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Cloning & Transgenesis, 2015; 4(2):1000136-1-1000136-4

Originally published at:- http://dx.doi.org/10.4172/2168-9849.1000136

Harrison et al., Clon Transgen 2015, 4:2
http://dx.doi.org/10.4172/2168-9849.1000136

Cloning & Transgenesis

Research Article Open Access

Multipotent Cell Types in Primary Fibroblast Cell Lines Used to Clone Pigs using Somatic Cell Nuclear Transfer

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Abstract

We have previously demonstrated that the use of porcine mesenchymal stem cells (MSCs) isolated from the bone marrow can increase the proportion of somatic cell nuclear transfer (SCNT) embryos that develop to the blastocyst stage compared with adult fibroblasts obtained from the same animal. The aim of the present study was to determine if MSCs are also present in primary cultures of adult fibroblasts which are commonly used for cloning live animals. To do this we chose a primary culture of adult fibroblasts that we had previously used to clone pigs. Single cell clones were isolated using low-density plating. After seven days of culture 63% of colonies displayed typical fibroblast morphology, while the remainder appeared cobblestone-like in appearance. Two of the 57 clones that displayed fibroblast morphology differentiated into adipocytes but not chondrocytes or osteocytes (uni-potent clones). Three of the 33 cobblestone-like clones differentiated into chondrocytes only, while 3 differentiated into adipocytes and chondrocytes but not osteocytes (bi-potent clones). One of the bi-potent cobblestone-like clones was then used for SCNT and *in vitro* development compared with a fibroblast-like clone which did not differentiate. Both cell types produced blastocysts at similar rates. In conclusion we have identified uni-potent and bi-potent cell types in primary cultures of adult fibroblasts used previously to clone live piglets.

Keywords: Mesenchymal stem cells; Stem cells; Somatic cell nuclear transfer; Cloning; Pig

Introduction

Current somatic cell nuclear transfer (SCNT) efficiencies for the livestock species are relatively low with only 1-3% development to term and number animals exhibiting abnormalities at birth due to incorrect reprogramming of the donor nucleus in some species [1,2]. We have previously reported the production of cloned pigs using fetal fibroblasts [3], genetically modified (α1,3-galactosyltransferase knockout) fetal fibroblasts [4] and adult fibroblasts isolated from live animals [5]. Based our experience and that of others, new approaches are required to increase these efficiencies, particularly if animal cloning is to be used commercially for breeding purposes. In this regard there is evidence to suggest that a less differentiated cell can increase the efficiency of SCNT compared with terminally differentiated cell types because these are more easily programmed. For example, Hochedlinger et al. [6] suggested that cloned animals are derived from rare somatic stem cells selected by chance from heterogeneous donor cell populations. Furthermore clones reconstructed with embryonic stem cells have been shown to result in an increase in the number of viable offspring compared with those reconstructed with adult cells [7]. We have also reported that the use of porcine mesenchymal stem cells (MSCs) isolated from the bone marrow can increase the proportion of SCNT embryos that develop to the blastocyst stage compared with adult fibroblasts obtained from the same animal (Faast et al.). Subsequent studies by Lee et al. [8] and Li et al. [9] have suggested that blastocyst cell number and development to term is also increased when MSCs are used compared with fibroblasts. MSCs are found in the stromal compartment of the bone marrow and can be differentiated into adipocytes, chondrocytes and osteocytes [10]. They are also present at other sites including the peripheral circulation [1]. The aim of the present study was to determine if MSCs are present in primary cultures of skin fibroblasts which are commonly used for cloning live animals. To do this we chose a primary cell line of adult fibroblasts derived from ear tissue that had previously produced two litters of one and three live born piglets from 6 transfers of between 91 to 146 one cell SCNT embryos.

Materials and Methods

Clonal isolation and differentiation of adult porcine fibroblasts

All procedures were carried out in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes [11] and approved by the University of Adelaide Animal Ethics Committee. All chemicals were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. Skin fibroblasts were obtained from ear punch tissue isolated from adult male pigs. Finely minced ear tissue was incubated in 0.1% trypsin (Invitrogen) at 38.5°C for 1 h, and resuspending in high-glucose DMEM (Invitrogen, Grand Island, NY) supplemented with 40% fetal bovine serum (FBS: JRH Biosciences, Lenexa, KS) and 100 units/mL penicillin G sodium, 100 µg/mL streptomycin sulfate and 0.25 µg/ml amphotericin B (Gibco, Invitrogen Corporation, Grand Island, NY) and cultured at 5% CO_2/2% O_2 at 38.5°C. Following 3 days of culture, medium was replaced with DMEM/20% FBS and antibiotics. After 7 days culture, cells were 70% confluent and were passaged by rinsing twice with PBS for 5 min and incubating with trypsin, 0.05 mg/ml, (Gibco, Invitrogen Corporation, Grand Island, NY) for 5 min at 38.5°C. Cells were subsequently passaged at 5000 cells/cm^2 and frozen stocks prepared.

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Received April 23, 2015; **Accepted** May 26, 2015; **Published** May 29, 2015

Citation: Harrison SJ, Beebe LFS, Vassiliev I, McIlfatrick SM, Nottle MB (2015) Multipotent Cell Types in Primary Fibroblast Cell Lines Used to Clone Pigs using Somatic Cell Nuclear Transfer. Clon Transgen 4: 136. doi:10.4172/2168-9849.1000136

Clonal isolation was achieved by plating cells at low density (1 cell/cm^2) and culturing for 7-10 days. Individual colonies (clones) were isolated and cultured in duplicate wells of 24-well trays to 80% confluence. An aliquot of each clone was frozen down and the remaining cells used for multilineage differentiation studies. Multilineage differentiation potential of selected clones was demonstrated by culturing cells in ambient oxygen and 5% CO_2 under conditions that stimulate osteogenic [12], adipogenic [13] or chondrogenic development (micromass culture [14]) overlayed with chondrogenic induction medium [15]. Histochemical assays were used to detect osteogenic precursors (von Kossa), adipocytes (Oil Red O) and chondrocytes (Alcian blue). Adipocytes were identified morphologically by staining with Oil Red O, whilst culturing in osteogenic medium resulted in the deposition of calcium, detected by von Kossa stain. Chondrogenic induction produced spheres containing cells that stained positive for Alcian blue, signifying the production of cartilage.

Somatic cell nuclear transfer

To determine the SCNT potential of the bi-potent clones identified in experiment one, a second experiment was undertaken to determine whether cells from one bi-potent clone from the cobblestone-like group could increase the rate at which SCNT embryos developed to the blastocyst stage compared with a fibroblast-like clone that did not differentiate. Fibroblasts were seeded concurrently at 1-2 × 10^4 cells/cm^2 into 4 well trays and maintained at confluence for 5-10 days, prior to harvesting for SCNT. The fusion before activation protocol used was essentially as described previously by us [4] with the exception that enucleation was performed using Hoechst 33342. Cumulus oocyte complexes were obtained from adult ovaries and matured in TCM-199 (Invitrogen) supplemented with 5 μg/ml insulin, 10 ng/mL EGF, 0.5 μM cysteamine, 0.2 mM Na-pyruvate, 5 μg/mL FSH, 75 μg/ml penicillin-G, 50 μg/mL streptomycin sulphate and 10% sow follicular fluid in a humidified atmosphere of 5% CO_2 in air. After approximately 38 h maturation, oocytes were freed of the cumulus cells by brief exposure to 0.1% hyaluronidase and manual pipetting. Oocytes with even cytoplasm, intact plasma membrane and a visible polar body were stained with Hoechst 33342 and enucleated in HEPES buffered NCSU23 (HNCSU23) containing 7.5 μg/ml cytochalasin B. The first polar body and the nearby cytoplasm was removed with a micropipette with an inner diameter of about 25 μm. Successful enucleation was confirmed by a brief exposure to UV light. The cytoplasts were held in NSCU23 containing 5% FBS until reconstruction. Reconstruction was performed in calcium free HNCSU23. A single donor cell was placed in the perivitelline space. The couplets were washed in the fusion medium (0.28 M mannitol, 0.2 mM MgSO4.7H_2O, and 0.01% polyvinylalcohol (PVA)), placed between two stainless steel electrodes (BTX, San Diego, CA), and 0.5 mm apart, which were overlaid with the fusion medium, and then manually aligned so that the plane of contact between the donor and recipient cells was parallel with the electrodes. Fusion was performed with one DC pulse of 220 V/mm for 60 μsec using a BTX Electro Cell Manipulator 2001 (BTX). After the electrical pulse, the couplets were returned to the calcium free HNCSU23 for 15 min and then held in NCSU23 containing 5% FBS until activated (at least 1.5 hr). Fusion was evaluated after 30 min and unfused couplets were discarded. Fused couplets were activated by two 60 μsec DC pulses of 150 V/mm, 1 sec apart in activation medium (0.28 M mannitol, 0.2 mM $MgSO_4.7H_2O$, 0.1 mM $CaCl_2.2H_2O$ and 0.01% PVA) while between two stainless steel electrodes, 1 mm apart. The prospective zygotes were cultured in a modified NCSU23 (containing 0.6 mM glucose, 5.7 mM lactate, 0.2 mM pyruvate and MEM non-essential amino acids) at 38.5°C in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2 for approximately 60 h, when morphology was assessed. They were then cultured for the remaining time in NCSU23 (5.6 mM glucose) containing MEM non-essential amino acids and MEM essential amino acids (Invitrogen). The number of SCNT embryos that developed to the blastocyst stage was determined on day 6.

Results

Experiment 1: Clonal isolation and differentiation of adult porcine fibroblasts

Seven days after plating two distinct cell morphologies were observed with colonies existing entirely of cells with typical fibroblast (fibroblast-like) morphology or cells with a more rounded appearance which we termed cobblestone-like. Fifty-seven of 90 (63%) colonies displayed typical fibroblast morphology, while 33 (37%) were cobblestone-like in appearance (Figure 1). Cells were then differentiated using protocols previously used by us for MSCs. Two of the 57 clones that displayed fibroblast morphology differentiated into adipocytes but not chondrocytes or osteocytes (uni-potent). Three of the 33 cobblestone-like clones differentiated into both adipocytes and chondrocytes (bi-potent) but not osteocytes, and three differentiated into chondrocytes only (Table 1 and Figure 2). A porcine MSC line, isolated from bone marrow [1] was used as a positive control for the differentiation experiment and was positive for adipocytes, osteocytes and chondrocytes (results not shown).

Experiment 2: *In vitro* potential of bi-potent fibroblasts cells for SCNT

The development of SCNT embryos reconstructed using a non-differentiating fibroblast cell clone and one of the cobblestone-like cell clones that differentiated into adipocytes and chondrocytes is shown in Table 2. There was no difference in cleavage rates between the two cell types. Blastocyst rates were also not different between the two clones. For the bi-potent cobblestone-like clone, 26 of the 128 fused couplets reconstructed developed to the blastocyst stage by Day 6 (20%)

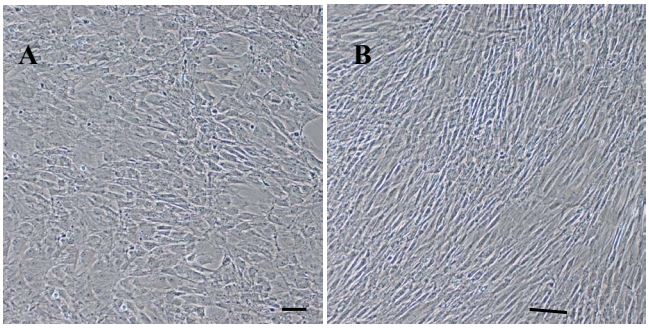

Figure 1: Typical morphology of the two types of adult porcine fibroblast clones isolated A). Cobblestone-like. B). Fibroblast-like. Scale bars=200 μm. Skin fibroblasts were obtained from ear punch tissue isolated from adult male pigs. Clonal isolation was achieved by plating cells at low density (1 cell/cm^2) and culturing for 7-10 days. Following plating two distinct cell morphologies were observed with colonies existing entirely of cells with typical fibroblast (fibroblast-like) morphology or cells with a more rounded appearance which we termed cobblestone-like.

Cell type	Adipocyte only	Chondrocyte only	Osteocyte only	Adipocyte and chondrocyte
Fibroblast-like	2/57	0/57	0/57	0/57
Cobblestone-like	0/33	3/33	0/33	3/33

Table 1: Differentiation of fibroblast and cobblestone-like cell clones.

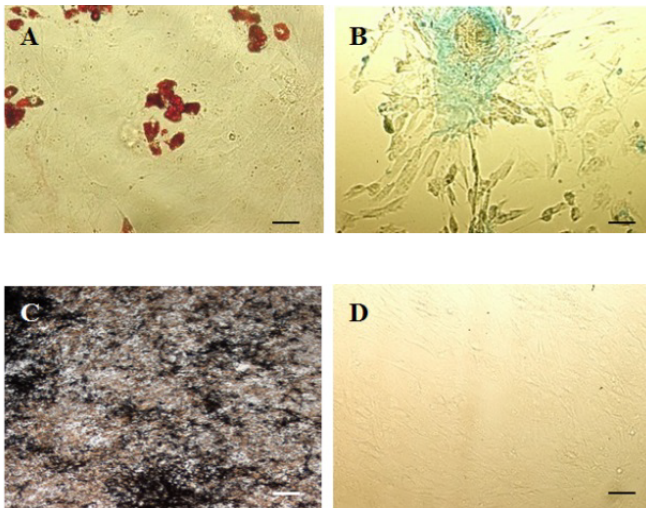

Figure 2: Differentiation of a clonally isolated porcine adult fibroblast cell line showing bi-potency. A). Oil Red O staining following adipogenic induction and B). Alcian Blue staining following chondrogenic induction. C). Von Kossa staining following osteogenic induction, using a porcine MSC line as a positive control. D). untreated. Scale bars=150 µm. Multilineage differentiation potential of selected clones was demonstrated by culturing cells in ambient oxygen and 5% CO_2 under conditions that stimulate osteogenic [12], adipogenic [13] or chondrogenic development (micromass culture [14]) overlayed with chondrogenic induction medium [15]. Histochemical assays were used to detect osteogenic precursors (von Kossa), adipocytes (Oil Red O) and chondrocytes (Alcian blue).

Donor cell	n[1]	Cleaved (%)	Day 6 Blastocysts (%)
Non differentiating fibroblast-like clone	140	83 (65)	23 (16)
Bi-potent cobblestone-like clone	128	81 (63)	26 (20)
[1]Number of fused couplets placed into culture.			

Table 2: Development of SCNT embryos using fibroblast and cobblestone-like cell clones.

Similarly for the non-differentiating fibroblast-like clone, 23 of the 140 fused couplets developed to the blastocyst stage (16%).

Discussion

The present study was undertaken to determine if MSCs present in bone marrow and blood, are also present in primary cultures of adult fibroblasts that are normally used to clone live animals. To examine this hypothesis we chose a primary culture that had been used previously by us to produce cloned pigs using SCNT. Following low plating density, 54 per cent of single cell derived colonies (clones) displayed typical fibroblast morphology while the remainders of cells were cobblestone-like in their appearance. This is the first time to our knowledge that primary cultures of porcine adult fibroblasts have been shown to consist of two morphologically distinct subpopulations. The significance of these two populations remains unclear, although the cobblestone morphology we observed is typical of endothelial cells which are likely to be present in primary cultures derived from skin tissue [16]. These cells were then differentiated using protocols used previously by us to identify MSCs. Two of the clones from the fibroblast-like group differentiated into adipocytes. In contrast, three of the 33 clones that displayed a cobblestone-like morphology differentiated into adipocytes and chondrocytes and three differentiated into chondrocytes. However none of the clones examined differentiated into osteocytes or all three cell types suggesting that these cells may not be MSCs. Alternatively these cells may have been MSCs but had lost some of their multipotency in culture as evidenced by their lack of ability to differentiate into osteocytes [17]. Nevertheless, we have shown that primary cultures of adult fibroblasts contain at least two distinct cell populations which can be classified according to their morphology namely fibroblast and cobblestone-like, and that the later contains multipotent cells which can be differentiated using protocols for MSCs. How many different multipotent cell types are present in these primary cultures remains to be determined as we only used differentiation protocols for identifying MSCs. However, other workers have reported the presence of other stem cells in fetal and adult tissues. Zhu et al. [18] reported that the use of porcine skin-derived fetal stem cells increased blastocyst rates compared with fetal fibroblasts and suggested that this was due to the stem cell-like nature of these cells. Kues et al. [19] also identified a population of cells in mice and pigs, termed fetal somatic stem cells, which exhibit an increased proliferative capacity, compared with fetal fibroblasts and can form chimeric fetuses when injected into mouse blastocysts.

To examine the developmental potential of two cell types we used one fibroblast-like clone which failed to differentiate and one of the cobblestone clones that differentiated into adipocytes and chondrocytes for SCNT. Both clones produced blastocysts at similar rates despite differences in their differentiation capacity. Further studies are needed to confirm these findings as we only examined two cell clones. Furthermore whether blastocyst rates reflect the ability of SCNT embryos to develop to term remains unclear. Wakayama et al. [20] reported that embryos reconstructed using mouse ES cells resulted in lower blastocyst rates but gave higher rates of liveborn compared with cumulus cells clones. Instead these workers suggested that the development of cloned embryos should be assessed in terms of the number of blastocyst that develop to term rather than the number of embryos that develop to the blastocyst stage. Interestingly Lee et al. [8] and Li et al. [9] have reported that porcine MSCs increase blastocyst development and cell number as well as live born when these cells are used for SCNT compared with fibroblasts.

In conclusion, we have identified that primary cultures of porcine adult fibroblasts used to clone pigs contain two morphologically distinct populations. These populations contain cells which can be differentiated into adipocytes and/or chondrocytes using protocols designed to identify MSCs. These findings need to be confirmed using additional cell lines including those which have been shown not to produce cloned pigs. However based on our initial findings and those of other workers, we speculate that primary cultures of fibroblasts contain populations of adult stem cells which when used for SCNT are more likely to result in the production of live offspring. Further evidence is required to support this suggestion. Such studies will require not only *in vitro* developmental data but also large scale embryo transfer experiments which examine development to term. Although prohibitive, such studies have the potential to improve current animal cloning efficiencies.

Conflict of Interests

The authors declare no conflict of interest

Author's Contribution

SJ Harrison, LFS Beebe, I Vassiliev and SM McIlfatrick contributed to the experimental work. SJ Harrison and MB Nottle were responsible for the experimental design and wrote the manuscript and all authors approved the final version.

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