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Experimental embryonic-somatic chimaerism in the sheep confirmed by random amplified polymorphic DNA assay

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ABSTRACT Developmental potencies of sheep somatic cells (foetal fibroblasts, FFs) in chimaeric animals were analysed. FFs from pigmented Polish Heatherhead (wrzosowka) breed were microsurgically injected into morulae or blastocysts of white Polish Merino breed (5 cells to each embryo). In one experiment the cells were stained with vital fluorescent dye PKH26, and chimaeric blastocysts were cultured *in vitro* to confirm the presence of fluorescent cells. In the majority of experiments the blastocysts were transferred to synchronized recipient ewes for development until term. Cultured embryonic cells (CEC), earlier known to produce chimaeras, were injected into blastocysts in control experiments. Seven young were born from FF-injected embryos and three were born from CEC-injected ones. All of them were white, but all three control lambs and three experimental lambs showed small areas of skin pigmentation, which indicated Heatherhead CEC or FF contribution. Tissue samples originating from three germ layers were taken from two FFs-originating presumably chimaeric lambs (male and female) at the age of one month for DNA analysis. The random amplified polymorphic DNA-PCR method supplied two markers of chimaerism, which were amplification products of 643 bp and 615 bp long DNA fragments, found in tissues of experimental lambs as well as in FFs, but not in the blood of parents of blastocysts. The 643 bp marker was found in the majority of tissues of both lambs. The 615 bp amplicon was detected in the skin and lungs of the female lamb and in the hooves of the male lamb. Our data show that foetal fibroblasts introduced to sheep blastocysts can participate in development and can contribute to all tissue lineages up to at least one month of age.

KEY WORDS: *sheep, chimaera, fibroblast, RAPD-PCR*

Introduction

Two lines of evidence suggest that mouse somatic cells (foetal fibroblasts) can be reprogrammed to regain totipotency and to participate in embryonic development. First, experimental reprogramming of cells *in vitro* culminated in the results by Takahashi and Yamanaka (2006), who have shown that the four genes introduced to foetal or adult fibroblasts caused a small fraction of fibroblasts to become totipotent ES-like cells. The second line of evidence is chimaera formation after injecting foetal fibroblasts to cleaving mouse embryos (Piliszek *et al.,* 2007). Progeny of injected somatic cells could be found in many organs of adult mice, suggesting their reprogramming *in vivo* and subsequent participation in development.

Mouse was the first species to give rise to experimental chimaeras (Tarkowski, 1961; Mintz, 1962). Also in the mouse (and primates) totipotent embryo-derived ES cells are available. Once injected into blastocysts these cells join to form chimaeras, and participate in all tissues of embryonic body, germ line included (Beddington and Robertson, 1989). None of these forefront heralds of easily forming adult chimaeras are available in the sheep.

Therefore we aimed at producing embryonic-somatic chimaeras in the sheep. It was ealier known that ovine embryo-derived cultured cell lines, also referred to as embryonic stem cell-like cell lines, can contribute to sheep chimaeras (Modlinski *et al.,* 1996). In the presented experiments, such cultured embryonic cells (CECs) were injected as control, and foetal fibroblasts (Fig. 1) were

Abbreviations used in this paper: cec, cultured embryonic cell; ff, foetal fibroblast; RAPD, random amplified polymorphic DNA (assay); rs, removing sponge.

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injected to the embryos as somatic cells. The use of RAPD-PCR assay supplied two markers of chimaerism, which allowed to confirm contribution from fibroblasts to animals born.

Results

Presence of PKH26-stained fibroblasts in blastocysts

In a single experiment observation of the embryos *in vitro* confirmed the presence of injected cells. The day after injection three and 5 cells were found. On the third day 2, 7 and 7 cells were found. In few cases the embryos cultured for one week displayed but one marked cell. The observations are not in disagreement with the results of detailed analysis in the mouse (Piliszek *et al.,* 2007), showing that FFs resume divisions at day 3 after injection and that cell loses happen at any stage of development.

Postimplantation development of presumed chimaeric embryos

Lambing efficiency was about 12% (7 lambs out of 58 blastocysts) when presumably chimaeric blastocysts were transferred to recipient ewes (Table 1). Six transfers terminated at full term, giving rise to 7 young out of 17 blastocysts with the introduced fibroblasts (Table 2). Six lambs were perfectly normal. One (female No 5) was born dead. It was a large lamb, with shortened head. In two transfers terminated at term, 3 young were born of 4 blastocysts harbouring cultured embryonic cells. However, one lamb (male No 10) was born dead. Out of eight lambs born alive, two (female No 6 and male No 7, both originating from FFs-injected embryos) were sacrificed early (at the age of one month) to take tissue samples for DNA analysis. Of the remaining six, one (female No 9) broke leg and died. The survivors: one male and four females, grew normally and they are from two to three years old now.

Overt chimaerism

Lack of skin pigmentation and curly wool type in recipient Merino breed allows to discriminate between non-chimaeric lambs and those displaying areas of Heatherhead dark pigmented skin and slightly wavy hair that can indicate for chimaerism. These morphological features served as markers of overt chimaerism.

In the present experiments all three control lambs were born with patches of dark pigmentation on mouth and legs (Fig. 2), including male lamb Nr 10 that was born dead and had dark patches at face and on the top of its hooves (Table 2). One female additionally had areas of donor-type hair on her legs.

Three lambs derived from FFs-injected blastocysts had at birth areas of pigmented skin (dark skin of nose and lips, some dark hair nearby, but also dark hooves; Fig. 2). At the age of about one year

TABLE 1

DEVELOPMENT OF BLASTOCYSTS INJECTED WITH CELLS, AFTER TRANSFER TO RECIPIENTS

Type of cells	No. transfers (blastocysts)	No. term pregnancies (blastocysts)	No. young born	No. live young
FFs	20 (58)	6 (17)	7 (5♀,2♂)	6 (4♀,2♂)
CECs	9 (26)	2 (4)	3 (2♀,1♂)	2 (2♀)

CECs - cultured embryonic cells (control)

FFs - fibroblasts

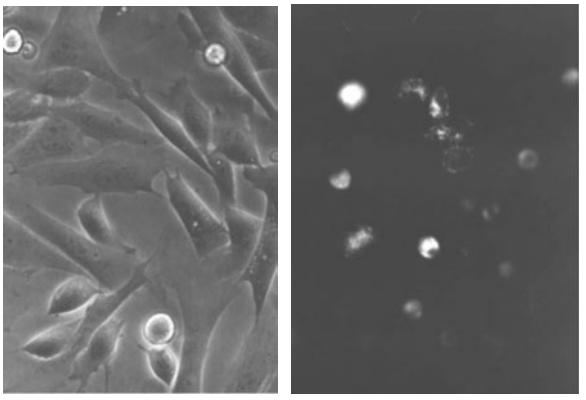

Fig.1. Sheep foetal fibroblasts growing *in vitro* (left; phase contrast) and seeded after PKH26 staining (right, UV light).

the fourth lamb of this group who was white, in its general appearance resembled Heatherhead sheep (slim head, the posture), with legs covered by wavy hair. Also at the sides of the body patches of donor type hair were evident.

Two lambs with no signs of overt chimaerism (No 6 and 7) were sacrificed at 1 month of age to supply tissue samples for DNA analysis.

RAPD DNA analysis

Fourteen different tissues (blood, thymus, liver, kidney, gonads, skeletal muscles, heart muscle, small intestine, brain, skin of nose, lungs, cloven hooves, thyroid, pancreas) from lambs No 6 and 7 (a total of 27 samples) as well as samples of blood from the parents of blastocysts and the sample of the same Heatherhead fibroblasts that had been injected to blastocysts, were analysed.

Band sharing values (BS) between FFs and parents of blastocysts (Table 3) calculated from the electrophorograms, confirm otherwise obvious features of experimental design: high BS value between male and female parent of blastocysts reveals their

TABLE 2

LAMBS BORN AFTER INTRODUCING FIBROBLASTS OR CULTURED EMBRYONIC CELLS FROM HEATHERHEAD PIGMENTED SHEEP INTO BLASTOCYSTS OF WHITE MERINO BREED

No.	Sex	Cells (passage)	Pigmented skin*	Heatherhead type of hair**	Comments
1	♀	FF (6)	+	+	-
2	♀	FF (4)	-	+	Slim head and posture of Heatherhead
3	♀	FF (4)	-	-	-
4	♂	FF (5)	+	-	-
5	♀	FF (14)	+	-	Born dead, puggish head
6	♀	FF (11)	-	-	-
7	♂	FF (11)	-	-	-
8	♀	CEC (4)	+	+	-
9	♀	CEC (4)	+	-	-
10	♂	CEC (7)	+	-	Born dead

CEC, cultured embryonic cells; FF, foetal fibroblasts; * lips, nose and/or hooves; ** legs, sides

common breed identity (Merino), whereas lower BS values between any parent and FFs indicate other breed of the cells (Heatherhead).

In Table 4, RAPD markers identified in lambs No 6 and 7 are summarized. The majority of them (6 and 8) are type I markers, which denote bands found in any material studied (parents of blastocysts, lambs born and injected fibroblasts). Type IV marker (male parent and offspring) is only found in the female lamb, thus excluding its localization to chromosome Y. The absence of common amplification products for father and male lamb indicates that no Y chromosome specific sequence has been revealed by RAPD analysis with the primers used.

Amplification of DNA fragments originating from donor fibroblasts, using arbitrarily chosen primers revealed the presence of two absolute RAPD markers, i.e. such that they are present only in band pattern of fibroblasts and not in DNA originating from parents of blastocysts (lambs). These markers are *loci* that are 615 and 643 bp long, respectively (Fig. 3). Since both bands were found in both individuals analysed (lamb No 6 and 7), thus their chimaerism has been confirmed in the form of coexistence of donor (fibroblasts) and recipient cells.

Analysis of tissue distribution (cf. Fig. 3) has revealed that all ectodermal tissues as well as tissues originating from mesoderm and/or endoderm (with the exceptions of thyroid, liver and blood) of lamb 2 (male No 7) contained marker amplification product of 643 bp long DNA fragment. Based on the quantity of this amplification product, the lamb could be characterized as showing varying tissue chimaerism. The other lamb studied (female No 6) displayed amplification product of 643 bp long DNA fragment in all tissues analysed. Also in this individual, tissue-specific variation in quantity of the product indicates for varying chimaerism (cf. Fig. 3).

The second type V marker was the amplification product of 615 bp long DNA fragment. The presence of this fragment was found among amplification products of DNA isolated from the skin on the nose as well as from lungs of lamb 1 (female No 6) and in DNA isolated from the hooves of lamb 2 (male No 7).

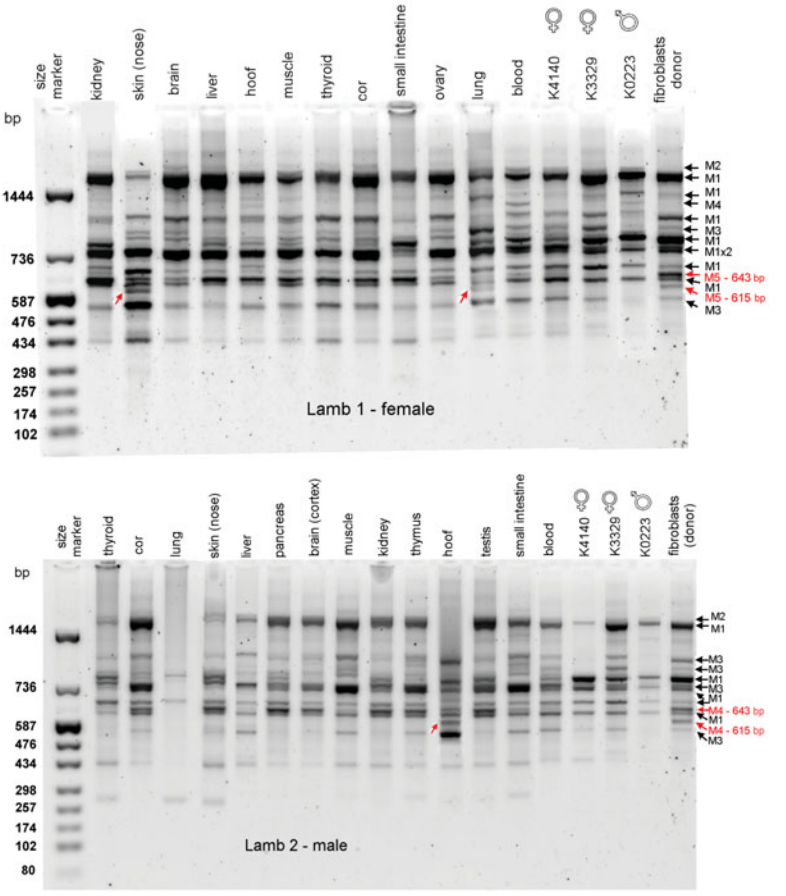

Fig. 3. Electrophorograms of RAPD molecular marker patterns generated with a primer in the tissues of the lambs as recipients, the blood of their parents (K) and in donor fibroblast cells. *Two markers of chimaerism, which are amplification products of 643 bp and 615 bp long DNA fragments, are indicated with red arrows. In the tissue samples of the female lamb (top panel) the 643 bp marker was commonly detected. The other 615 bp amplicon appeared in the skin and lungs. In the tissues of the male lamb (bottom panel), the 643 bp amplicon was also omnipresent, and the 615 bp marker appeared in the hooves. The data confirm embryonic-somatic chimaerism of the lambs at the molecular level.*

Sex identification

An accurate, sensitive, and quick method for determining the sex of ovine fibroblasts consisted in using polymerase chain reaction (PCR) primers derived from an ovine-specific SRY gene (Fig. 4). The fibroblasts used in the experiments proved to be male.

Fig. 2. Dark pigmentation of lips (lambs 8 and 9) and cloven hooves (lamb 5) is the marker of overt chimaerism, *indicating the donor contribution (Heatherhead fibroblasts) to recipient blastocysts of white breed, and confirming the chimaerism of the animals.*

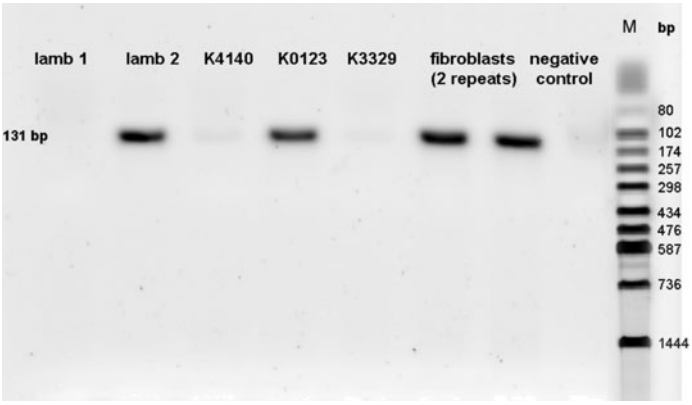

Fig. 4. Electrophorogram showing the product of ovine SRY fragment amplification (131 bp in length), as found in male parent (K0123), male lamb (lamb 2) and in foetal fibroblasts. *The band was absent from female lamb (lamb 1) and female parents (K4140 and K3329). Taken together, the data are indicative of the male sex of the fibroblasts used.*

Discussion

Experimental chimaerism in mammals

Mouse embryonic chimaera formation was first described by Tarkowski (1961) and Mintz (1962) and this technique spread gradually on other mammalian species. In the cow, chimaera production has been applied to rescue parthenogenetic embryos by aggregating them with fertilized ones (Boediono *et al.,* 1999). In the sheep embryonic chimaeras were produced between cleaving embryos (Fehilly *et al.,* 1984) and by introducing inner cell masses to blastocysts (Butler *et al.,* 1987). Cultured embryonic cells derived from early embryos have been tested for their pluripotency by chimaera formation in many species (mouse – Beddington and Robertson, 1989; cow – Cibelli *et al.,* 1998; Iwasaki *et al.,* 2000; Saito *et al.,* 2003; sheep – Modlinski *et al.,* 1996; pig – Notarianni *et al.,* 1997; Onishi *et al.,* 2000; rabbit – Schoonjans *et al.,* 1996; Bodo *et al.,* 2004).

The first to test developmental potential of differentiated somatic cells by chimaera formation was Brinster (1974), who injected bone marrow cells into mouse blastocysts. In the experiments conducted later, various somatic stem cells have been proved able to contribute to foetal or adult tissue after being introduced into blastocysts (hematopoetic stem cells - Geiger *et al.,* 1998; neural stem cells - Clarke *et al.,* 2000; mesenchymal multipotent progenitor cells - Jiang *et al.,* 2002; foetal somatic stem cells - Kues *et al.,* 2005).

Foetal fibroblasts which are non-stem somatic cells, participated in the formation of the majority of tissues in adult mice after being introduced to cleaving embryos (Piliszek *et al.,* 2007). The mice obtained were thus adult embryonic-somatic chimaeras. In the present paper adult embryonic- somatic chimaeras are for the first time obtained in the sheep.

Markers of chimaerism in the sheep

Dark pigmentation of hooves, skin on the nose and/or lips can be used as the marker of chimaerism in white Polish Merino sheep, since pigmentation has been eliminated from this breed to such an extent, that small patches of pigmentation at birth happen to less than 0.5% lambs (Niznikowski, personal information). All three lambs born from our CECs-injected embryos, and three out of seven lambs born from FFs-injected embryos, had dark patches. This frequency is so high, that random appearance of this phenomenon can be excluded, and the skin chimaerism of these lambs can be safely concluded.

Biochemical polymorphism of glucosephosphate isomerase (GPI) has been an excellent marker of chimaerism in the mouse for over thirty years now (Chapman *et al.,* 1972; Gearhart and Mintz, 1972a, b) and is still in use (e.g. West and Flockhart, 1994; Wang *et al.,* 1997; Tarkowski *et al.,* 2001; Suwinska *et al.,* 2005). Unfortunately, isozymes of glucose-6-phosphate isomerase could not be used as the marker of chimaerism in the sheep, since two (Beatty, 1983) or three (Rasero *et al.,* 1993) isozymes are found in each breed and there are only quantitative differences in their expression between the breeds. Therefore, for identifying molecular markers of chimaerism, random amplified polymorphic DNA (RAPD) method was used. In this method, by using a single arbitrary primer (10 mer) and amplifying DNA in polymerase chain reaction (PCR), DNA markers appear that can be separated easily on an agarose gel by electrophoresis (Williams *et al.,* 1990). The advantages of RAPD is its simplicity, rapidity, the requirement for only a small quantity of DNA, which allows to detect small numbers of donor cells, i.e. the low level of chimaerism, and the ability to generate numerous polymorphisms (for review see: Hadrys *et al*., 1992; Bassam and Bentley, 1994).

RAPDs can be generated with minimal cost and investment, but the limitation is that many more dominant than codominant markers are needed to accomplish the same resolution (Huff *et al.,* 1993) due to difficulties in distinguishing between homozygous (++) and heterozygous individuals (+-); recessive (—) individuals are unambiguous. However, as soon as at least one marker of chimaerism is found, the prove of donor contribution to

TABLE 3

BAND SHARING (BS) VALUES BETWEEN FIBROBLASTS (FFS) AND PARENTS OF BLASTOCYSTS, CALCULATED ON THE BASIS OF RAPD PROFILES

Source of DNA (number of bands)	BS	
	female parent/(12 bands)	male parent/(11 bands)
FFs (11)	0.783	0.636
male parent (11)	0.956	-

TABLE 4

RAPD MARKERS IDENTIFIED IN LAMBS NO 6 AND 7 (TYPE V - MARKERS OF CHIMAERISM)

	Bands found in*				Number of RAPD markers	
Type of marker	Mo	Fa	FFs	La	Lamb 7 (♂)	Lamb 6 (♀)
I	+	+	+	+	6	8
II	+	+		+	1	1
III	+		+	+	4	2
IV		+		+	0	1
V			+	+	2	2
Total					13	14

* Mo – mother of blastocysts; Fa – father of blastocysts; FFs – fibroblasts; La - lambs

an individual can be accomplished. The other limitation is not always high reproducibility of the results obtained by RAPD. Unlike two-primer mediated PCR, RAPD assay is performed using low stringency conditions. By interference, mismatches may occur between the primer and its target sequence in the amplification reaction (MacPherson *et al.,* 1993). However, with standardized methodology: the same thermal cycler, temperature profiles, the brand of DNA polymerase, and the concentration of $MgCl_2$, primer and template DNA, we obtained a high repeatability of RAPD assay, so that the final result is reliable.

Among RAPD markers, the band patterns in the chimaeras were found to be fully additive. The offspring revealed 100% shared markers with parents of blastocysts. The presence of RAPD markers of type V allowed identifying chimaerism in both lambs analysed. The occurrence of such markers in band patterns indicated that fibroblasts introduced into morulae or blastocysts were able to contribute to tissues originating not only from mesoderm, which is the layer of origin of fibroblasts, but from two other germ layers as well.

Type III and IV markers are particularly important to identify the polymorphisms in RAPD *loci*. Sources of polymorphisms in RAPD assay may include base change within priming site sequence, deletions of priming site, insertions that render priming sites too distant to support amplification, and deletions or insertions that change the size of a DNA fragment without preventing its amplification (Williams *et al.,* 1990).

RAPD method proved effective in the studies of farm animal species (buffalo, Indian Zebu cattle, sheep and goat) (Cushwa *et al.,* 1996; Rao *et al.,* 1996). However, as far as we know, RAPD has not been earlier applied to study experimental chimaerism in mammals. The effects of our attempt show that this method can possibly be used to study tissue-distribution of chimaerism and/or transplantation efficiency.

Somatic-embryonic chimaerism in the sheep

Areas of pigmented skin in born lambs prove that somatic cells introduced to very early sheep embryos can participate in development until term. Persistant location of dark patches on mouth and legs of all lambs may suggests an ordered mechanism of somatic cell positioning in development or loosening of the morphogenetic control in distal parts of the body.

Earlier studies on embryonic goat-sheep chimaeras have shown that skin fibroblasts explanted from sites covered with goat hair as opposed to sheep wool, show chromosomal composition of the goat as opposed to the sheep, respectively (Jaszczak *et al.,* 1999). Therefore we suggest that a lamb obtained in our study, carrying patches of unpigmented wavy Heatherhead hair, is a skin chimaera as well.

Two polymorphic DNA markers revealed a broad range of tissue chimaerism in our study, including all embryonic lineages (ectoderm, endoderm and mesoderm). From life-long cytogenetic analysis of an embryonic goat-sheep chimaera a conclusion was drawn, that cell contributions from both partners were established during primary colonization but later on they changed with the age of the animal, so that the dominant contribution increased, actually approaching 100%. The exception among tissues studied was lung epithelium in which the proportion of sheep to goat cells was 1:1 in the 10 years old chimaera (Jaszczak *et al.,* 1999).

Two markers of chimaerism were found in each lamb studied. It remains to be explained why *locus* 615 bp long could not be found in as many tissues of lambs as the other amplified locus. A hypothesis can be offered, that loss of heterozygosity (LOH) occurs in fibroblasts introduced to embryos, causing silencing of a presumably polymorphic allel in many tissues. Indeed, LOH was detected *in vitro*, when mouse foetal fibroblasts at early passage were cultured for up to 24 hours (Larson *et al.,* 2006), and can possibly occur *in vivo* as well

Most lambs born after receiving male fibroblasts were females. Female embryos must have been the recipients of male fibroblasts to produce female lambs. It can be further expected that gonadal chimaerism was low in at least RAPD-analysed female lamb, since normal ovary was present in this individual, contrary to what is found in embryonic XX/XY sheep chimaeras, who develop into males (Tucker *et al.,* 1974).

Two presumably chimaeric lambs were born dead: one from the blastocyst that had obtained cultured embryonic cells (the control), and the other one after fibroblast injection. In both cases the passage of donor cells *in vitro* was the highest in the group. High passage might be the reason for lower vitality of the chimaeras. Even mouse embryonic stem cells, which are best suited to form germ-line chimaeras, do so most effectively, when they undergo not more than 15 passages *in vitro* (Fedorov *et al.,* 1997).

In the mouse – the only mammalian species studied before - somatic-embryonic chimaerism lead to the presence of hybrid cells in foetuses and the young born (Piliszek *et al.,* 2007). The suggested origin of hybrid cells was by cell fusion of somatic cells (fibroblasts) and embryonic recipient cells. The animals were thus chimaeras between recipient cells and tetraploid hybrid cells with contributions from the donor fibroblasts. Therefore, in further studies of somatic-embryonic sheep chimaeras, cytogenetic studies and possibly also analysis of polymorphic forms of proteins should aid RAPD method.

Materials and Methods

The experiments were performed according to the rules of the Polish Governmental Act for Animal Care and were approved (No. 6/2003) by the Third Local Ethics Committee for Animal Care.

Obtaining of morulae/blastocysts from hormonally stimulated ewes

Ewes of Polish Merino breed were synchronized for 14 days with fluorogestone acetate sponges (40 mg, Chronogest, Intervet, Holland). Superovulation was induced by four injections of follicle-stimulating hormone (FSH, Sigma): 6 mg twenty four hours before removing sponges (r.s.), 5 mg twelve hours before r.s., 3 mg on the morning of r.s., 2 mg twelve hours after r.s. First signs of oestrus were observed 24 - 48 hours after r.s. Rams of Polish merino were used for natural mating starting from the onset of oestrus and then every 12 h until the oestrus (usually 2-4 times). Embryos were flushed from the oviducts and uterine horns at day 6-7. They were quickly transferred to warm M2 medium under paraffin oil in a Petri dish on a heated stage (38.5ºC).

Foetal fibroblasts

Foetal fibroblasts were derived from a 35-day Polish Heatherhead (wrzosowka) foetus. Briefly, the carcass obtained by dissecting out and discarding the soft tissues (liver, heart and other viscera) was washed in phosphate buffered saline, minced into small pieces of tissue and incubated 30 min in 0.25% trypsin (Sigma) at 38ºC. After adding DMEM medium (Sigma) with 10% foetal calf serum (Gibco) the suspension was centrifuged at 200g, and the pellet was resuspended in the culture

medium and seeded on Petri tissue culture dishes. After the plates became confluent the cultures were expanded and cells were frozen. The appearance of these cells is fibroblastic (Fig. 1). The cells were used between the 4th and 14th passage. For the experiment the cells were thawed, and after they reached confluence, they were trypsinised out from the dish and suspended in M2.

Cultured embryonic cells

Polish Heatherhead embryonic cell lines have been produced from Day 12 embryonic discs, essentially as described earlier (Karasiewicz *et al.,* 1996) and were cultured in Dulbecco's modified Eagle medium (high glucose: 4.5 g/l, Sigma), supplemented with 0.1 mM β-mercaptoethanol, 0.1 mM nonessential amino acids, and nucleosides (Robertson, 1987), with 20% foetal calf serum. The appearance of these cell lines is epithelioid. The cells were used between the 4th and 7th passage. For the experiment the cells were trypsinised out from the dish and suspended in M2.

PKH26 staining

In some experiments fibroblasts were stained with PKH26 dye (Sigma) according to the manufacturer's instructions. Briefly, freshly diluted dye was added to the pellet of cells at a concentration of 2 x 10^{-6}M for 2-5 min at room temperature. Extensive rinsing of cells with culture medium containing 10% foetal calf serum, together with three rounds of pelleting them by mild centrifugation, prevented any cell deterioration. In fact, when followed up *in vitro* the stained cells survived at least next two passages. The cells were red fluorescent upon the use of filter set for rhodamine, in Opton inverted fluorescent microscope IM35 (Zeiss, Germany). The same dye was earlier used to mark cow blastomeres (Rho *et al.,* 2001).

Injection of fibroblasts or embryonic cells into the embryos

For manipulation the embryos were placed together with fibroblasts or embryonic cells in micromanipulation chamber, in M2 medium. The chamber was placed in Leitz Fluovert inverted microscope equipped with Nomarski Differential Interference Contrast under which all micromanipulation procedures were carried out. Leitz mechanical micromanipulators were used. Pipettes were pulled at P-97 puller (Sutter Instruments Ltd., USA) of 1 mm capillaries (Sutter Instruments Ltd., USA). Five cells were placed in the middle of a morula or within the cavity of blastocyst.

***Co-culture* in vitro**

After manipulation the embryos were placed on Vero/BRL monolayer (Duszewska *et al.,* 2000) or STO feeder layer to be cultured overnight (for transfer) or longer. The culture medium was DMEM, which was supplemented like for embryonic cells.

Transfer to recipient ewes

After co-culture the blastocysts (in groups of 2 to 5) were transferred to Heatherhead ewes, that were synchronized and FSH-injected due to the same procedures as the embryo donors, but applied one day in advance. Pregnancy was diagnosed by ultrasonography.

Tissue samples from born lambs

Two lambs were slaughtered in the slaughterhouse of Institute of Genetics and Animal Breeding. Tissues samples were immediately taken from them and were frozen at -70ºC until the time of DNA isolation.

DNA isolation

Total cellular DNA from frozen tissues of recipients (lambs), from fibroblasts and blood samples of parents, was isolated using DNeasy Blood & Tissue Kit (Qiagen, Japan) according to the manufacturer's instructions and was stored at -70ºC. The DNA concentration and quality was measured using spectrophotometry (FT-IR BIO-RAD FTS 135; USA) (at 270, 320 and 360 nm) and by electrophoresis, and the DNA samples were adjusted to a final concentration of 100 μg per ml.

RAPD method

PCR reactions were performed by using 25 μl of a mixture containing 10 mM Tris-HCl pH 8.0, 1.5 mM $MgCl_2$, with four dNTPs (0.2 mM each), 0.2 μM primers, 1.5 units of *Taq* DNA polymerase (Polgen, Poland) and 10 ng genomic DNA. 10-mer primer (CCBC – 5'AAACGGGCGG 3') synthesised in DNA Sequencing and Oligonucleotide Synthesis Laboratory, Institute of Biochemistry and Biophysics (Warsaw, Poland) and previously tested in other experiments was used. Reactions were carried out using DNA thermocycler (MJ Research, USA) programmed as follows: incubation at 94ºC for 3 min; 40 cycles at 94ºC for 1 min, 40ºC for 1 min 45 s, and 72ºC for 1 min 30 sec, followed by one final extension cycle of 10 min at 72ºC. A negative control reaction omitting template DNA was prepared each time. All amplification reactions were repeated twice to test reproducibility.

Fragment visualisation

Amplified RAPD products were separated and visualized in 2% agarose gels (20 x 10-cm) stained with ethidium bromide, with Tris-borate (0.089 M Tris, 0.089 M borate, 2 mM EDTA, pH 8.3) tank and gel buffers. An additional molecular-weight marker *1444-80* (pUC/*Bsp* R1/*Taq*) was added in the outside lane and gel running times were set so that fragments > 80bp were retained on the gels.

Computational analysis

Visual analysis of the band patterns was aided by DNA-ProScan software (DNA ProScan Inc., USA). The results were derived from the analysis performed by two researchers (inter-researcher results correlation was close to 1). The analysis included only bands representing fragments ranging from 500 to 1500 base pairs (bp). Amplified DNA fragments (bands) were scored as 1 (fragment present) or 0 (fragment absent) for each sample. Banding patterns were compared between the lanes to identify shared and non-shared bands. Bands were regarded as non-shared if they differed in their position more than half of band width and if intensity ratio was less than 1:4.

Statistic calculations

Based on the results of patterns generated by RAPD the calculations were done to compare analysed individuals and to seek genetic distance between them. Major statistical parameters of band patterns, such as band sharing (BS), based on the number of common bands between two individual samples, were used to describe the similarity between profiles of RAPD. BS was estimated with the following assumptions: a) All RAPD loci show complete dominance, b) All loci have two alleles, c) The marker alleles from different loci do not comigrate to the same position on the gel. This parameter was calculated from the formula:

$$BS = \frac{2N_{ab}}{N_a + N_b}$$, where N_{ab} is the number of bands shared and N_a and N_b are the total number of bands scored in samples A and B, respectively (Jeffreys and Morton, 1987).

Sex identification in fibroblasts and its confirmation in lambs and their parents

DNA was extracted from fibroblasts and from blood of parents of blastocysts and of lambs, using DNeasy® Blood & Tissue Kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturers' handbook. Isolated DNA was diluted in 50 μl distilled H2O and used for further analyses.

We used one set of primers for amplifying the sheep SRY gene. The DNA sequences of the primers were:
F: 5'-CTCGTGAACGAAGACGAAAGG-3') and

R: 5'-CTGTGCCTCCTCAAAGAAATGG-3'. Amplifications were performed in a final volume of 20 μl in 10 X PCR buffer (15 mM MgCl2, pH 8.3), 100 μM for each dNTP (Polgen, Lodz, Poland), with 1 M Taq DNA Polymerase (Polgen, Lodz, Poland) and 10 pmol of each primer synthesised in DNA Sequencing and Oligonucleotide Synthesis Laboratory, Institute of Biochemistry and Biophysics, Warsaw, Poland. Four μl of the DNA-extract were added to the PCR mix. The amplification was carried out with initial denaturation at 95ºC for 10 min, followed by 35 cycles of one denaturation step at 94ºC for 50 sec, primer annealing at 56ºC for 45 sec and primer extension at 72ºC for 50 sec in a MJ Research thermocycler (MJ Research, Waltham, USA). A final extension cycle was 10 min at 72ºC. PCR-products were purified using the Gen Elute^{TM}PCR Clean-up kit (Sigma, Japan) according to the manufacturers' instructions.

Amplified products were separated and visualized in 2% agarose gels (10 x 10-cm) stained with ethidium bromide, with Tris-borate (0.089 M Tris, 0.089 M borate, 2 mM EDTA, pH 8.3) tank and gel buffers. An additional molecular-weight marker *1444-80* pUC/*Bsp* R1/*Taq* (Polgen, Lodz, Poland) was added in the outside lane. The product of SRY fragment amplification was 131 bp in length.

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References

BASSAM, B.J., BENTLEY, S. (1994). DNA fingerprinting using arbitrary primer technology (APT): a tool or a torment. *Australas. Biotechnol*. 4: 232-236.

BEATTY, E.M., DOXELY, D.L., FITZSIMONS, J. (1983). Glucosephosphate isomerase polymorphism in sheep. *Anim. Blood Groups and Bioechem. Genet.* 14: 213-218.

BEDDINGTON, R.S., and ROBERTSON, E.J. (1989). An assessment of the developmental potential of embryonic stem cells in the midgestation mouse embryo. *Development* 105: 733-737.

BOEDIONO, A., SUZUKI, T., LI, L.Y., GODKE, R.A. (1999). Offspring born from chimaeras reconstructed from parthenogenetic and *in vitro* fertilized bovine embryos. *Mol. Reprod. Dev.* 53: 159-70.

BODO, S., GOCZA, E., REVAY, T., HIRIPI, L., CARSTEA, B., KOVACS, A., BODROGI, L., BOSZE, Z. (2004). Production of transgenic chimaeric rabbits and transmission of the transgene through the germline. *Mol. Reprod. Dev*. 68: 435-40.

BRINSTER, R.L. (1974). The effect of cells transferred into the mouse blastocyst on subsequent development. *J. Exp. Medicine* 140: 1049-1056.

BUTLER, J.E., ANDERSON, G.B., BONDURANT, R.H., PASHEN, R.L., PENEDO, M.C.T. (1987). Production of ovine chimaeras by inner cell mass transplantation. *J. Anim. Sci.* 65: 317-324.

CHAPMAN, V.M., ANSELL, J.D., MCLAREN, A. (1972). Trophoblast giant cell differentiation in the mouse: expression of glucose phosphate isomerase (GPI-1) electrophoretic variants in transferred and chimaeric embryos. *Dev. Biol.* 29: 48-54.

CIBELLI, J.B., STICE, S.L., GOLUEKE, P.J., KANE, J.J., JERRY, J., BLACKWELL, C., PONCE DE LEON, F.A., ROBL, J.M. (1998). Transgenic bovine chimaeric offspring produced from somatic cell-derived stem-like cells. *Nat. Biotechnol*. 16: 642-646.

CLARKE, D.L., JOHANSSON, C.B., WILBERTZ, J., VERESS, B., NILSSON, E., KALSTROM, H., LENDAHL, U., FRIESEN, J. (2000). Generalized potential of adult neural stem cells. *Science* 288: 1660-1663.

CUSHWA, W.T., DODDS, K.G., CRAWFORD, A.M., MEDRANO, J.F. (1996). Identification and genetic mapping of random amplified polymorphic DNA (RAPD) markers to the sheep genome. *Mamm. Gen*. 7: 580–585.

DUSZEWSKA, A.M., REKLEWSKI, Z., PIENKOWSKI, M., KARASIEWICZ, J., MODLINSKI, J.A. (2000). Development of bovine embryos on Vero/BRL cell monolayers (mixed co-culture). *Theriogenology* 54: 1239-1247.

FEHILLY, C.B., WILLADSEN, S.M., TUCKER, E.M. (1984). Experimental chimaerism in sheep. *J. Reprod. Fertil.* 70: 347-51.

FEDOROV, L.M., HAEGEL-KRONENBERGER, H., HIRCHENHAIN, J. (1997). A comparison of the germline potential of differently aged ES cell lines and their transfected descendants. *Transgenic Res*. 6: 223-31.

GEARHART, J.D., MINTZ, B. (1972a). Glucose phosphate isomerase subunit-reassociation tests for maternal-foetal and foetal-foetal cell fusion in the mouse placenta. *Dev. Biol.* 29: 55-64.

GEARHART, J.D. and MINTZ, B. (1972b). Clonal origins of somites and their muscle derivatives: evidence from allophenic mice. *Dev. Biol.* 29: 27-37.

GEIGER, H., SICK, S., BONIFER, C., MUELLER, A.M. (1998). Globin gene expression is reprogrammed in chimaeras generated by injection adult hematopoietic stem cells into mouse blastocysts. *Cell* 93: 1055-1065.

HADRYS, H., BALICK, M., SCHIERWATER, B. (1992). Applications of random amplified polymorphic DNA (RAPD) in molecular ecology. *Mol. Ecol*. 1: 55-63

HUFF, D.R., PEAKALL, R., SMOUSE, P.E. (1993). RAPD variation within and among natural populations of out-crossing bufalograss [Buchloee dactyloides (Nutt.) Engelm.]. *Theor. Appl. Genet*. 86: 927-934.

IWASAKI, S., CAMPBELL, K.H., GALLI, C., AKIYAMA, K. (2000). Production of live calves derived from embryonic stem-like cells aggregated with tetraploid embryos. *Biol. Reprod.* 62: 470-5

JASZCZAK, K., PARADA, R., GUSZKIEWICZ, A. (1999). Cytogenetic study of some tissues and age-related changes in cell proportions in a goat-sheep chimera. *Cytogenet. Cell Genet*. 84: 55-7.

JEFFREYS, A.J., MORTON, D.B. (1987). DNA fingerprints of dogs and cats. *Animal Genet.* 18: 1–15

JIANG, Y., JAHAGIRDAR, B.N., REINHARDT, R.L., SCHWARTZ, R., KEENE, C.D., ORTIZ-GONZALEZ, X.R., REYES, M., LENVIK, T., LUND, T., BLACKSTAD, M., DU, J., ALDRICH, S., LISBERG, A., LOW, W.C. (2002). Pluripotency of mesenchymal stem cells derived from adult marrow. *Nature* 418, 41-49.

KARASIEWICZ, J., SZABLISTY, E., GUSZKIEWICZ, A., KOSSAKOWSKI, M., STEFANSKI, G., REED, M., MODLINSKI, J.A. (1996). Development of isolated sheep inner cell masses/embryonic discs in vitro. *Roux's Arch. Dev. Biol.* 205: 437-442.

KUES, W.A., PETERSEN, B., MYSEGADES, W., CARNWATH, J.W., NIEMANN, H. (2005). Isolation of murine and porcine foetal stem cells from somatic tissue. *Biol. Reprod*. 72: 1020-1028.

LARSON, J.S., YIN, M., FISCHER, J.M., STRINGER, S.L., STRINGER, J.R. (2006). Expression and loss of alleles in cultured mouse embryonic fibroblasts and stem cells carrying allelic fluorescent protein genes. *BMC Mol. Biol*. 7: 36-50.

MACPHERSON, J.M., ECKSTEIN, P.E., SCOLES, G.J., GAJADHAR, A.A. (1993). Variability of the random amplified polymorphic DNA assay among thermal cyclers, and effects of primer and DNA concentration. *Mol. Cell. Probes* 7: 293-299.

MINTZ, B. (1962). Formation of genotypically mosaic mouse embryos. *Am. Zool.* 2: 432

MODLINSKI, J.A., REED, M.A., WAGNER, T.E., KARASIEWICZ, J. (1996). Embryonic stem cells: developmental capabilities and their possible use in mammalian embryo cloning. *Anim. Reprod. Sci.* 42: 437–446.

NOTARIANNI, E., LAURIE, S., NG, A., SATHASIVAM, K. (1997). Incorporation of cultured embryonic cells into transgenic and chimeric, porcine fetuses. *Int. J. Dev. Biol.* 41: 537-40.

ONISHI, A., TAKEDA, K., AKITA, T., HANADA, H. (2000). Production of chimaeric pigs by injection of primary cultured inner cell mass cells. *Theriogenology* 53: 237.

PILISZEK, A., MODLINSKI, J.A., PYSNIAK, K., KARASIEWICZ, J. (2007). Foetal fibroblasts introduced to cleaving mouse embryos contribute to full-term development. *Reproduction* 133: 207-218.

RAO, K.B.C., BHAT, K.V., TOTEY, S.M. (1996). Detection of species-specific genetic markers in farm animals through random amplified polymorphic DNA (RAPD). *Genet. Anal.* 13: 135-138.

RASERO, R., DI STASIO, L., BERRA, G.P. (1993). Polymorphism of erythrocyte glucosephosphate isomerase in sheep. *Anim. Genet.* 24: 63-64.

RHO, G-J., KANG, T-Y., KOCCHAR, H.P.S., HAHNEL, A.C., BETTERIDGE, K.J. (2001). Effects of blastomere sex and fluorescent labelling on the development of bovine chimaeric embryos reconstituted at four-cell stage. *Mol. Reprod. Dev.* 60: 202-207.

ROBERTSON, E.J. (1987). Embryo derived stem cell lines, In: *Teratocarcinomas and embryonic stem cells. A practical approach* (Ed. E.J.Robertson), IRL Press, Oxford, Washingon DC, pp 71-112.

SAITO, S., SAWAI, K., UGAI, H., MORIYASU, S., MINAMIHASHI, A., YAMAMOTO, Y., HIRAYAMA, H., KAGEYAMA, S., PAN, J., MURATA, T., KOBAYASHI, Y., OBATA, Y., YOKOYAMA, K.K. (2003). Generation of cloned calves and transgenic chimaeric embryos from bovine embryonic stem-like cells. *Biochem. Biophys. Res. Commun.* 309: 104-13.

SCHOONJANS, L., ALBRIGHT, G.M., LI, J.L., COLLEN, D., MOREADITH, R.W. (1996). Pluripotential rabbit embryonic stem (ES) cells are capable of forming overt coat color chimeras following injection into blastocysts. *Mol. Reprod. Dev.* 45: 439-43.

SUWINSKA, A., OZDZENSKI, W., WAKSMUNDZKA, M., TARKOWSKI, A.K. (2005). Experimentally produced diploid-triploid mouse chimaeras develop up to adulthood. *Mol. Reprod. Dev.* 72: 362-76.

TAKAHASHI, K., YAMANAKA, S. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* 126: 663-76.

TARKOWSKI, A.K. (1961). Mouse chimaeras developed from fused eggs. *Nature* 190: 857-860.

TARKOWSKI, A.K., OZDZENSKI, W., CZOLOWSKA, R. (2001). Mouse singletons and twins developed from isolated diploid blastomeres supported with tetraploid blastomeres. *Int. J. Dev. Biol.* 45: 591-596.

TUCKER, E.M., MOOR, R.M., ROWSON, L.E. (1974). Tetraparental sheep chimaeras induced by blastomere transplantation. *Immunology* 26: 613-21.

WANG, Z.Q., Kiefer, F., Urbanek, P., Wagner, E.F. (1997) Generation of completely embryonic stem cell-derived mutant mice using tetraploid blastocyst injection. *Mech. Develop.* 62: 137-145.

WEST, J.D., Flockhart, J.H. (1994). Genotypically unbalanced diploid–diploid foetal mouse chimaeras: possible relevance to human confined mosaicism. *Genet. Res.* 63: 87–99. Erratum in: *Genet. Res.* (1994) 63:†236.

WILLIAMS, J., Kubelik, A., Livak, K., Rafalski, J.A., Tingey, S. (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res.* 18: 6531–6535.