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In vitro Optimization of White Yak (*Bos grunniens*) Oviduct Epithelial Cells Culture

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Abstract: Epithelial cells of the mammalian oviduct play an important role in reproductive and developmental events. Therefore, the objectives of the present study was to establish a method for isolation and culture of White yak (*Bos grunniens*) oviduct epithelial cells *in vitro* and to determine the effect of Fetal Calf Serum (FCS), Epidermal Growth Factor (EGF) and Transforming Growth Factor-β_1 (TGF-β_1) and their combination on White yak oviduct epithelial cells. The results showed and 100 ng mL^{-1} of EGF in TCM 199 with 10% FCS could significantly increased cell numbers of yak oviduct epithelial. Alone no addition, EGF (10 ng mL^{-1}) and TGF-β_1 (1 ng mL^{-1}) had no effect on cell number. However, when included with 10% FCS in medium yak oviduct epithelial cell numbers significantly increased on day 6 of culture. The combination of FCS (10%) and EGF (10 ng mL^{-1}) is the ideal formula to culture yak oviduct epithelial cells. Co-culture with yak oviduct epithelial cells the developmental rate of blastocyst was 8.9%. The results support the culture environment that oviduct epithelial cell may have direct effects on yak embryo developmental block *in vitro* production.

Key words: White yak, oviduct epithelial cell, embryo, culture *in vitro*, China

INTRODUCTION

Yaks (*Bos grunniens*) are regarded as one of the world's most interesting domestic animals since they not only thrive in conditions of extreme harshness and deprivation but also provide respectable amounts of meat, milk, wool and draft power for people (Guo *et al*., 2012). About 15 million or >90% of the world's total yak population are currently herded in Chinese territories which are the major source of livelihood for the nomadic Tibetans in the highland plains (Ding *et al*., 2010). The White yak is the rare local breeding and found in Tianzhu County of Gansu Province in China which is located in the Eastern end of the Qilian mountains and the Northern edge of the Qinghai-Tibetan Plateau (102°02'-103°29'E; 36°29'-37°41'N). Because the White yak hair is easily dyed into different colours, it has been highly valued in local markets. Currently, there are about 4000 purebred of the White yak individuals. Furthermore, the yak suffers from certain inherent reproductive problems, such as late maturity, seasonality of oestrus, long post partum calving intervals and low reproduction which limits its reproductive efficiency (Sarkar *et al*., 2008a, b). On account of this, the White yak is thin and rare. Hence, it is a focus to develop and utilize genetic resources of White yak.

In vitro Production (IVP) is a well-established embryonic biotechnology with a variety of applications in basic and applied sciences. IVP has been made great progress in cattle which is becoming one of the most exciting and progressive procedures available for today's producers but the efficiency of yak IVP is still low (Zi *et al*., 2008; Guo *et al*., 2012). There are still many problems need to be solved or improved, such as developmental block, unknown factors affecting embryo developmental potential and so on. The purpose of this was to overcome early embryo development block *in vitro* fertilization to establish co-culture system in favor of preimplantation embryo development *in vitro* for conservation genetic resources of White yak, through dissociation and culture of oviduct epithelial cells and analysis influence factors, such as Fetal Calf Serum (FCS), Epidermal Growth Factor (EGF), Transforming Growth Factor-β_1 (TGF-β_1) and their combination.

MATERIALS AND METHODS

Sample collection and treatment: The ovaries and oviducts of White yak were collected from yaks at a local slaughter house and brought to the laboratory in physiological saline at 32-38°C within 3 h during October

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to December. The Cumulus-Oocyte Complexes (COCs) were collected from follicles of 2-8 mm in diameter with an 18-gauge needle attached to a 10 mL disposable syringe. The 2-cell embryos were produced by IVF (Guo *et al*., 2012). Yak Oviduct Epithelial Cells (YOEC) were isolated by opening the oviduct longitudinally and scraping the mucosal epithelial layer with a sterile glass slide and were further processed as described by Reischl *et al*. (1999).

Isolation and culture of yak oviduct epithelial cell: Epithelial cells were collected in 2.5 mL Hepes-buffered TCM 199 (Gibco, Cat.31100-035, USA) with 10% FCS (HyClone) and were poured from three oviducts before being washed twice by centrifugation at 200 g for 5 min each. The cell pellet was incubated in 2 mL 0.25% (w/v) trypsin -0.02% (v/v) EDTA (Ethylene Diamine Tetraacetic Acid) solution (Gibco, Lot.939422, Canada) for 8 min at 38.5°C. Finally, the cells were washed in TCM 199 with 10% FCS, centrifuged at 170 g for 5 min and counted before plating (Rief *et al*., 2002). Cells (5×10^3 mL^{-1}) were inoculated into 4-well culture plates. Cell were incubated for 8-10 days at 38.5°C in a humidified atmosphere of 5% CO_2. The growth medium was changed every 48 h until the cultures became a growing monolayer.

Experiment 1, 5 different concentration EGF (0, 0.1, 1, 10 and 100 ng mL^{-1}) (Sigma) on the proliferation of oviduct epithelial cells were designed in TCM 199 with Earle's salts and L-glutamine supplemented with 10% FCS.

Experiment 2, 7 different factor combination (no addition, FCS, EGF, TGF-β_1 (Sigma), FCS+EGF, FCS+TGF-β_1, FCS+EGF+TGF-β_1) on the proliferation of oviduct epithelial cells were defined in TCM 199 with Earle's salts and L-glutamine supplemented. Concentrations of FCS (10%), EGF (10 ng mL^{-1}) and TGF-β_1 (1 ng mL^{-1}) were tested.

Experiment 3, collection yak cleavage cells (2-cell), randomly divided into two groups, one group co-cultured with yak oviduct epithelial cells and the other had no addition in culture medium. The medium were all TCM199 with 10% FCS and 10 ng mL^{-1} EGF. All of the culture media used were supplemented with antibiotics (100 iu penicillin mL^{-1} and 100 mg streptomycin mL^{-1}).

Morphological analysis and statistics: The growth mode and morphological character of yak oviduct epithelial cells were evaluated by visual examination under phase-contrast microscopy. Values of cell number and embryo development rate are expressed as the Mean±SE of triplicate cultures from multiple trials. Data were analyzed by one-way ANOVA analysis of variance, using the statistical program SPSS.

RESULTS

Characterization and morphology of cultured yak oviduct epithelial cells: Yak oviduct epithelial cells exhibited multilateral morphology and cluster. Phase contrast microscopy studies of the cultured cell showed epithelial characteristics including microvilli in the surface of the cells and tight junction between neighbouring cells (Fig. 1). Yak oviduct epithelial cell monolayer was formed at the 6th day after primary culture (Fig. 2). At the 10th day, epithelial cell density increased, arrangement overlapped and kytoplasm vacuolus appeared (Fig. 3). These cells in primary culture showed a typical epithelial morphology with highly packed polygonal cells. Ciliated cells were recognized by an active movement of cilia for the first several days of primary culture but these cells disappeared in the secondary culture.

Effect of epidermal growth factor on the proliferation of oviduct epithelial cells: To determine the effect of EGF on yak oviduct epithelial cell proliferation, cells were

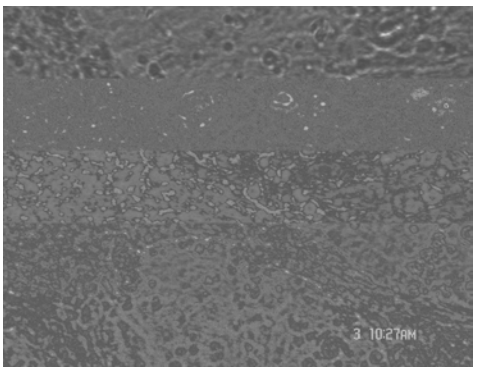

Fig. 1: Yak oviduct epithelial cells are ciliated and aggregate (250x)

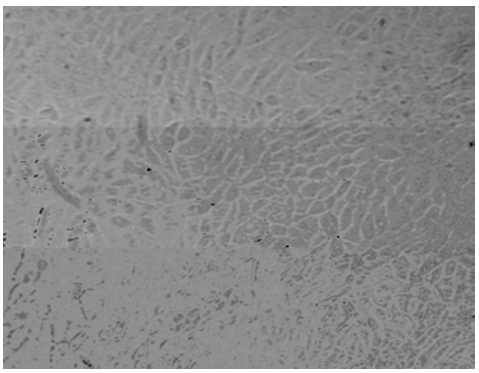

Fig. 2: Yak oviduct epithelial cells formed monolayer at the 6th day after primary culture (250x)

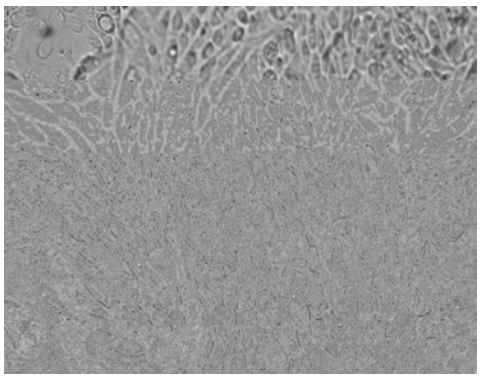

Fig. 3: Yak oviduct epithelial cell cultured at the 10th day after primary culture (250x)

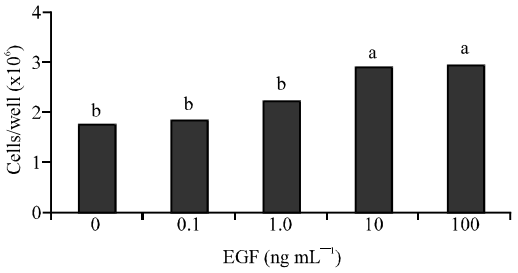

Fig. 4: Effect of epidermal growth factor on the proliferation of yak oviduct epithelial cells; values with different superscripts are significantly different ($p<0.05$)

obtained from oviduct, cultured for 48 h in 10% FCS and then cultured with EGF. Cultures were maintained for 6 day and medium was collected at day 6. Cell numbers were counted at day 6. The dose of 10 and 100 ng mL^{-1} EGF caused significant increases in cell numbers, compared with 0, 0.1 and 1 ng mL^{-1} EGF, respectively (Fig. 4). Cell numbers of added 100 ng mL^{-1} EGF is higher than the one of 10 ng mL^{-1} EGF but there is no significantly between 10 and 100 ng mL^{-1}.

Effect of fetal calf serum, epidermal growth factor, transforming growth factor-β_1 and their combinations on the proliferation of oviduct epithelial cells: Concentrations of FCS (10%), EGF (10 ng mL^{-1}) and TGF-β_1 (1 ng mL^{-1}) were tested. Cell numbers of FCS and EGF group is the highest than other groups and there were significant difference, respectively. Except for FCS and EGF, FCS is the higher than other groups and there were also significant difference, respectively. Added TGF-β_1 on the proliferate of yak oviduct epithelia cells is no significant difference. No addition any component in medium, oviduct epithelial cells could be normal growth and proliferated (Fig. 5).

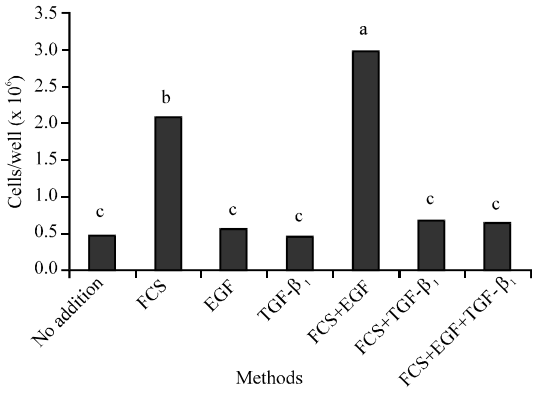

Fig. 5: Effect of FCS, EGF, TGF-β_1 and their combinations on the proliferation of oviduct epithelial cells; values with different superscripts are significantly different ($p<0.05$)

Table 1: Effects of co-culture on yak early embryo *in vitro* development

Culture system	No. of cleavage (2-cell)	(Mean±SE) of total (2-cell embryo) examined (%) Morulae	Blastocysts
Co-culture with yak oviduct epithelial cells	212	28.1±1.9[a]	8.9±0.8[a]
TCM199	206	0[b]	0[b]

Values with different superscripts are highly significantly different ($p<0.01$)

Effects of co-culture system on *in vitro* development of yak blastocyst: Development into blastocyst was significantly ($p<0.01$) affected by the addition of yak oviduct epithelial cells compared with control, the result in Table 1. At 5-6 and 7-8 days after IVF development to morulae and blastocysts were 28.1 and 8.9% in co-culture system with yak oviduct epithelial cells, respectively.

DISCUSSION

The oviduct is an active secretory part of the female reproductive system and provides suitable microenvironment in oviductal lumen for oocyte maturation, sperm capacitation, fertilization, early embryonic development and transport (Das *et al*., 2012). The oviduct of mammals is a highly specialized structure and it assumes one of the most fundamental roles in the reproductive process. The mammalian oviduct functions as more than a simple conduit for the transport of ovulated ova, spermatozoa or developing embryos between ovary and uterus. The functional oviduct is an active organ that maintains and modulates a dynamic fluid-filled milieu. Tubal (Oviductal) fluid provides a suitable environment for sperm capacitation, fertilization and early embryonic development. Oviductal epithelial cell is a kind of important physiological function of the adult

cells because of its in embryonic development process play an important role which has attracted the attention of the researchers. At present, it has obtained many kinds of oviductal epithelial cell lines, such as human (Ling *et al*., 2005), mouse, rat, pig, cattle and so on (Yaniz *et al*., 2006) but it has not reported in yak research.

Success in epithelial cell cultures is related to the presence of homogenous populations of epithelial cells and their proliferation potential (Drewa *et al*., 2009). No selective media are commercially available for Bovine Oviduct Epithelial Cell (BOEC) culture system. In literature, many different optional media are suggested to raise the oviduct epithelial cells (Gao *et al*., 2005; Ebers *et al*., 2009). However, researchers chose a common cell culture medium, TCM 199 which is typically used for culturing oocyte and embryo in cattle, supplemented with different growth factors and serum. The cells treated this way seem to require about 6 days more to completely adhere to the bottom of the culture dish and to form clearly visible and proliferating colonies. The growth character of oviduct epithelial cells in yak was polygonal in shape and the growth mode was monolayer cluster. About 144-192 h after culture, the monolayer was formed. There are many factors effect development of oviductal epithelial cell. EGF and TGF-β_1 are two important members of growth factors, belonging to EGF family and TGF-β superfamily, respectively. EGF which was initially identified as a potent mitogenic peptide (Groenen *et al*., 1994) has various non-mitogenic activities including regulation of cellular migration, differentiation proliferation and maintenance of karyocyte both in the developing and mature system. EGF has been considered a potential regulator of meiotic and cytoplasmic maturation in mammalian oocytes but inconsistencies exist between earlier studies, probably due to differences in the culture conditions used (Uhm *et al*., 2010).

Transforming Growth Factor Beta's (TGF-βs) are known as multifunctional growth factors which participate in the regulation of key events of development, disease and tissue repair. Transforming Growth Factor-Beta 1 (TGF-β_1) an isoform of TGF-βs is an important chemical component among the most widespread and versatile cytokines (Bottner *et al*., 2000). It functions in the regulation of key events, such as cell-cycle control, regulation of early development and differentiation, neuronal survival and orchestration of repair processes in the nervous system (Gomes *et al*., 2005). In the study, FCS, EGF, TGF-β_1 and their combination were studied. FCS was superior when oviduct epithelial cells were present, perhaps because it has factors which promote the development of the cells. In the study, EGF could significantly increase the quantity of YOEC but TGF-β_1 did not increase. The study showed TGF-β_1 did not play a role in yak oviduct epithelial cells culture *in vitro*.

Studies in sheep (Rexroad and Powell, 1988), bovine (Eyestone and First, 1989) and pigs (White *et al*., 1989) have shown that co-culture of oviductal epithelial cells increases embryonic development. Oviductal epithelial cells of several mammalian species have been isolated and cultured *in vitro* (Takeuchi *et al*., 1991). They have suggested that some of the oviduct specific factors play important roles in normal embryonic development. In the bovine species, oviductal epithelial cell cultures have been reported by several investigators (Hoshi *et al*., 1992). BOEC co-culture enhanced bovine embryos developmental rates and/or pregnancy rates (Ellington *et al*., 1990). These cells have been popularly used as culture support for embryonic development either in co-culture systems or for conditioning of embryo culture media. Co-culture systems using oviductal epithelial cell monolayers have been shown to alleviate developmental blocks in the embryo and increase the rate of blastocyst formation during extended culture periods *in vitro* (Thibodeaux and Godke, 1992). The co-culture system with BOEC could be a useful tool to investigate the mechanisms of reproductive events that occur in the oviduct.

CONCLUSION

The present study shows that co-culture with oviduct epithelial cell in the IVC media improve the blastocyst development rate in yak. Oviduct epithelial cells could be used to co-culture system in early embryo development in yak.

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