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Erratum

Retinoic acid induces differentiation of buffalo (*Bubalus bubalis*) embryonic stem cells into germ cells

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ABSTRACT

Development of precise and reproducible culture system for *in vitro* differentiation of embryonic stem (ES) cells into germ cells counts as a major leap forward for understanding not only the remarkable process of gametogenesis, otherwise obscured by limited availability of precursor primordial germ cells (PGCs), but in finally treating the catastrophic infertility. Taking into account the significant role of retinoic acid (RA) during *in vivo* gametogenesis, we designed the present study to investigate the effects of its stimulation on directing the differentiation of ES cells into germ cells. The effects of RA were analyzed across dose-and-time upon various stages of gametogenesis like PGC induction, meiosis initiation and completion, haploid cell formation and development of the final gamete (oocyte and spermatozoa). Out of the series of RA doses (2, 4, 8, 16, 20 and 30 μM), 16 μM RA for 8 day culture interval was found to induce highest expression of PGC- and meiosis-associated genes like *DAZL*, *VASA*, *SYCP*3, *MLH*1, *TNP*1/2 and *PRM*2, while mature germ cell genes like *BOULE* and *TEKT*1 (Spermatocyte markers), *GDF*9 and *ZP*2 (Oocyte markers) showed higher expression at 2 μM RA dose, suggesting functional concentration-gradient of RA activity. Immunocytochemistry revealed expression of germ lineage-specific markers like: c-KIT, DAZL and VASA (PGC-markers); SYCP3, MLH1 and PROTAMINE1 (Meiotic-markers); ACROSIN and HAPRIN (Spermatocyte-markers); and GDF9 and ZP4 (Oocyte-markers) in optimally differentiated embryoid bodies (EBs) and adherent cultures. We observed significantly reduced ($p < 0.05$) concentration of 5-methyl-2-deoxycytidine in RA-differentiated EBs which is suggestive of the occurrence of methylation erasure. FACS analysis of optimally differentiated cultures detected 3.07% haploid cell population, indicating completion of meiosis. Oocyte-like structures (OLS) were obtained in adherent differentiated cultures. They had a big nucleus and a zona pellucida (ZP4) coat. They showed progression through 2-cell, 4-cell, 8-cell, morula and blastocyst-like structures upon extended culture beyond 14 days.

1. Introduction

The origin of germ cells (sperm and oocytes) can be traced back to primordial germ cells (PGCs) which undergo a long and eventful journey till their differentiation into functional gametes. To fulfill their role as carriers of genetic information from generation to generation, germ cells undergo a unique process of meiosis to reduce their genetic material to half. The origin, properties and unique capabilities of germ cells are a special focus to reproductive biologists interested in *in vitro* generation of germ cells (artificial gametes). To elucidate molecular mechanisms and signaling pathways of germ cell formation, the ideal starting material would be the progenitor cells of germ lineage. The study of these progenitor cells, known as PGCs, is rather difficult owing to their limited number, embedded nature within the embryo and paucity of the primary tissue, first trimester fetal gonads (Aflatoonian et al., 2009). In view of these limitations, embryonic stem cells could provide a direct system for experimental examination of the landmark events and genetic requirements in germ-cell formation, epigenetic reprogramming, meiosis and post-meiotic progression to gametogenesis (Kee et al., 2009). A number of studies have been conducted to screen the potential inducers of PGC formation from ES cells; most of them identifying bone morphogenetic protein (BMP) family members like BMP4, BMP2, BMP8b (Panula et al., 2011; Aflatoonian et al., 2009; Wei et al., 2008), retinoic acid (Cai et al., 2013; Zhu et al., 2012;

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Abbreviations: IVF, *In vitro* fertilization; ESC, Embryonic stem cells; AFP, Alpha fetoprotein; BMP4, Bone morphogenetic protein4; EB, Embryoid body; HNF4, Hepatocyte Nuclear Factor 4; GATA4, Global Transcription Factor Alpha 4; MSX1, Msh Homeobox 1; NF-68, Neurofilament Light peptide 68; RA, Retinoic acid; 5mC, 5-methylcytosine; RAR, Retinoic acid receptors

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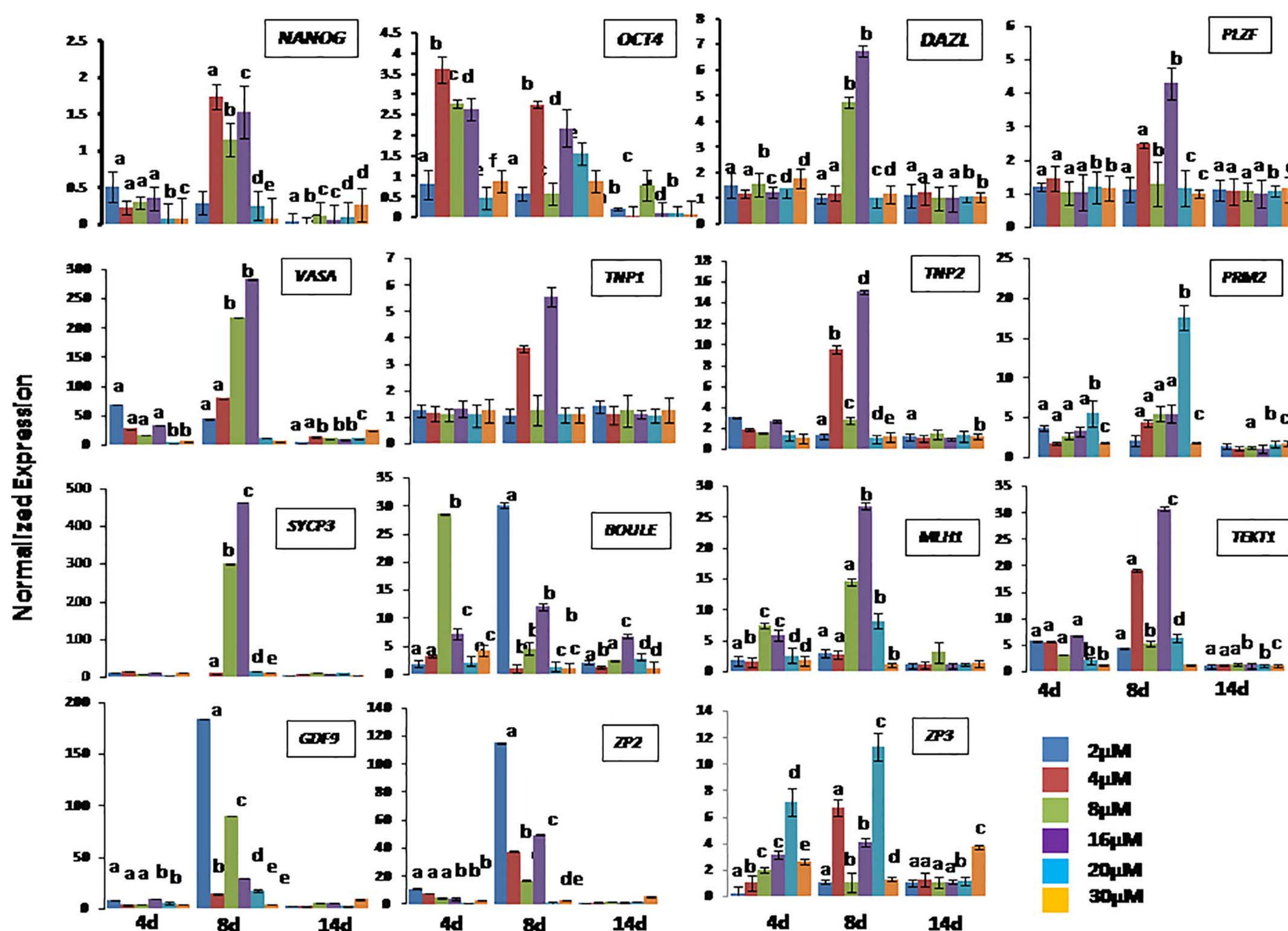

Fig. 1. qPCR analysis of normalized expression of key genes involved in ES cell differentiation to germ cell lineage upon RA induction over 14 days of culture period. Bars represent $2^{-\Delta\Delta cT}$ values ± S.E. of mean, and are calibrated against the corresponding normalized values of undifferentiated ES cell colonies. Bars with different superscripts differ significantly ($p < 0.05$).

Aflatoonian et al., 2009), testicular cell conditioned media (Lacham-Kaplan et al., 2006), cumulus cell conditioned media (Shah et al., 2015b) and other growth factors like bFGF, KIT-ligand and Wnt proteins *etc.* (Park et al., 2009; Wei et al., 2008; West et al., 2008; Tilgner et al., 2008). Most of these studies were carried in murine ES cells, and hence, warrant a caution in their extrapolation to human and higher mammalian species, because inferences from murine studies may not always translate to advances in higher mammalian germ cell development. This highlights the need to investigate germ cell signaling factors and their role in PGC specification, meiosis and mature germ cell formation either directly in the species of interest or in an ontogenetically closer one. On the basis of some preliminary reports that retinoic acid stimulation induces ES cell differentiation to germ cell lineage; we designed this study to investigate the effects of all-trans retinoic acid (RA), in different dose- and time- combinations, on ES cell differentiation to germ cell lineage. RA has been demonstrated to be produced in both male and female mesonephroi and has been implicated in *in vivo* gametogenesis (Bowles et al., 2006). A source/sink system of RA has also been demonstrated in developing gonads which directs meiotic progression or inhibition, depending upon sex of the embryo (McCaffery et al., 1999; Romand et al., 2006). We employed the two most commonly used strategies: i) differentiation in floating cultures *via* embryoid body (EB) formation; and ii) differentiation in monolayer adherent cultures in absence of feeder cells. We initially adopted EB differentiation protocol based on the understanding that 3D culture would more closely resemble to *in situ* conditions than the monolayer cultures (Aflatoonian et al., 2009). The differentiation was assayed for protein markers and gene expression profile consistent with germ lineage cells. For example, immunocytochemical analysis for PGC-specific, meiosis-specific, spermatocyte-specific as well as oocyte-specific markers was performed in both EB and monolayer adherent cultures, differentiated at the optimum dose- and time-period. Methylation erasure was assayed by Global DNA methylation analysis for quantification of 5-methyl-2-deoxycytidine at optimum EB differentiation conditions. The monolayer differentiation cultures were subjected to FACS analysis for determination of haploid cell population. This study is, as per our knowledge, the first of its kind in farm animals, especially bubaline species. It would provide for understanding genetics, epigenetics and biochemistry of gametogenesis, especially of the bubaline species. In addition to its future applications in transgenesis, elite animal conservation and propagation as well as in development of designer gametes, it would provide a higher mammalian model for understanding human gametogenesis *in vitro*.

2. Materials and methods

2.1. Chemicals

Chemicals and plastic ware were purchased from Sigma Aldrich (St. Louis, MO) and Falcon (Paignton, UK), respectively, unless stated otherwise.

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