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In Vitro Culture of Fibroblast-Like Cells From Sheep Ear Skin Stored at 25-26°C for 10 Days After Animal Death

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Abstract

Successful somatic cell nuclear transfer *aka* cloning requires good quality undamaged nuclear DNA from desired cell types. *In vitro* culture of cells is one way of ensuring nuclear integrity. Cellular contents including nucleus gradually decompose postmortem, if not preserved within a reasonable time, leading to cell and ultimately nuclear DNA damage. The goal of this study was to determine time limits within which live and culturable cells can be obtained, after death of an animal, using sheep as a model. How long the somatic cells are alive and have potential to replicate after the animal death is not precisely known. Here we show, for the first time, that the sheep ear skin stored at 25-26°C after animal death can be cultured up to 10 days postmortem. The culture confluence is inversely correlated with increasing postmortem time interval. The cultured fibroblast-like cells have 95±5.2 % post cryopreservation cell-viability; have normal karyotype, and a comparable growth profile to that of fresh tissue derived cells. This study shows that sheep skin has potential for *in vitro* culture of its cells up to 10 days postmortem. Cultured cells can be successfully used for preservation of biodiversity for possible future cloning of animals.

Keywords: fibroblast, postmortem recovery, skin stem cells, cryopreservation, skin banking, animal cloning

1. Introduction

Cryopreservation of tissues from various breeds/species and/or animals with superior traits, both domesticated and wild relatives, has been suggested to conserve genetic diversity, before it is too late. These preserved genetic resources could be utilized globally, to meet the climatic and/or other challenges in future, to meet the global demand for food, especially protein source. However, obtaining viable and culturable tissues/cells from live animals for cryopreservation presents ethical challenges. Recent studies show effective preservation of postmortem tissues and producing live animals from these tissues by somatic cell nuclear transfer *aka* cloning. However, for success of a cloning experiment, nuclear integrity of donor cells is a key requirement (Hoshino et al., 2009). *In vitro* culture of cells ensures nuclear integrity and enhances the success rate of animal cloning (Mastromonaco et al., 2006). *In vitro* culture of cells from live or dead animal tissues, preserved at sub-zero temperatures, has been discussed in several studies (Palmer et al., 2001; Viel et al., 2001; Wakayama et al., 2008; Hoshino et al., 2009; Erker et al., 2010). The nucleus from these cultured cells from preserved tissues has been used to clone the animals even after many years of their death (Hoshino et al., 2009; Wakayama et al., 2008). However, in all these cases the tissues used to obtain viable cells as a source of nucleus were preserved within few hours of animal death. Delays in preservation of tissues within a reasonable time may compromise *in vitro* culture potential, and thus the cloning efficiency, due to cellular death. How long live and culturable cells can be recovered from postmortem tissues in mammalian species is not precisely known. Hasegawa and his colleagues have shown that 2% neurosphere-forming activity remained in a postmortem rat brain even after 25 h of storage at 25°C (Hasegawa et al., 2009). Neural stem and progenitor cells were recovered from postmortem rat brains which were stored at 4°C for a week (Xu et al., 2003). Cells in postmortem tissues stored at room temperature remain culturable up to 4 days after animal death in goat and sheep (Silvestre et al., 2004) and up to 6 days in goat (Singh et al., 2012) and rabbits and pigs (Silvestre et al., 2003). Do cells remain culturable beyond 6 days after animal death in carcasses lying at room temperature (25-26°C) is not known? Here we show, *in vitro* culture of cells after

10 days of animal death, in sheep skin tissues stored at 25-26°C, and demonstrate that these cell cultures are genetically stable and have similar growth profile as that of fresh tissue derived cells.

2. Material and Methods

2.1 Tissue Sample Collection:

The tissue samples were procured from the University slaughter house. Ears of individual healthy Katahdin sheep (1.0-1.5 years of age) were excised from the animal head and brought to the laboratory within an hour. The ears were cleaned with 70% alcohol swabs, wrapped in clean sterile paper towels, and stored in plastic bags in the laboratory at room temperature (25-26°C).

2.2 Explant Preparation, Primary Cell Cultures, and Data Collection:

The explants were prepared and cultured as described earlier (Singh et al., 2011). In brief, inner side of the ear was cleaned with 70% ethanol swabs, and the skin samples excised aseptically after 0, 2, 4, 6, 8, and 10 days of postmortem storage. Excised samples were chopped into 2-3 mm^2 size pieces (explants) and adhered onto 60 mm diameter dishes (Falcon, B. D. Biosciences, Oxnard, CA, USA). Each dish contained 5 explants placed at a uniform distance. Adhered explants were cultured in P116, a fibroblast culture media (Cell Applications Inc., San Diego, CA) at 37°C, 5% CO_2 in a humidified environment. The media in dishes was changed on Mondays, Wednesdays and Fridays. At the same time the dishes were also observed for any microbial or fungal contamination, explant dislodging, and for outgrowth of cells around explants, under an inverted microscope. In our experience, we never observed outgrowth around explants that dislodged within first 3-4 days of culture and, therefore, such explants were removed from the dish as soon as observed. Explants dislodged after day 4 of culture were observed for any outgrowth at their foot-prints and the results included in final analysis. Presence or absence of the outgrowth of cells around explants (clusters of > 50 cells) was recorded and the level of confluence was compared on day 10-12 of culture for different postmortem time intervals.

2.3 Establishing Secondary Cultures and Cell Storage:

Primary outgrowing cells (80-90% confluence) around the explants were trypsinized and secondary cultures established as described (Singh et al., 2011). Briefly, the cells in dishes were washed twice with 2.0 mL of the balanced salt solution without calcium and magnesium (Gibco@ Life Technologies, Grand Island, NY, USA), and incubated with 2.0 mL of 0.125% trypsin for 5-10 min at 37°C. The trypsinized cells were neutralized with 5 volumes of complete growth media, counted to assess cell-viability using Trypan Blue Dye Exclusion Method (Strober, 2001), and pelleted at 200 X g for 7 min. The pellets were suspended in Synth-a-Freeze® (Life Technologies Corp., Carlsbad, CA) media, aliquoted into cryogenic storage vials (1.0 X 10^5 cells / vial) and frozen at -80°C deep freezer o/n using Nalgene™ Cryo 1°C Freezing Container (Nalgene, Rochester, NY). Next day the vials were transferred to liquid nitrogen tank and stored till used for further experiments. To establish secondary cultures, and to expand cell numbers, the frozen vials were quickly thawed at 37°C, mixed slowly with 9.0 mL of the media, pelleted at 200 X g for 7 min, dissolved in complete growth media, and cultured in appropriate (T25 or T75) culture flasks. To determine post cryopreservation cell-viability, representative vials in triplicate were thawed after 10 months of cryopreservation and viable cells were counted manually using a hemocytometer. Cell-viability was expressed as % of live cells from total cell count.

2.4 Generating Growth Curves:

Growth curves were generated from passage 4 cultures using a 24-well micro titer plate format as described earlier (Singh et al., 2012). Briefly, 20, 000 cells were inoculated in each well in 0.5 mL of growth media to initiate the culture. The wells were trypsinized (in triplicate) after day 1, 3, 5, 7, 9 and 11 of culture. The viable cells were counted in each well using a hemocytometer. Mean and standard deviation of the cells / mL in 3 wells were plotted against time using Excel program to generate growth curves.

2.5 Cytogenetic Analysis:

Sheep cells cultured from 10 days postmortem tissues stored at 25-26°C were processed for cytogenetic analysis, at passage 2 level, using previously established methodologies (Meisner & Johnson, 2008) by Cell Line Genetics (Madison, WI; www.clgenetics.com). The chromosome assignments were made according to the Atlas of Mammalian Cytogenetics (O'Brien, Menninger, & Nash, 2006).

3. Results and Discussion

3.1 In Vitro Culture of Cells After 10 Days of Animal Death:

We studied healthy sheep of Katahdin breed for postmortem survival of their skin cells, using an *in vitro* culture approach. *In vitro* cell culture ensures nuclear integrity, a key requirement of successful animal cloning. Sheep ears

were chosen as a source of skin sample because they are easily accessible. Ear skin stored in the laboratory at 25-26°C postmortem was sliced into small explants and cultured in complete growth media. Forty explants were cultured in 8 dishes of 60 mm diameter (5 explants / dish) after 0, 2, 4, 6, 8, and 10 days of animal death. Outgrowth of fibroblast-like cells around the explants was scored after 10-12 days of culture for each time point. Our results show outgrowth of fibroblast-like cells around all explants (except one) that adhered to dish surface, even from 10 days postmortem tissues (Table 1). To our knowledge this is the first report of *in vitro* culture of postmortem tissues after 10 days of animal death in mammals from their carcass exposed to 25-26°C. Earlier studies have shown outgrowth of fibroblast-like cells up to 4 days of animal death in goats and sheep (Silvestre et al., 2004), six days in goats (Singh et al., 2012), and up to 6 days in rabbits and pigs (Silvestre et al., 2003) when the skin tissue was exposed to around 25°C. It should be noted that in all these earlier studies, outgrowth was observed in all the time points studied, including the last time point, although the number of explants that responded varied. In fact, that inspired us to explore, if the postmortem tissues exposed to room temperature beyond six days, still have live cells that can be cultured *in vitro*. It is presumed that the individual cells in skin tissues die gradually over time. It is perhaps correlated with gradual reduction in live adult stem cells in skin tissues with increasing postmortem time interval. What made these skin cells to survive for such a long time of 10 days postmortem is not clear. It is possible that diffusion of environmental oxygen into skin tissues kept them alive for such a longer period at room temperature (Fife et al., 2009). The time taken to have complete death of all cells in a tissue may depend upon environmental temperature, humidity, age, and health of an animal. Although for normal practical purposes, it is unlikely that the animal will remain in field without notice for such a long time after its death, this study shows that the live cells can be recovered after several days postmortem, and effectively cryopreserved for future use.

Table 1. Outgrowth of fibroblast-like cells around skin explants after different days postmortem

Sheep	Breed / age / weight	Number of outgrowth positive / total explants adhered to dish surface					
		0-dpm	2-dpm	4-dpm	6-dpm	8-dpm	10-dpm
S1	Katahdin / 1.5 year / 120 lb	25/25	20/20	16/16	15/15	17/17	10/10
S2	Katahdin / 1.0 year / 100 lb	15/15	10/10	15/15	10/10	15/15	9/10
Total		40/40	30/30	31/31	25/25	32/32	19/20
% outgrowth		100	100	100	100	100	95

Note: Forty explants were used for each time point. The explants floated within first 3 days of culture (non-adhered explants) and the contaminated dishes during experimentation were removed from the study.

3.2 Comparison of Confluence of Outgrowth Around Tissues Cultured After Different Postmortem Time Intervals:

Confluence in cell biology is defined as the number of cells per unit area in a culture dish or flask. We show in Figure 1 that the confluence level of outgrowing cells is decreasing with increasing postmortem time interval on a given day. For example, the number of cells around skin explants of 0-dpm (first panel in Figure 1) is more than 2-dpm, which intern has more cells than 4-dpm. It is known that oxygen deprivation, due to cessation of blood circulation, progressively leads to decomposition of tissues (Zdravkovic, 2010). This happens due to action of lysosomal enzymes present in the cells which ultimately results in cellular death. It is conceivable that longer the interval from animal death to cell culture, lesser the number of live skin adult stem cells available to produce outgrowth. This could explain the observed reduction in outgrowth with increasing postmortem time interval in this study. Our results are in agreement with similar reduction in cells with extended postmortem time interval in neurosphere cell cultures in adult human retina (Carter et al., 2007), and fibroblast-like cell cultures in goat ear skin (Singh et al., 2012).

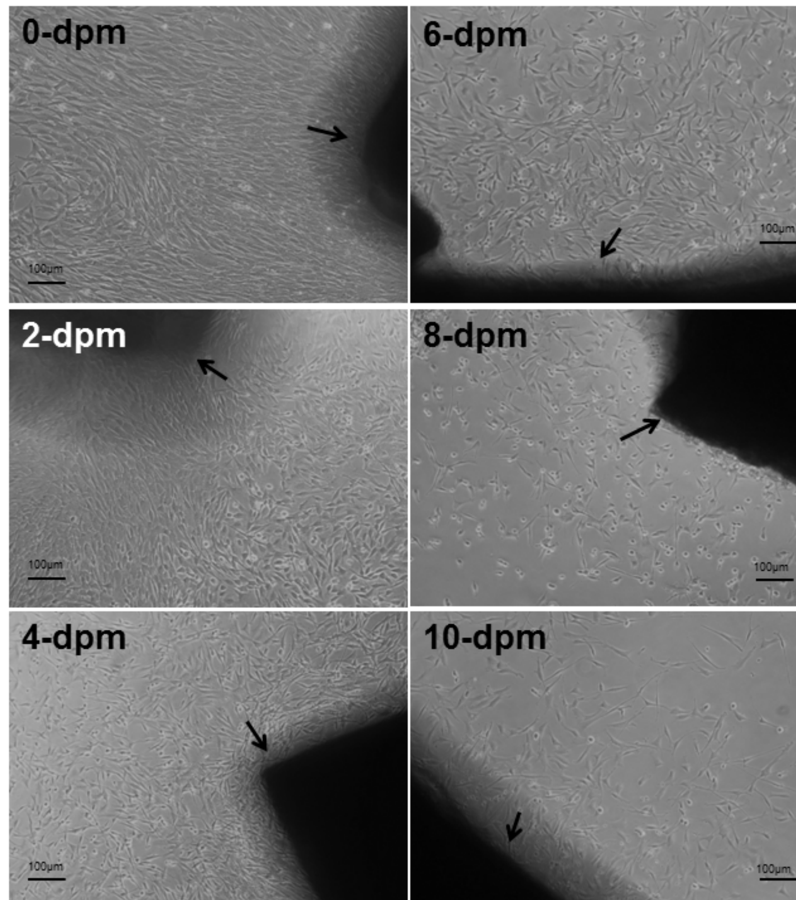

Figure 1. A representation of comparative cell confluence of different days postmortem tissues after a fixed interval of 10-12 days of *in vitro* culture (note that except 6-dpm, rest images here were after 10 days of culture). Arrow marked dark areas are skin explants adhered on dish surface. Nikon Eclipse TS100 inverted microscope was used to capture the images. Dpm = days postmortem

3.3 Establishment of Secondary Cultures, Their Cryopreservation, and Growth Profiles:

Although, the confluence level of primary outgrowth reduces with increasing postmortem time interval, how the cell populations in subsequent passages differ, with respect to their growth profile, is not known. To find out the differences between these cell populations, we established secondary cultures from the primary outgrowth in 0-dpm (control) and 10-dpm tissues, by serial passaging. It was observed that secondary cultures of these cell populations grow much faster as compared to the primary outgrowth. These cultures became 50-60% confluent in 3-4 days (Figure 2; upper panels) as opposed to the primary outgrowth (Figure 1), which took 2-5 times longer in reaching same level of confluence. Faster growth of secondary cultures may be related to their adaptation to the culture environment.

The cells harvested from passage 2 and passage 3 cultures of 0-dpm and 10-dpm tissues were preserved in liquid nitrogen in freezing media. Representative Cryovials, in triplicate, were tested for their post-freezing cell-viability and *in vitro* culture ability after 10 months of cryopreservation. Results show 96.67±1.53 % and 95.00±5.20 % post-freezing cell-viability for 0-dpm and 10-dpm cell cultures, respectively (Figure 2; lower right panel). All tested cryovials showed normal growth in *in vitro* cultures, which was indistinguishable for 0-dpm and 10-dpm cell populations.

To determine the similarities / differences between two cell populations (*i.e.* 0-dpm and 10-dpm), we performed sequential passaging of cells so as to obtain pure fibroblast-like cells (note that some epithelial cell colonies also appear in primary outgrowth which are eliminated by partial trypsinization during sequential passages). Subsequently, these cell populations were compared at passage 4 level for their, a) growth curves, and b) growth morphology. Growth curves were generated using a 24-well micro titer plate format. The results show typical mammalian growth curves, which are similar but not identical, for both 0-dpm and 10-dpm cell populations

(Figure 2; lower left panel). Comparative images of growing cell cultures on day 4 were captured to study their morphology. As shown in Figure 2 (upper panels), images display typical elongated, bipolar fibroblast-like morphology which is indistinguishable for both cell populations. It suggests their similarity, despite of the time lapse between animal death and the cell culture. It would be interesting to see reprogramming of their nuclear DNA by enucleated oocyte cytoplasm, embryo development, and ultimately cloning of sheep that was dead for 10 days.

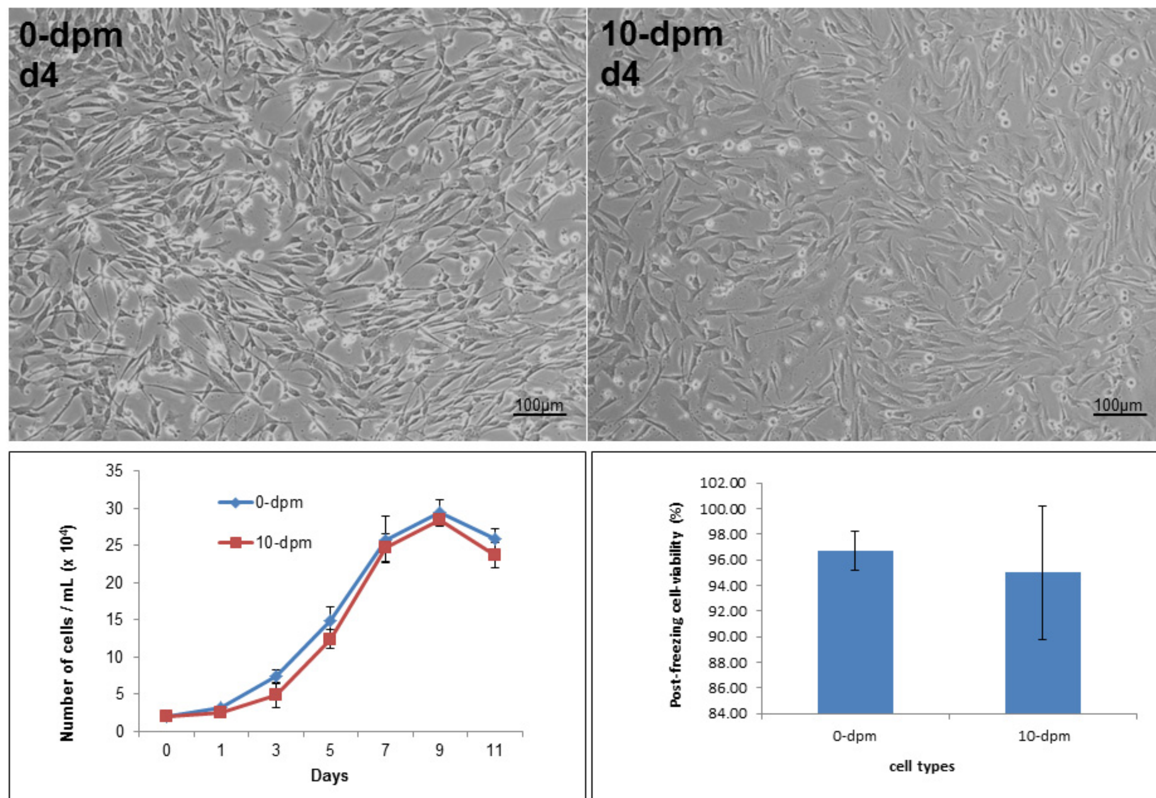

Figure 2. Figure shows comparative cell morphology and confluence of 0-dpm (control) and 10-dpm cell-lines at passage 4 level on day 4 of culture (upper panels); growth curves show mean ± SD of cells in triplicate wells (lower left panel); and post-freezing cell-viability show mean ± SD of 3 experiments (lower right panel)

3.4 Cytogenetic Stability of 10 Days Postmortem Tissue Derived Cell-Line:

To determine chromosomal stability of the cells cultured from 10 days postmortem tissues, a cytogenetic analysis was performed. The diploid (2x) number of the chromosomes in the cell-line was determined to be 54, XY chromosomes. As can be seen in Figure 3 (panel B), it consisted of 52 autosomes and two (X and Y) sex chromosomes, which is consistent with earlier studies on sheep cytogenetics (Ansari et al., 1996; Iannuzzi et al., 2009). Cytogenetic analysis was performed on twenty G-banded metaphase cells of 10-dpm (SSFRT-10DPM, p2) cell-line. Nineteen cells exhibited an apparently normal male karyotype, while one cell showed a non-clonal chromosomal aberration which is most likely an artifact of culture. Thus these results are apparently consistent with a normal male sheep karyotype.

In conclusion, we have shown for the first time that sheep ear skin stored at 25-26°C has *in vitro* culture potential up to 10 days of animal death. The postmortem tissue-derived cells are effectively cryopreserved with > 95% of post-freezing cell-viability, are indistinguishable from normal control cells, with respect to their growth profiles, and are cytogenetically stable. Future studies will determine their potential for reprogramming and animal cloning.

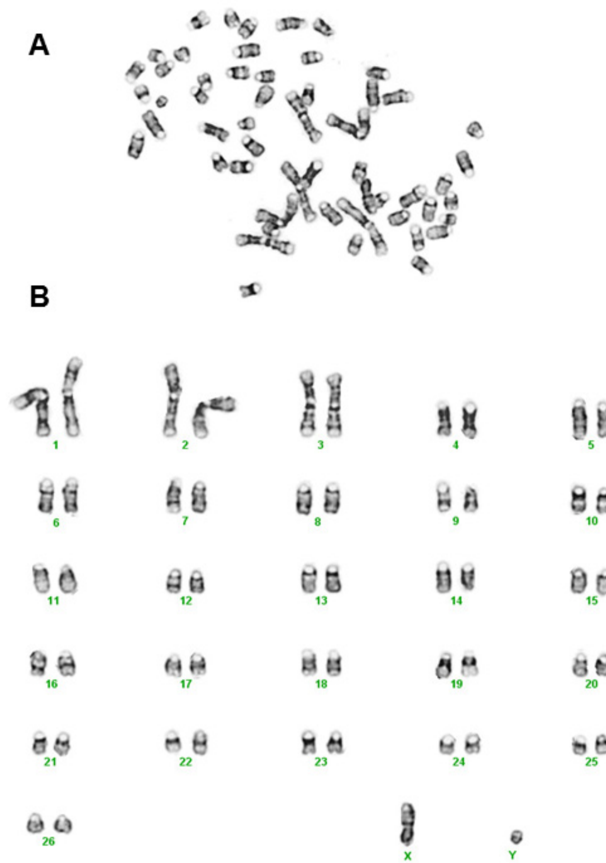

Figure 3. Cytogenetic analysis of SSFRT-10DPM cell-line: Panel "A" show a representative single cell spread, and panel "B" show karyotype

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