Hypodiploidy as a prominent attributor to chromosomal aneuploidy in transgenic rabbit embryos

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ABSTRACT: Transgenic animals play a vital role in basic research, agriculture and pharmaceutical industries. Rabbits have the advantage of other large laboratory species in that they have a short gestation period and yield large numbers of embryos. Production of transgenic rabbits has been directed towards using the rabbit as a model for large domestic animals or as a basic biological model for studying the mammalian gene regulation. In connection with their use, developmental and health disorders have also been reported in genetically modified animals. Random integration of a transgene can disrupt the function or regulation of an endogenous gene, resulting in insertion mutations or chromosomal aneuploidy. Chromosomal abnormalities affect the developmental potential of early embryos and serve as potential predictors of developmental outcome. This study was aimed at analyzing the cytogenetic profile of transgenic rabbit embryos, which is necessary for selecting optimal lines for dissemination in order to eliminate animals with chromosomal aberrations. Conventional Giemsa stained c-metaphase spreads obtained from blastomeres of intact as well as microinjected transgenic (EGFP and hFVIII) and non-transgenic embryos revealed a significantly higher ($P < 0.01$) rate of aneuploid cells in transgenic rabbits compared to non-transgenic animals. However, microinjection did not seem to influence the rate of aneuploidy, as the incidence of aneuploidy in non-transgenic blastomeres was significantly lower ($P < 0.01$) in comparison with intact ones (14.3 vs. 73.33%). The findings suggest hypodiploidy as the prominent attributor to the occurrence of aneuploidy. This is the first report of 100% chromosomal aneuploidy in the embryos of both EGFP and hFVIII transgenic rabbits.

Keywords: chromosomal aneuploidy; EGFP; embryo; hFVIII; rabbit; transgenic

The latest progress of biological sciences is intensively related with the development of genetic technologies focused on regulation and modification of biological processes in farm animals. Today, transgenic animals embody one of the most potent and exciting research tools in biological sciences. Transgenic animals represent unique models that are custom tailored to address specific biological questions. Hence, the ability to introduce functional genes into animals provides a very powerful tool for dissecting complex biological processes and systems. This has made it possible to explore the regulation of gene expression as well as the regulation of cellular and physiological processes. Significant uses of live transgenic mammals are in the areas of agricultural, biological, biotechnological and biomedical sciences including the production of pharmaceuticals and in the field of organ transfer from transgenic animals to humans. Experimental designs have taken advantage of the ability to direct specific expression (including cell types, tissue, organ type, and a multiplicity of inter-

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nal targets) and ubiquitous, whole-body expression *in vivo*. Transgenic farm animals are important in human medicine as sources of biologically active proteins, as donors in xenotransplantation, and for research in cell and gene therapy. Typical agricultural applications include improved carcass composition, lactation performance and wool production, as well as enhanced disease resistance and reduced environmental impact.

Transgenic rabbits provide a useful biological model for the study of the regulation of mammalian genes. Transgenic rabbits have thus become a useful organism in genome, gene expression, gene regulation studies and also in recombinant protein production from the mammary gland (Stromqvist et al., 1997). Reciprocal chromosome painting has revealed that rabbit chromosomes 12, 19 and X are completely homologous to human chromosomes 6, 17 and X, respectively (Korstanje et al., 1999). They have also shown that all other human chromosomes are homologous to two or sometimes three rabbit chromosomes. Many other conserved chromosome segments found previously in other mammals (e.g. cat, pig, cattle, Indian muntjac) were also found to be conserved in rabbit chromosomes (Korstanje et al., 1999).

However, the effects of genetic engineering on animal health and welfare are of significant public concern (Mench, 1999). Developmental and health disorders in genetically modified animals have also been reported in conjunction with its use; therefore, researchers must take care to minimize animal suffering. It has been reported that genetically modified animals may exhibit health problems throughout their life. They may age prematurely as Dolly was diagnosed with arthritis at a seemingly young age and cloned mice had a shorter than normal life span. On the other hand, incidental integration of transgene construct may affect the function and regulation abilities of endogenous genes, which leads to insertion mutations (Palmiter and Brinster, 1986) or chromosomal aneuploidy (Goepfert et al., 2000). Aneuploidy means gain or loss of chromosomes caused by a meiotic or mitotic non-disjunction event, which may lead to violation in gene stability or to heterozygous loss (Goepfert et al., 2000). Aneuploidy is also a common feature of human tumours, often correlating with poor prognosis. As a group, aneuploid individuals have distinct and characteristic phenotypic features. The phenotypic effects range from minor physical symptoms to devastating and lethal deficiencies in major organ systems. These are often accompanied by behavioural deficits and mental retardation. Chromosomal aberrations accompanied by the production of transgenic animals could be among the causes of their health problems. Therefore, the chromosomal aneuploidy analysis of transgenic rabbits can be viewed as a potential technique for detecting and controlling the health status of transgenic offspring and their potential use for production of transgenic lines.

Based on the considerations mentioned above, the present investigation aimed to compare the frequency of chromosomal aneuploidy between transgenic (EGFP and hFVIII positive) and non-transgenic rabbit embryos at blastocyst stage.

MATERIAL AND METHODS

Biological material

Our experiments were conducted on the rabbits of broiler lines of New Zealand White breed having a weight of 3.5–4.0 kg at an age of 4–5 months from a rabbit facility at the Slovak Agricultural Research Centre in Nitra. The animals were stabled in air-conditioned halls in single-deck individual metallic cages of standard size. Water was supplied through automatic feed-pumps and animals were fed complete feed mixture KK V1. The light conditions were maintained at 14 h of daylight and 10 h of darkness.

19–20 h after the mating of hormonally treated donors (PMSG and HCG; Chrenek et al., 2005), the pronuclear stage eggs were flushed from the oviducts of the animals with PBS. The selection of flushed eggs was done in CIM medium + 10% FCS (Gibco BRL, USA). The embryos were cultured in *in vitro* conditions (5% CO_2, 39°C, k-DMEM + 10% FCS; Gibco BRL, USA) as reported by Chrenek et al. (2005).

Gene constructs

An EGFP (Enhanced Green Fluorescence Protein) reporter gene commercially obtained from Clontech of size 4.7 kb (Genebank U55762) with CMVIE (Cytomegalovirus) promoter was used for microinjection into fertilized rabbit eggs after linearization of the plasmid (Clontech, USA) with *Cla*I. Gene construct was prepared at a concentration of 5 µg/ml, at a copy number of 350/pl.

The second construct was used for microinjection consisting of a 2.5 kb murine whey acidic protein promoter (mWAP), 7.2 kb cDNA of the human clotting factor VIII (hFVIII), and 4.6 kb of 3' flanking sequences of the mWAP gene. This gene was provided by the late H. Lubon (American Red Cross, Rockville, USA). The plasmid was digested with *Not* I to release the 14.3 kb insert and purified in a Qiaex II gel extraction kit (Qiagen, USA). Gene construct was prepared at a concentration of 5 µg/ml, with a copy number of ca. 350/pl. Aliquots of the same DNA preparation were used for all injections.

Microinjection of foreign DNA and culture of rabbit transgenic embryos *in vitro*

After the evaluation of the flushed ova, eggs with both pronuclei were subjected to microinjection in CIM medium + 10% foetal bovine serum (FBS, both from Gibco BRL, USA) using an Olympus microscope equipped with micromanipulation units (Alcatel, France) and microinjector (Eppendorf, Femto Jet, Germany). The eggs were fixed by suction with a holding pipette, and 5 µg/ml of DNA (EGFP) in 1–2 pl was microinjected using air pressure (Pc – compensation and Pi – injection pressure, with injection time) into one pro-nucleus (single microinjection, SM) or into both pronuclei (double microinjection, DM), as described (Kupriyanov et al., 1998; Chrenek et al., 2005). Swelling of the pronuclei by 10% indicated successful microinjection. Then the eggs were transferred to four well dishes (NUNC, Denmark) and cultured in k-DMEM medium supplemented with 10% FBS (Gibco BRL, USA) at 5% CO_2 and 39°C up to the blastocyst stage (72 h PC) for the analysis of transgene (EGFP) integration and expression and chromosomal aneuploidy.

Chromosomal analysis from embryos

Individual transgenic embryos carrying either hFVIII gene (detected by PCR analysis as described by Chrenek et al., 2005) or EGFP gene (detected by fluorescence analysis as described by Ikawa et al., 1995 and Chrenek et al., 2005) as well as non-transgenic control embryos for analysis were taken from *in vivo* or *in vitro* fertilization. The first step involved synchronization of embryo donor females using PMSG and HCG hormones for superovulation purposes. Donors of embryos were slaughtered 24 h after HCG treatment. Mating process for obtaining ovaries and oviducts with uterus was performed. In laboratory conditions embryos were flushed out from oviducts using PBS medium (Gibco BRL, USA) supplemented with FBS, transferred to CO_2 independent medium (Gibco BRL, USA) for selection purposes and then immediately transferred to *in vitro* "knock-out" (Gibco BRL, USA) culture medium and stored in a CO_2 incubator at 39°C. Then they were either used for transgenesis in pronuclear stage or kept for natural development to higher stages in CO_2 incubator conditions. Embryos at various stages were known to be used for chromosomal evaluation. In blastomeres, the nuclear cycle (embryo cells up to blastocyst stage) for chromosomal aneuploidy detection was synchronized into metaphase stage. Synchronization was done by the addition of colcemide into the "knock-out" culture medium, where approximately 30 µl of colcemide was added into 500 µl of culture medium with embryos within at least 1 h of incubation. Another possible way was the addition of nocodazole (Sigma, USA) 15 µl or more from diluted solution into culture medium for the same purpose as colcemide but the incubation took at least 12 h, which is an approximate time for embryos to reach the subsequent higher stage. After the culture of transgenic embryos for 60 min in 0.6 µg/ml colcemide at 39°C (Life Technologies, USA), they were treated with 0.5% pronase solution (Sigma, USA) for 3.5 min in order to facilitate the removal of *zona pellucida*. After the removal of *zona pellucida*, embryos were transferred to 0.5 ml hypotonic solution of KCl (0.075 M) for cultivation in thermobox conditions for approximately 20 min at a temperature of 37°C. In advance, microslides were cleaned with benzyl alcohol, and a small drop of a fixative solution of methanol:distilled water:acetic acid (5:4:1) was put on the slide and hypotonized blastomeres were transferred into the fixative 5:4:1 solution for 5 min. After five min, drops with embryos on slides were recovered with 5 µl of modified Carnoy's solution (methanol:acetic acid at 3:1). It is important that both the fixative solutions be freshly prepared and cooled to 4°C. Dried microscopic slides with embryonic cell samples were then stained with 2% Giemsa solution for 7 min.

Chromosomal staining

Chromosomes were stained with 2% Giemsa solution prepared from 98 ml MILI Q water sup-

Table 1. Distribution of c-metaphases of rabbit transgenic and non-transgenic embryos according to ploidy

Blastomeres	Diploidy	Polyploidy	Hypodiploidy	Hyperdiploidy	Aneuploidy Σ
Non-transgenic microinjected (*n* = 14)	12 (85.7%)	0 (0%)	1 (14.3%)	0 (0%)	1 (14.3%)
EGFP (*n* = 20)	0 (0%)	0 (0%)	20 (100%)	0 (0%)	20 (100%)
hFVIII (*n* = 28)	0 (0%)	0 (0%)	28 (100%)	0 (0%)	28 (100%)
Intact (*n* = 30)	8 (26.67%)	0 (0%)	22 (73.33%)	0 (0%)	22 (73.33%)

plemented with 2 ml of Giemsa (Gibco BRL, USA) pH = 6.8 for 5–10 min. After staining, coloured slides were gently washed with distilled water and dried at room temperature.

Microscopic slide preparation

Microscopic slides were cleaned with benzyl alcohol. Clean and dried microscopic slides were stored in freezer or in clean box at room temperature, depending on the purpose of use.

Microscopic observations

Stained slides were checked under the Leica microscope and chromosomal analysis was carried out from microphotographs.

Statistical analysis

The χ^2-test was used to compare the chromosomal aneuploidy between transgenic and non-transgenic rabbit embryos.

RESULTS AND DISCUSSION

Cytogenetic analysis was carried out from blastomeres of intact as well as microinjected transgenic (EGFP and hFVIII) and non-transgenic rabbit embryos by conventional Giemsa stained c-metaphase spreads. A significantly higher ($P < 0.01$) rate of aneuploid cells was observed in transgenic rabbits compared to non-transgenic animals (Table 1). On the other hand, non-transgenic rabbits exhibited a significantly higher rate ($P < 0.01$) of diploid somatic cells (85.7% in non-transgenic microinjected and 26.67% in intact blastomeres as against 0% in the case of both EGFP and hFVIII transgenes) (Figure 1).

Microinjection did not seem to influence the rate of aneuploidy, as the incidence of aneuploidy in non-transgenic blastomeres was significantly lower ($P < 0.01$) in comparison with intact ones (14.3 vs. 73.33%) (Figures 1B, 2A and 2B). All the embryos microinjected with EGFP and hFVIII gene were found to be aneuploid (Figures 3 and 4), with the total occurrence of hypodiploidy (100%). In fact, hypodiploidy emerged out to be the primary attributor to the occurrence of aneuploidy in all the groups of blastomeres. Also, transgenic rabbits exhibited lower numbers of diploid somatic cells than their non-transgenic counterparts ($P < 0.01$).

Chromosomes have a supreme importance because they carry the coded messages or genes that transmit inherited characteristics from one generation to another. Morphological examination provides information about various parameters of embryo quality. In 7-day embryos of good quality the total cell number, mitotic index, karyotype and the DNA content of the interphase nuclei were studied (Diskin, 1986). This is because while the evaluation of embryos based on morphological characteristics remains subjective, the study of cytological parameters may result in a better estimation of the embryo quality (Diskin, 1986). By establishing the total cell number and the mitotic index, King et al. (1979) acquired more information about the stage of development and the mitotic activity of the embryo. Karyotype analysis before embryo transfer may provide opportunities for sex determination (Singh and Hare, 1980) and for the detection of chromosomal abnormalities (Hare et al., 1980; King et al., 1981). The study of DNA contents of interphase nuclei may also lead to additional information about the mitotic activity and to the identification of polyploid embryos (Diskin, 1986). Cytogenetic analyses are also a good marker for the mutagenicity test in transgenic laboratory animals (Shephard et al., 1994). This can be explained on the basis of the fact that chromosomal abnormalities affect the developmental potential of

early embryos and may serve as potential predictors of developmental outcome (King et al., 2006).

In the present study, cytogenetic analysis was carried out for transgenic and non-transgenic rabbits. Our data demonstrates that there is a significant increase in chromosomal aberrations in transgenics compared to the non-transgenic rabbit offspring. This report of higher numerical chromosomal aberrations, or aneuploidies, in transgenic rabbits than in non-transgenic rabbits is consistent with the reports of Parkanyi et al. (2004) and Chrenek et al. (2005) as well as Zartman and Fechheimer (1967), who claimed that the rate of aneuploid cells in mammals was genetically determined. Obviously, transgenic rabbits are genetically different from non-transgenic ones. The differential rates of aneuploidy may arise due to differences in the used cultures and methods of recording. The work of Karp and Smith (1975) suggested the probable influence of temperature on *in vitro* cultured oocytes which resulted in abnormal numbers of chromosomes. In general, the incidence of chromosomal aneuploidies may depend on many factors, such as the source of the cells, animal species, age of animal, cell culture conditions, etc.

Non-transgenic rabbits exhibited a higher diploid somatic cell content. This is so probably because transgenic rabbits have a significantly lower level of mitotic non-disjunction (Goepfert et al., 2000). According to these authors, the molecular events that interfere with appropriate chromosome segregation and result in aneuploidy are not well understood and are likely to involve multiple targets. For instance, it is assumed that aneuploidy is generated by numerous factors including interference with mitotic spindle dynamics, abnormal centrosomes, duplication, altered chromosome condensation and cohesion, defective centromeres, and the loss of mitotic checkpoints (Goepfert et al., 2000). In this study, all the aneuploid cells in transgenic rabbits were attributed by hypodiploidy. However, hypodiploid cells occurred in both transgenic (100%) and non-transgenic (14.3% in microinjected and 73.33% in intact, respectively) rabbit blastocysts at different frequencies. This report is consistent with the works of Gómez et al. (2006), who compared the incidence of aneuploidy in *in vitro* fertilized domestic cat embryos (DSH-IVF) with that of African wildcat (AWC) cloned embryos reconstructed with AWC fibroblast donor cells from different passages (AWC-NT). They reported that hypoploidy was the most common chromosomal abnormality in either AWC-NT or DSH-IVF embryos, and it occurred more frequently than hyperploidy. In another study carried out using peripheral blood lymphocytes and bone marrows, Curlej et al. (2007) also reported a higher frequency of hypodiploid cells in transgenic as well as non-transgenic rabbits.

A higher proportion of hypodiploid cells in rabbit intact blastocysts against a relatively low proportion of such cells in microinjected, non-transgenic blastocysts could be the result of random selection of embryos. These embryos were not selected according to any specialized criteria for quality, while microinjected and transgenic embryos were of high quality because they were selected keeping in mind all the criteria to perform further manipulation in this investigation of microinjection. However, the low level of chromosomally abnormal cells is not thought to be detrimental, because these cells can be eliminated in early development or diverted to extraembryonic structures (Ward et al., 1993). Also, extensive and efficient reprogramming steps occur soon after fertilization and also probably during early embryogenesis to reverse completely the differentiated state of the gamete and to achieve totipotency or later on pluripotency of embryonic cells (Fulka et al., 2008). Parkanyi and his colleagues (2004) reported 34–66% of hypodiploidy from peripheral blood lymphocytes of transgenic rabbits. In general, *in vitro* produced embryos exhibit a higher rate of abnormalities than *in vivo* produced embryos (Viuff et al., 1999; Slimane et al., 2000). In bovines, the embryo culture alone doubled the chromosomal abnormality frequency (Slimane et al., 2000). The rate of aneuploidy in different studies also varied depending on the cell source. Thus, about 17% of cells from rabbit blood cultures were aneuploid (Zartman and Fechheimer, 1967), 18% from rabbit lymphocytes, about 35% from bone marrow (Parkanyi, 1981), 63% from rabbit blastocysts (Austin, 1967) and as high as 83% from cumulus cell nuclear transfer-derived rabbit embryos (Shi et al., 2004). Chromosomal anomalies were also observed in about 46% of *in vivo* fertilized rabbit embryos at the blastocyst stage and in more than 75% of rabbit nuclear transfer-derived embryos (Shi et al., 2004). Karp and Smith (1975) reported that 46.4% of oocytes cooled to 23°C had abnormal numbers of chromosomes. Moreover, chromosomal abnormalities were recorded as a cause of lower fertilization *in vitro* (Mahmoud, 2001) and early embryonic death (Mahmoud et al., 2002).

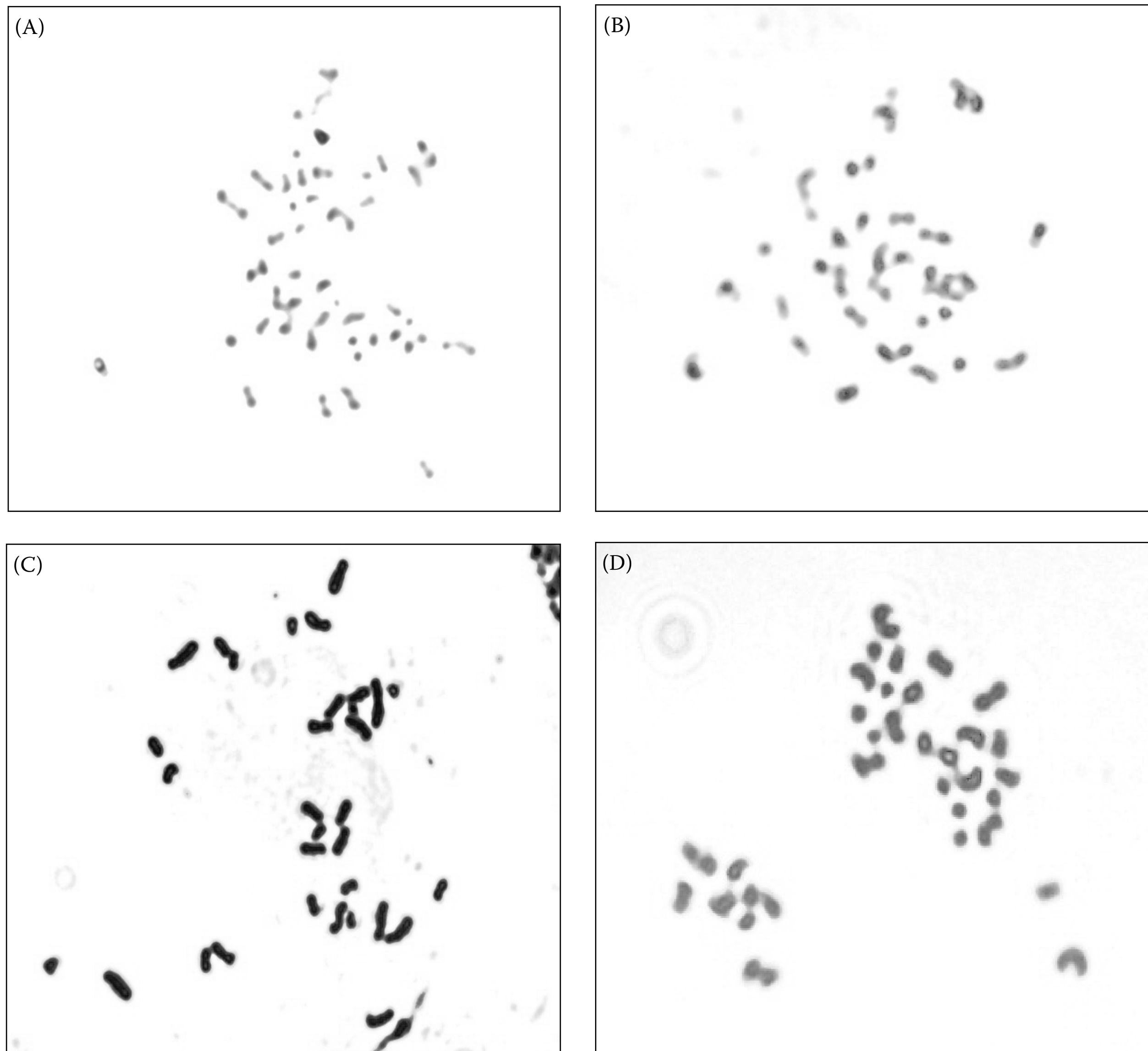

Figure 1. (A) Diploid c-metaphase of a non-transgenic microinjected rabbit detected from embryo, 2n = 44; (B) Hypodiploid c-metaphase of an intact non-transgenic rabbit detected from embryo, 2n < 44; (C) Hypodiploid c-metaphase of an EGFP transgenic rabbit detected from embryo, 2n < 44; (D) Hypodiploid c-metaphase of an hFVIII transgenic rabbit detected from embryo, 2n < 44

Furthermore, DNA aneuploidy of single blastomeres is common in rabbits and embryonic viability was not affected by the presence of aneuploid blastomeres; thus a limited number of abnormal blastomeres was compatible with normal preimplantation development (Schumacher et al., 1993). Shi et al. (2004) demonstrated significantly higher chromosomal aneuploidy rates in cumulus cell-nuclear transfer rabbit embryos and embryos derived from the nuclear transfer of rabbit fibroblasts into bovine oocytes, compared to *in vivo* fertilized rabbit embryos. The incidence of chromosomal abnormalities was correlated with subsequent developmental failure. This suggested that the genetic manipulation of embryos may also lead to increased aneuploidy. But the mode of microinjection (SM or DM) did not have any considerable impact on the rates of aneuploidy (Parkanyi et al., 2004; Chrenek et al., 2005). The type of medium used to grow rabbit cells was also found to influence their chromosomal stability (Earley, 1976). The karyotype stability of rabbit cells *in vitro* is well known to correlate with the origin of the cell lines as well as with their age in culture (Yang and Rosanoff,

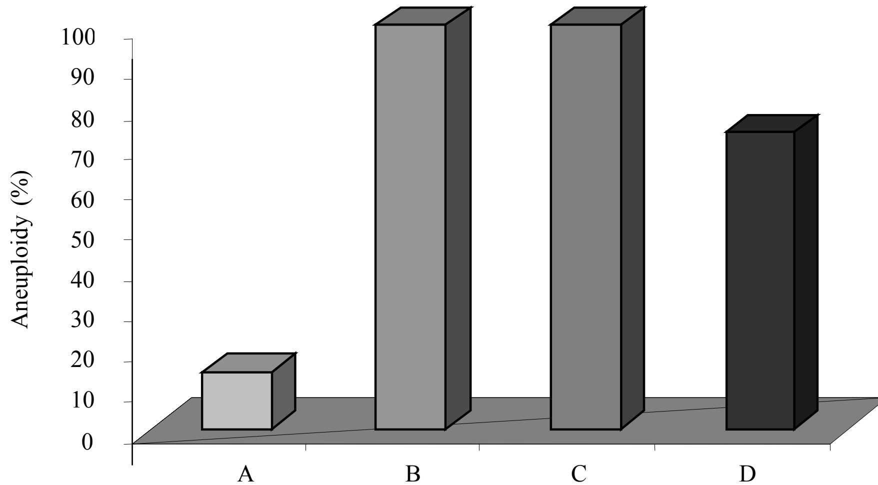

Figure 2A. Comparison of the average frequency of aneuploidy in non-transgenic microinjected and intact rabbit blastocysts

1977). Lines derived from the cornea, spleen and kidney also showed high frequencies of polyploidy in early passages and in later passages some cells became pseudodiploid and hyperdiploid. Thus, the higher percentage of chromosomal aneuploidy may be explained by the older age of rabbits and different stabling conditions such as air ventilation, the way of slurry removal (Parkanyi et al., 2004). A similar reason pertaining to the age of the animals was reported by Hughes (1968), who found in the experiments with Syrian hamsters of different ages that aneuploidy was present only in 16-months-old hamsters. Another possible cause of chromosomal anomalies may be the detrimental effect of superovulation on chromosomal complements as shown by mouse preimplantation embryos and human unfertilized oocytes (Ma, 1995). This resulted from a disturbance at maturation division as well as from a simple dose-response relationship between the PMSG dose and the incidence of polyploidy, including triploidy and tetraploidy in CD-1 mouse embryos after superovulation or the blockage of the meiosis process at the meiosis I level after superovulation in human unfertilized oocytes.

In her investigation using multi-colour FISH DNA probes Bielanska (2002) addressed the identification of the chromosomally mosaic human embryos at all stages of preimplantation development, from the 2-cell stage to the blastocyst stage, which comprised diploid, aneuploid, "chaotic", haploid, and polyploid chromosome patterns. It was further reported that compared to blastocysts, arrested embryos or embryos at the earlier stages of development showed a much higher incidence of mosaicism involving "chaotic" imbalances for multiple

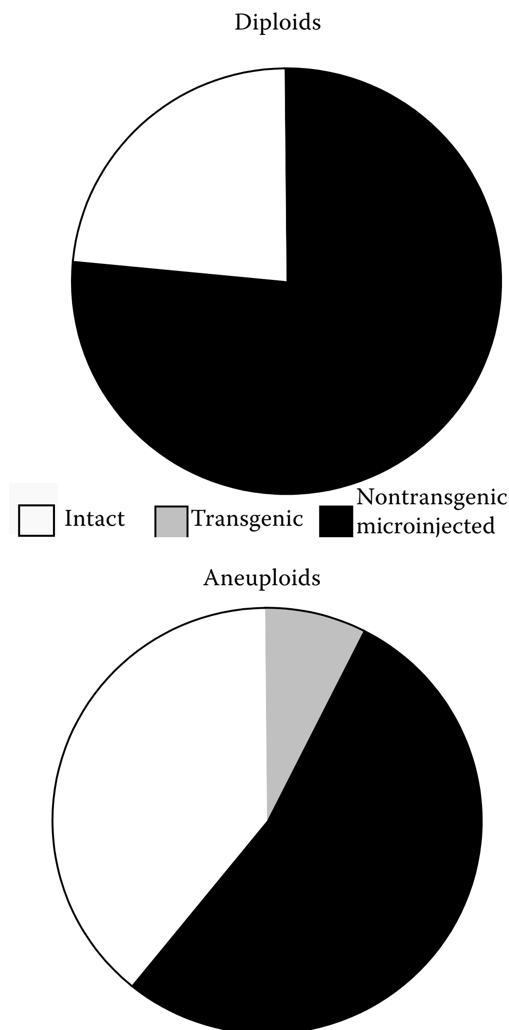

Figure 2B. Comparison of the average frequency of diploid (a) and aneuploid (b) cells between transgenic and non-transgenic rabbit embryos

chromosomes and/or high percentages of abnormal cells (Bielanska, 2002). Bronson et al. (1995) revealed aneuploidy in humans from several embryonic stem cell-derived male offspring. Treatment of pregnant mice with low, environmentally relevant doses of BPA during mid-gestation translated into an increase in aneuploid eggs and embryos in the mature female (Susiarjo et al., 2007).

A significant difference was found in aneuploidies between hFVIII transgenic and non-transgenic peripheral blood cells (Parkanyi et al., 2004; unpublished observations) but without any deleterious effect on the health or reproduction of animals. On the other hand, Curlej et al. (2006) reported 37.78% aneuploidy in hFVIII transgenic rabbits from peripheral blood culture in comparison with 37.22% in non-transgenic ones and hence concluded that there were no significant differences in the rate of aneuploidy between transgenic and non-transgenic rabbits. Roychoudhury et al. (2007) also analyzed chromosomal aneuploidy in the F4 generation transgenic rabbits with mWAP-hFVIII fusion gene detected from bone marrow lymphocytes with no significant difference between transgenic and non-transgenic animals. They also suggested that aneuploidy was caused mainly by the hypodiploid cells ($2n < 44$), and hyperdiploid cells ($2n > 44$) occurred at lower (3–30%) frequencies in both transgenic and non-transgenic animals, while the occurrence of polyploid cells ($\geq 3n$) was very low in the transgenic as well as non-transgenic rabbits (0–20%) studied. All these reports are in accordance with the current findings which support the occurrence of a considerably high proportion of aneuploidy, particularly hypodiploidy in transgenic rabbits.

CONCLUSION

Information about the aneuploidy level evaluated in various cell divisions could be a good marker to show possible genetic defects which might have serious negative effects on the health, production and reproduction abilities of animals. Neither embryos nor offspring with a higher aneuploidy level should be used for production or reproduction purposes. Therefore, it is important to analyze the chromosomal aneuploidy of genetically modified animals in order to be used for the production of transgenic lines. As chromosomal abnormalities affect the developmental potential of early embryos and serve as potential predictors of developmental outcome, the analysis of chromosomal aneuploidy becomes vital as a means of detecting and controlling the health status of transgenic animals. Therefore, the analysis of cytogenetic profile of the transgenic rabbits becomes necessary for selecting the optimal lines for dissemination in order to eliminate animals with chromosomal aberrations.

However, there are insufficient reports of aneuploidy from the embryo. Further research is also required to improve the effectiveness of chromosomal aneuploidy detection from embryonic cells. This is the first report that demonstrates 100% occurrence of chromosomal aneuploidy from embryos predominantly attributed by hypodiploid cells in both EGFP and hFVIII transgenic rabbits, as compared to non-transgenic animals.

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