanoate, but decreased at more than 200 μM , failing to 13% at 800 μM . Further well-designed studies are needed to find proper formulations of culture media including fatty acids and unknown components.

(ii) Predictive factors for pregnancy using spent culture medium

Several studies have aimed to find good predictive factors for pregnancy to prevent abortions. In a murine study, higher glucose consumption, a lower glycolytic rate (expressed as the percentage of glucose converted to lactate), and more aspartate and little or no produced glutamate in spent culture media were reported to correlate with a larger inner cell mass (ICM) number, and thereafter higher fetal post-implantation survival rate [32].

In human studies, the spent culture media in which viable embryos were cultured from 24 h after oocyte retrieval until day 3 was compared with that of non-viable embryos. Detection and quantitation of the alpha-1 haptoglobin fragment of the culture medium proved to be a useful additional method for identifying nonviable embryos, increasing the success rate from 25–30% to 50% [33].

In addition, increased concentrations of microRNAs, miR-20a and miR-30c, in spent blastocyst culture media have been useful for predicting the success rate of implantation. These miRNA functions are predicted *in silico* to be involved in 23 implantation-related pathways

that are related to blastocyst–endometrial communication: cell-to-cell communication/ cell signaling, cell-to-cell adhesion, and cell growth. Based on bioinformatic analysis, miR-20a is predicted to regulate two transcripts (SOS1, TCF7L1) involved in endometrial cell proliferation and growth through the modulation of five genes (PTEN, NRAS, MAPK1, MYC, and CCND1). Also, miR-30c is predicted to regulate five distinct transcripts (APC, KRAS, PIK3CD, SOS1, and FOXO3) that are involved in endometrial cell proliferation and growth [34].

Latest Knowledge from Somatic Cell Nuclear Transfer (SCNT) Studies of Preimplantation Embryo Culture Medium

(i) Zygotic genome activation (ZGA) is indispensable for human preimplantation embryo development and pluripotent stem cell isolation after SCNT

SCNT with human eggs offers a good model for reprogramming. Nuclear transfer embryonic stem cells (ntESCs) are assumed to be closer to embryonic stem cells (ESCs) than induced pluripotent stem cells (iPSCs). Because SCNT can reprogram somatic cells following an embryonic developmental path, their gene expression resembles that of embryos [35].

Initial attempts to produce human stem cells using SCNT failed, as most of the reconstructed oocytes arrested at the cleavage stages. In 2011, Noggle and colleagues revealed that one of the reasons for this failure is that only 16% (124 of 761) of ZGA transcripts were upregulated after genome exchange in 2n-SCNT human embryos (Fig. 2A). In contrast, triploid embryos containing a diploid genome derived from the somatic cell and a haploid genome derived from the oocyte (3n-SCNT human embryo) can transcribe ZGA products and pluripotent stem cells [36]. These results suggest that ZGA is indispensable for the reprogramming and isolation of ESCs.

To induce proper zygotic transcription in 2n-SCNT embryos, Yamada and colleagues improved the SCNT protocol using a calcium pulse induced by a calcium ionophore and a combination of a kinase and a translation inhibitor to stimulate exit from meiosis. This resulted in developing blastocysts in which the GFP transgene from the transferred somatic cell genome was expressed (Fig. 2B), and using this method, they successfully derived stem cells from a female patient with type 1 diabetes (age 32 years, age of onset 10 years) [37]. Therefore, ZGA can be deemed indispensable for blastocyst development and stem cell derivation through SCNT.

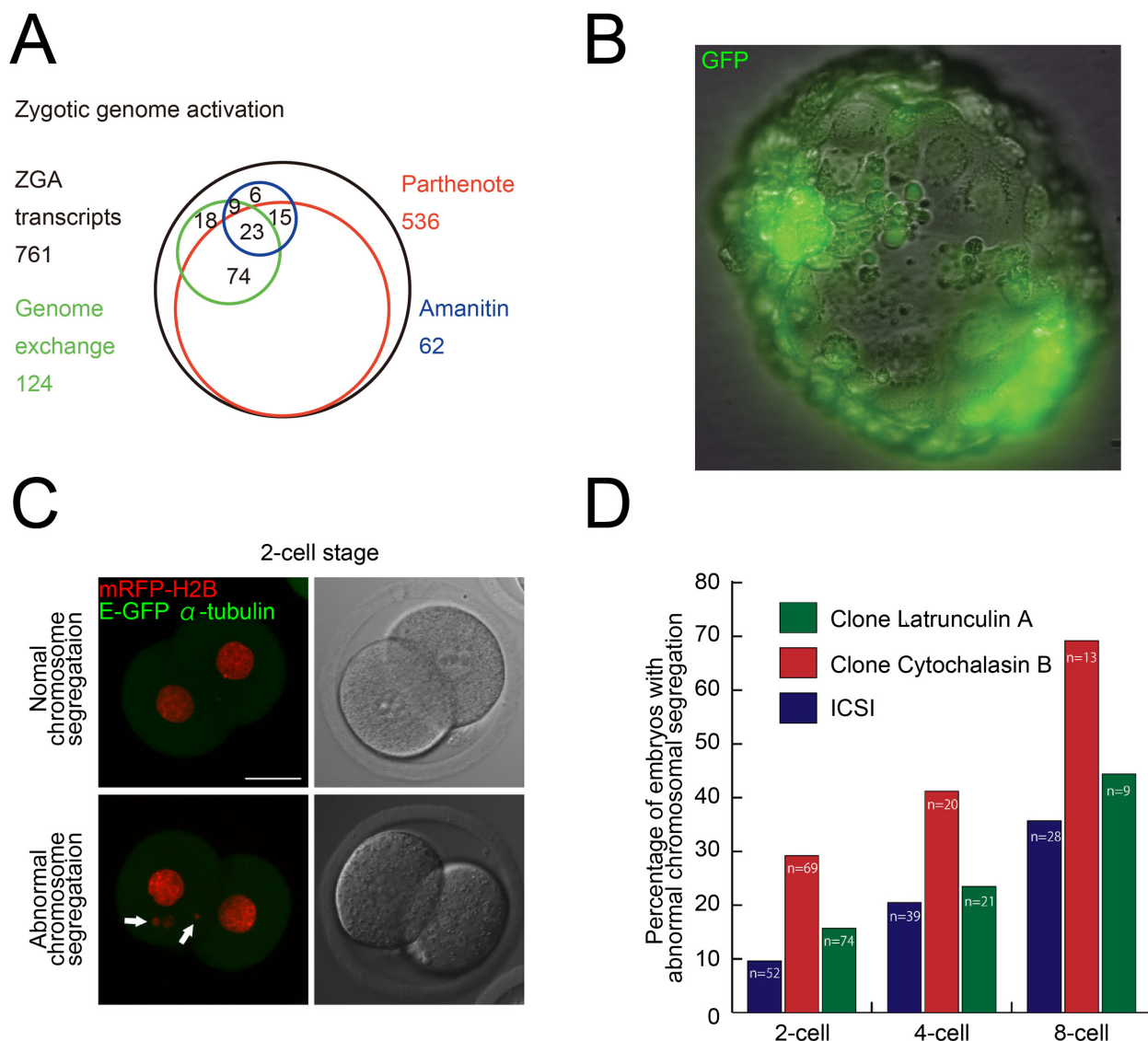


Fig. 2. ZGA and chromosome segregation after SCNT. a) Venn diagram of transcripts elevated in IVF samples on day 3–4 of development (black circle) in comparison with oocytes. The overlaps with parthenotes, amanitin-treated samples and genome exchange samples are shown (from [36]). Amanitin is an RNA polymerase II inhibitor, and can inhibit ZGA. b) Expression of a GFP transgene from the transferred somatic cell genome at the blastocyst stage (modified from [37]). c) Embryos with normal chromosomal segregation and abnormal chromosomal segregation (modified from [55]). d) The percentages of embryos with abnormal chromosome segregation at the 2-cell, 4-cell and 8-cell stages in intracytoplasmic sperm injection (ICSI)-generated and cloned embryos (modified from [55]).

(ii) Histone deacetylase inhibitors enhance the activation of embryo genomes and improve reprogramming efficiency after SCNT

The treatment of embryos with histone deacetylase (HDAC) inhibitors, such as trichostatin A (TSA) [38], valproic acid (VPA) [39] and scriptaid after SCNT has proven effective in the improvement of the reprogramming efficiency in pigs [40], mice [41], and humans [37,

42, 43]. Although the mechanism underlying this treatment is not completely understood [44], the effect of the HDAC inhibitor on ZGA is thought to benefit the normal development of cloned embryos.

Yamada and colleagues showed that embryo culture in the presence of an HDAC inhibitor during the first cell cycle allowed the derivation of stem cell lines from neonatal and adult somatic cells of a woman with type 1 dia-

betes. The resulting stem cells could give rise to all three germ layers, including insulin-producing cells. Whereas previous nuclear transfer protocols did not result in expression of a green fluorescent protein (GFP) transgene contained in the somatic cell genome [36], 58% (14 of 24) of the nuclear transfer cells treated with an HDAC inhibitor were GFP positive, and had a global gene expression profile similar to IVF embryos, demonstrating that transcriptional reprogramming was extensive [37] (for review, see [45]). Furthermore, Chung and colleagues used a protocol including TSA treatment similar to the one developed by Tachibana [42] to derive stem cells from adult somatic cells, including from a 35- and a 75 year old [43].

(iii) Other components improving reprogramming efficiency after SCNT

So far several compounds have been reported to improve reprogramming efficiency after SCNT by demethylating reprogramming resistant regions (RRR), changing energy substrates, reducing reactive oxygen species (ROS), relaxing chromosome complex, or inhibiting the cytoskeleton.

RRRs are enriched by the repressive histone modification histone H3 lysine 9 trimethylation (H3K9me3). H3K9me3 is reported to be an “epigenetic barrier” preventing transcriptional reprogramming by SCNT, leading to failure of ZGA and mouse preimplantation development [46]. This epigenetic barrier can be removed by injecting *Kdm4d* mRNA, an H3K9me3 demethylase. Removal of H3K9me3 facilitates ZGA and consequently enables more mouse SCNT embryos to reach the blastocyst stage (87% with *Kdm4d* injection, 26% without *Kdm4d* injection), leading to an increased rate of mouse ntESCs establishment (50% per MII oocytes with *Kdm4d* injection, 10% without *Kdm4d* injection) [46]. From these findings, Chung and colleagues applied an KDM4A mRNA injection protocol to human SCNT, and succeeded in generating human ntESCs from two adult patients with age-related macular degeneration (AMD) who had donated somatic nuclei (52- and 59- years old) [47].

Vitamin C, a micronutrient known for its anti-scurvy activity in humans, acts as an essential nutrient and antioxidant for many animal species [48]. Vitamin C promotes the efficiency of generating iPSCs through the activity of histone demethylating dioxygenases [49, 50], reducing p53 levels and attenuating cell senescence while maintaining intact DNA repair machinery. In this context, vitamin C is expected to be safe for reprogramming not only of iPSCs but also of SCNT embryos.

Histone acetylation, which loosens the compact chromatin structure and makes DNA more accessible to

regulatory proteins, can play a role not only in regulating gene expression, but also in DNA replication, repair and heterochromatin formation [51]. Histone H4 is one of the 5 main histone proteins and is involved in chromatin packaging, and lysine residue K5 follows its own specific pattern of acetylation during preimplantation development according to the rabbit study [52]. In a porcine SCNT experiment, the blastocyst development rate of SCNT embryos treated with 50 $\mu\text{g/ml}$ vitamin C 15 h after activation was significantly higher (36%) than that of untreated SCNT embryos (11%) [53]. This enhanced *in vitro* development rate of SCNT embryos was associated with an increased acetylation level of histone H4 lysine 5, which resulted in elevated *Oct4*, *Sox2* and *Klf4* expression levels in porcine blastocysts. Therefore, the acetylation of histone H4 lysine 5 by vitamin C treatment may be associated with more efficient genome activation during reprogramming.

Non-epigenetic factors also play an important role in determining the efficiency of cloning. Abnormal chromosome segregation is another major cause of the developmental failure of cloned embryos (Fig. 2C). Latrunculin A, an actin polymerization inhibitor, decreased chromosome segregation errors, and improved the birth rate of cloned embryos (Fig. 2D) [54, 55]. However, cloned embryo-specific epigenetic abnormalities such as dimethylation of H3K9me2 were not prevented by latrunculin A. Even when H3K9me2 was normalized using the G9a histone methyltransferase inhibitor, BIX-01294, the cloning success rate was not improved, implying that cloning failure occurs not only because of epigenetic abnormalities. In addition, the combined use of latrunculin A and 100 μM vitamin C for at least 16 h post-activation significantly increased mouse blastocyst formation and also development to term [56]. Glutamine and hypotaurine added in the culture medium improved intracellular oxidative status and *in vitro* development [57]. These components can be useful not only for SCNT embryo development but also for ART by inducing ZGA or preventing chromosomal abnormalities.

Toward Chemically Defined Culture Media

These results suggest that the optimization of preimplantation embryo culture media ameliorates ZGA quality and the cellular reprogramming efficiency, maximizing embryonic development. Further understanding of culture conditions obtained through metabolomic, transcriptomic and methylation analyses should lead to development of chemically defined culture media, which would exclude unknown nutrients, prevent batch-to-batch vari-

ability in composition and contamination risks, and improve embryo development and development after birth. These efforts are certain to contribute to ART and the generation of personalized stem cells with therapeutic potential.

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