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Karyotyping as an important screen for suitable donor cells to generate cloned and cloned transgenic animals by nuclear transfer

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ABSTRACT

Modern reproductive technologies are using cultured cells for the reconstruction of embryos. Cultured cells, however, can acquire abnormal karyotypes and, if not diagnosed, will compromise correct embryonic development. This manuscript briefly describes the evaluation of the karyotypes of cells used either for nuclear transfer or blastocyst aggregation to reconstruct embryos. Tetraploidy was found to be the major deviation from the normal diploid set of chromosomes. Most of the primary bovine and ovine cell lines derived from adult skin tissue contained cells with an abnormal chromosome number (75%). The percentage of abnormal cells was in the range of 0-55%. A higher proportion of clonally derived foetal cell lines had a normal karyotype (74%). Although two abnormal cell lines of this class contained only tetraploid cells, which is most likely due to the clonal nature of their derivation. In contrast, all clonal mouse embryonic stem-cell lines (four) were karyotypically normal.

Keywords: nuclear transfer; cloning; chromosome; tetraploidy; karyotype.

INTRODUCTION

Nuclear transfer (NT) is a novel reproductive technology that is used to generate a whole new animal virtually from a single somatic donor cell (Campbell et al., 1996). The technique is also known as cloning and has applications in the rapid amplification of elite livestock animals and the production of transgenic animals (Gurdon & Colman, 1999). The somatic cells used as the donor cells in this process can be derived from embryonic, fetal or adult samples. Usually, they are established as a primary cell line and cultured *in vitro*. Such a primary cell line contains not a homogenous cell population but is rather a mix of a whole range of different cells. Under *in vitro* culture conditions, cells can occasionally acquire an abnormal karyotype (Freshney, 2000). Since single cells are used to reconstruct individual NT embryos, the success of the process depends upon a normal karyotype of the donor cell. A deviation from the normal chromosome number will almost certainly result in failure of embryonic development. The characterisation of the karyotype is even more important for transgenic cells. These cells have to go through a genetic modification step, which significantly prolongs the time the cells are cultured *in vitro* (Cibelli et al., 1998, Schnieke et al., 1997) and, thus, have an increased risk of acquiring chromosomal abnormalities. Moreover, transgenic cell lines are derived from a single cell that has been successfully transfected and are, thus, strictly dependent upon the karyotype of this parental cell.

The aim of this study was, firstly, to develop a method for the preparation of metaphase chromosome spreads from cultured bovine, ovine and mouse cells, and secondly, to apply this method to determine the occurrence of normal and abnormal chromosome numbers in these cells as a screen for suitable donor cell lines for nuclear transfer-generated cloned and cloned transgenic animals.

MATERIALS AND METHODS

Cell culture:

a) Mouse ES cell culture:

Mouse ES cells (male mouse strain R1) were cultured according to standard protocols on mitotically inactivated embryonic fibroblasts (STO puromycin-resistant) in DMEM-F12 (1:1) medium (Gibco) supplemented with 15% (v/v) fetal-calf serum (Gibco, Lot # 916) and 100 units/ml recombinant leukaemia inhibitory factor (ESGRO LIF, Gibco) (Wurst et al., 1994). Cells (passage 12) were transfected with a gene construct, selected with puromycin for one week, and puromycin-resistant colonies were isolated and further cultured (Udy et al., 1997). Cells had been in culture for 2-3 weeks when karyotyped.

b) Primary adult skin fibroblasts:

Sheep cells were isolated from young adult ram skin biopsies, cultured for two weeks, and karyotyped at passage four. Cattle cells were isolated from a three year old bull ear punch skin sample, cultured for two weeks and karyotyped at passage three.

c) Transgenic (TG) fetal fibroblasts:

Fetal fibroblasts were isolated from a day 60 female fetus, cultured *in vitro,* and passaged up to three times before being transfected with a gene construct. Cells were selected with puromycin for one week, and puromycin-resistant colonies were isolated and further cultured (Cibelli et al., 1998) for a total of 19 – 29 days before nuclear transfer.

Bovine and ovine cells were cultured using standard tissue culture techniques in 10 cm tissue culture plates with DMEM-F12 (1:1)(Gibco) culture media, supplemented with 10% (v/v) fetal-calf serum (FCS) (Gibco, Lot # 220861) and 1 mM sodium pyruvate. Incubations were carried out between 37-38.5°C in a humidified atmosphere of 5% (v/v) CO_2 in air.

Metaphase spreads: Cells were harvested for karyotyping following the basic techniques described by Rothfels (1958) and Rooney (1986), while the cells were still in the log phase of population growth (approximately 70% confluent) to ensure maximum number of cells in metaphase. Colcemid (Gibco) was added to the culture plates one hour prior to cell harvest at a final concentration

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of 0.1 μg/ml, incubated at normal culture temperature.

Cells were harvested using the standard 0.25% (w/v) trypsin-EGTA technique (Freshney, 2000). The supernatant was removed, and the cells were resuspended in 5 ml 0.56% (w/v) KCl solution. This was incubated for 15 min at 37°C to swell the cells. Five ml of fresh fixative (3:1 methanol:acetic acid) was added on top of the KCl solution, then centrifuged for 5 min at 1000 rpm. The supernatant was removed and a further 5 ml of fixative was added with gentle mixing, and incubated at 4°C for 30 min before centrifuging again. The fixative incubation and centrifuging steps were repeated two more times, the supernatant was removed, and 0.5 ml fresh fixative on ice was added. The cells were carefully mixed to ensure an even suspension.

Cells were spread onto pre-cleaned cold slides by pipetting three drops of cell suspension from a height of approximately 20 cm onto each slide and allowed to air dry.

Once dry, the slides were stained in 5% (v/v) (in 'Gurr's' buffer, pH 6.8) fresh Giemsa stain (Gibco) for 30 min, washed with PBS, dried, and the spreads examined under 1000X magnification, with oil optics.

Digital images were captured of suitable cell metaphase spreads, which were later examined and the chromosomes were counted in each cell.

RESULTS AND DISCUSSION

Bovine and ovine primary adult skin fibroblast cell lines, bovine clonal transgenic fetal lung fibroblast cell lines and mouse clonal transgenic embryonic stem-cell lines were analysed for their karyotype. Actively dividing cells were arrested in metaphase of the cell cycle and chromosome spreads of individual cells were prepared. Chromosomes were Giemsa stained and evaluated by light microscopy. The karyotyping screen revealed that most cell lines contained cells with abnormal karyotypes. Tetraploidy (4n) was observed as the main deviation from the normal diploid set of chromosomes (2n). Figure 1 shows representative metaphase spreads for normal karyotypes (2n) with 60 chromosomes for cattle and 54 chromosomes for sheep and tetraploid karyotypes (4n) with a duplicated set of chromosomes (120 for cattle and 108 for sheep).

Table 1 summarises the percentage of tetraploid cells found in the various cell lines studied.

These data are solely based on metaphase spreads in which all chromosomes were well separated and could be easily counted. In many spreads, chromosomes overlapped to various degrees. Evaluation of such spreads was, therefore, more difficult and they were not included in the analysis. Evaluation of spreads with few chromosomal overlaps, however, was consistent with the results given in Table 1 (data not shown).

Individual cell lines varied significantly in the number of polyploid cells ranging from 0% to 100% (Table 1). Moreover, striking differences between primary adult and clonal fetal cell lines were observed. In primary adult skin fibroblasts only 25% (4/16) of the cell lines analysed showed a completely normal karyotype. In contrast, the majority of these cell lines contained tetraploid cells to various degrees ranging from 3% to 55%.

The clonally derived transgenic cell lines had a much higher proportion of karyotypically normal cell lines. All

FIGURE 1: Metaphase chromosome spreads of cells with normal (2n) and tetraploid (4n) karyotypes. Bovine X chromosomes are the only submetacentric chromosomes and have been indicated by an arrow.

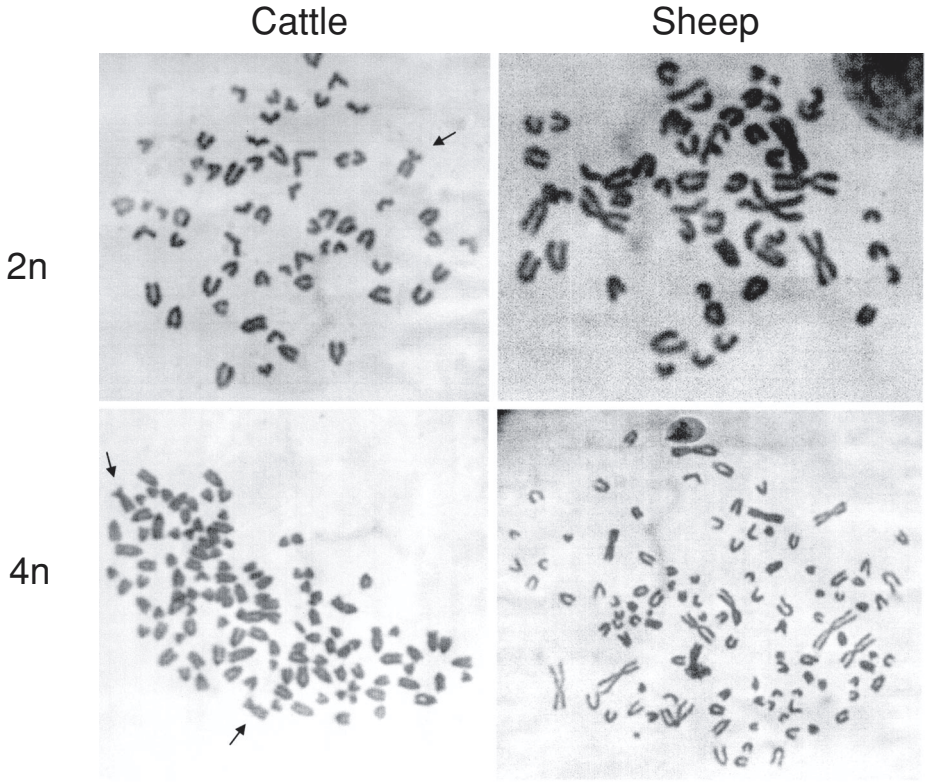

TABLE 1. Proportion of cells with polyploid karyotypes in donor cell lines used for the reconstruction of embryos. The data are based on metaphase spreads where all chromosomes were well separated. ES-cells: embryonic stem cells, *: one cell line in this class had a 59% monosomy X karyotype.

Cells	Sex	Species	No. of lines	Tetraploid spreads (%)	Spreads analysed
Primary		Cattle	3	11-17	9-24
Adult Skin			2	25-35	10-23
Fibroblasts	♂	Sheep	4	0	5-11
			3	3-8	12-36
			2	11-18	9-11
			4	21-33	12-26
			3	42-55	9-12
Transgenic	♀	Cattle	18	*0	5-17
(TG) Fetal			1	8	12
Fibroblasts			2	20	10-14
			2	100	5
TG ES-cells	♂	Mouse	4	0	3-6

of the analysed mouse embryonic stem-cell lines were normal (4/4) whereas 74% (17/23) of the transgenic cattle fetal fibroblast cell lines contained only cells with a normal karyotype. The latter category of cell lines, however, also featured two cell lines (2/23, 9%) at the other end of the spectrum. All the cells screened from these two cell lines were characterised as tetraploid. The tetraploid clonal cell lines were most likely derived from a single tetraploid cell that was transfected at the outset. One cell line (1/23, 4%) had a 59% monosomy X karyotype. Three cell lines (3/23, 13%) showed a mixed population of cells with 8-20% of cells having an abnormal karyotype.

Freshney (2000) described that cells acquired chromosomal abnormalities during *in vitro* culture. The increased incidence of abnormalities correlated with the length of time kept under *in vitro* culture conditions (Freshney, 2000). It is, therefore, important to minimise the length of time and number of cell divisions for which the cell lines are cultured. In our experiments, cattle and sheep primary adult skin fibroblast cell lines were kept the shortest time in culture. Nevertheless, these cells showed the highest incidence of tetraploidy, suggesting that cells derived from adult tissues might have a higher risk of becoming polyploid in culture. Due to the selection process, the embryonic and fetal derived clonal transgenic cell lines were kept substantially longer in culture. Yet, the majority of cell lines are of a normal karyotype. Since clonal cell lines are derived from a single cell, some of these cell lines might, however, be polyploidy, which was the case for two transgenic cattle cell lines. This would not be expected for primary adult fibroblast cell lines, which represent a mix of different cells, and no instances of complete tetraploidy were observed.

In order to avoid unsustainably low efficiencies in embryonic development, as has been observed with cell lines with a high percentage of cells with abnormal chromosome numbers, we now use only cell lines with more than 80% karyotypically normal cells for the reconstruction of NT embryos. Moreover, donor cells used for NT were further screened by selecting only the smaller cells indicative of a diploid chromosomal content.

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