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A comparative study on efficiency of adult fibroblast, putative embryonic stem cell and lymphocyte as donor cells for production of handmade cloned embryos in goat and characterization of putative ntES cells obtained from these embryos

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Abstract

The main purpose of the experiment was to compare the efficiency of three cell types, namely adult fibroblast, putative embryonic stem (ES) cell, and lymphocyte, as donor cells for somatic cell nuclear transfer by handmade cloning in goats. The outcome clearly shows that putative embryonic stem cells, with a cleavage and blastocyst production rate of 74.69% ± 3.92 and 39.75% ± 3.86, respectively, performs better in comparison to adult fibroblast cell and lymphocyte. Between adult fibroblast cell and lymphocyte no statistically significant difference exists at $P < 0.05$. An overall cleavage and blastocyst formation rate of 67.41% ± 3.92 and 26.96% ± 3.86 was obtained using adult fibroblast donor cells. The study establishes beyond doubt the reprogrammability of lymphocyte by handmade cloning (HMC) protocol with a cleavage and blastocyst production rate of 56.47% ± 3.92 and 24.70% ± 3.86, respectively. PCR analysis of highly polymorphic 286 bp fragment of MHC II DRB genes of cloned embryos and three donor cells were performed to verify the cloned embryos. The amplified PCR products were subjected to SSCP to confirm their genetic identity. The karyotyping of the cloned embryos showed normal chromosomal status as expected in goat. Significantly, in the second stage of the experiment, the produced cloned embryos were successfully used to derive ntES-like cells. The rate of primary colony formation rate was 62.50% ± 4.62 for fibroblast donor cell derived embryos. The same was 60.60% ± 4.62 for putative ES donor cell derived embryos and 66.66% ± 4.62 for lymphocyte donor cell derived embryos, respectively. The putative ntES colonies were positively characterized for alkaline phosphatase, Oct-4, TRA-1-60, TRA-1-81, Sox-2, and Nanog by Immunocytochemistry and Reverse Transcription PCR. To further validate the stem ness, the produced putative ntES colonies were differentiated to embryoid bodies. Immunocytochemistry revealed that embryoid bodies expressed NESTIN specific for ectodermal lineage; GATA-4 for endodermal lineage and smooth muscle actin-I, and troponin-I specific for mesodermal lineage. The study has established an efficient protocol for putative ntES cell derivation from HMC embryos. It could be of substantial significance as patient specific ntES cells have proven therapeutic significance.

Keywords: Blastocyst; Embryoid bodies; Handmade cloning; Nanog; ntES cell; Oct4

1. Introduction

Somatic Cell Nuclear Transfer (SCNT) by handmade cloning (HMC) technique in goats is very important for biomedical sciences on two accounts. First, handmade cloning is a relatively simple and inexpen-

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sive technique and second, dairy goats are ideal for production of diversified products of commercial value in milk when they are used as transgenic animals for the production of many recombinant therapeutic proteins for treatment of diseases. In fact, the first drug, called ATryn, is a human antithrombin protein, approved for human use and derived from a genetically engineered animal, was obtained from a goat. The goat is also a convenient domestic species for biological investigation and application because it has a relatively short gestation period (5 mo, vs. 9 mo for a cow), multiparous, reared in flock, and easy to maintain.

The developmental potential of adult somatic nuclei after nuclear transfer (NT) into enucleated, *in vitro*-matured oocytes has been demonstrated by using mammary gland cells [1], cumulus, and granulosa cells [2,3]. Comparative study on the efficiency of nuclear transfer with skin, kidney, gut, and muscle cells from female bovine fetuses, as well as skin, heart, kidney cells, etc. have shown that fibroblasts are not the only cells that can support development after nuclear transfer [4]. In goat species, successful cloning using adult granulose cells and fetal fibroblast cells has been reported [5]. Successful cloning has also been reported by using handmade cloning technique with cumulus and fetal fibroblast donor cell [6].

Several reports are available where researchers have successfully used embryonic stem (ES) cells and lymphocytes as donor cells. There have been reports describing 10–33% cloning efficiency with ES cells, which is up to 30 times higher than with commonly used somatic donors, such as cumulus cells and fibroblasts [7,8]. Considering the high incidence (20–80%) of non-reprogrammable karyotypic abnormalities in some targeted ES cell lines and sub-clones [9], the actual degree of ES cell reprogrammability was suggested to be even higher and comparable to *in vivo* derived zygotic nuclei [10]. For the first time, it was demonstrated that the genome of a unique T-cell population, natural killer T (NKT) cells, can be fully reprogrammed by a single-step NT and that direct SCNT of terminally differentiated post mitotic granulocytes leads to term development of cloned pups [11]. Successful cloning using terminally differentiated lymphocytes has demonstrated a high level of plasticity in the NKT-cell genome. Thus, differentiation of the genome is not always a barrier to NT cloning for either reproductive or therapeutic purposes [12].

The most exciting prospect of SCNT is therapeutic cloning. Reproductive cloning when used for the production of embryonic stem cells is referred to as therapeutic cloning. Previously, there have been many reports of nuclear transfer Embryonic Stem (ntES) cell lines in mice, humans, and primates. Primate ES cells have been produced successfully by SCNT [13] and there have been other attempts to produce human ES cells by SCNT [14–18]. Another report also described successful production of human ES cells by SCNT using a cumulus cell from the same oocyte donor as the donor cell [19]. In a successful demonstration of the applicability of the principle of therapeutic cloning a total of 187 ntES cell lines were derived from 24 parkinsonian mice and they were differentiated to dopamine neurons and transplanted into individually matched host mice showing therapeutic efficacy and lack of immunological response [20]. The findings support the notion that ES cell lines derived from cloned or fertilized blastocysts have an identical therapeutic potential.

The idea behind the present study was to compare the efficiency of adult fibroblast, lymphocyte, and embryonic stem cell donor cells for production of handmade cloned embryo and to produce nuclear transfer stem cells (ntES) from embryo. This is apparently the first comparative study between adult fibroblast, ES cell, and lymphocyte as donor cells for handmade cloning in goats. SCNT followed by ntES production is a very useful tool for transgenesis. The present research finding could be of significance for future studies on reproductive and therapeutic cloning for treatment of degenerative diseases.

2. Materials and methods

2.1. Chemicals, cell culture media, and supplements

The different media used in the present study for the culture of oocytes, for *in vitro* cloned embryo production, and for culture of putative ntES cells in various experiments were purchased from Sigma Chemical Co., St. Louis, MO, USA unless otherwise indicated. The media were filtered with 0.2 μm syringe filter (Nalgene) before using.

2.2. Establishment of somatic cell cultures for donor cells

2.2.1. Fibroblast cell culture

Skin biopsies were aseptically taken from the ear pinna of a healthy female goat using all aseptic measures. Norms regarding ethics and humane handling of animals were strictly followed during the whole operation. The tissues were held in sterile DPBS and trans-