Modifications of pig genome with expression gene constructs to produce organs resistant to acute transplant rejection

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ABSTRACT :

Among all animal species, the pig has the greatest number of anatomical and physiological traits similar to those of humans and thus it seems to be a promising donor of xenogenic organs. The greatest barrier for xenotransplantation in the pig-primate system is connected with the rejection of the transplanted organ via a cascade of immune mechanisms, including hyperacute rejection (HAR), acute humoral transplant rejection, acute cellular rejection and chronic rejection. Many different strategies of genetic modifications in pigs promote adaptation of their tissues and organs to functioning within organisms of their recipients following the transplantation procedure. These changes include e.g. expression of human factors CD39, CD47 and thrombomodulin (TM).

Three expression gene constructs were designed and prepared, containing coding sequences for factors CD47, CD39 and human thrombomodulin regulated with the EF-1α promoter. Gene constructs were used to transfect porcine embryonic fibroblasts by lipofection. Selected cell lines containing individual transgenes were subjected to molecular analysis, which showed integration of introduced transgenes. Cytogenetic analysis showed expression on the protein level in all tested lines and did not detect any chromosomal aberrations in the analyzed transgenic cell lines. Prepared gene constructs were prepared to produce transgenic animals by microinjection. Transgenic cell lines will provide nuclei to produce animals by somatic cloning.

Keywords: xenotransplantation, transplant rejection, gene construct, domestic pig.

INTRODUCTION:

Deficit of organs and tissues from deceased donors, required for transplantation purposes, is a considerable problem nowadays. The extending life expectancy in humans has led to an increase in the number of patients suffering from chronic diseases and end-stage organ failure. Additionally, organ failure is also observed in newborns and in infants. The gap between the number of available organs and the number of patients waiting for a transplantation has been increasing from year to year. Despite successful introduction of kidney and liver transplantation from related donors it seems hardly plausible to meet the needs of all patients waiting for transplantation [1].

What alternatives do we have for human organs and cells? Despite recent advances in stem cell biology and tissue engineering, clinical applications of techniques in the above mentioned areas are still to be developed. A readily available source of organs, tissues and cells of animal origin (transplantation between species, xenotransplantation) would solve the existing problem. Insight into barriers for xenotransplantation requires significant development of molecular techniques and progress in genetic engineering. The greatest advantage of xenotransplantation is connected with the potential for genetic modifications of the donor, facilitating control over problems related with rejection of the transplanted organ.

Genetically modified domestic pigs seem to be the

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most appropriate donors of organs for xenotransplantation [2]. It was found that due to the physiological similarity pigs are the most suitable organ donors for humans. Pigs are easy and cheap to breed, they reproduce rapidly and have numerous offspring. The diversity of breeds facilitates adaptation of organ size to different recipients. Those animals also have similar anatomical and physiological parameters, such as urine osmolarity or arterial pressure [3,4]. Due to genetic differences between the pig and the human a transplantation of organs from pigs would be rejected immediately, thus it is necessary to humanize these animals, which is a process consisting in the introduction of human genes to the pig genome.

Increasing availability of genetically modified pigs has facilitated progress in experiments connected with transplantations in the pig-primate system, excluding humans. Pharmacological treatment with the use of immunosuppresants made it possible to a considerable degree prevent graft rejection thanks to the application of T lymphocytes and cell-dependent antibody response. However, immune response after transplantation of porcine organs to primates turned out to be a significant obstacle, which has not been overcome to date [5]. F animals. However, production of transgenic pigs with the hCD39 gene to date has not provided expected results, since modified animals had survival periods of less than three months [6,7]. For this reason many research teams are currently attempting to modify GalT-KO pigs in terms of factors involved in acute vascular rejection (CD39, TM, TFPI), trying to show whether such changes lead to significant protection of transplanted organs against DXR [8].

The important factor limiting phagocytosis is CD47 - surface antigen that is recognized as a 'marker of self'. The interaction of CD47 and SIRPα gives a signal to macrophages that results in the phagocytosis inhibition. SIRPα is a receptor expressed on the surface of macrophages and it plays a significant role in the regulation of their activation [9,10]. It was shown that the SIRPα receptor interacts with factor CD47 in this way preventing phagocytosis. Although it was reported that hSIRPα might bind to pCD47, interaction of pCD47 with hSIRPα does not supply the inhibiting signal to human macrophages. It was demonstrated that expression of hCD47 in pig cells reduced susceptibility to phagocytosis by human macrophages *in vitro* [11]. Both *in vitro* and *in vivo* tests showed that the above mentioned modification may considerably reduce susceptibility of porcine cells to phagocytosis of murine macrophages. Studies indicate that that interspecies incompatibility in the case of factor CD47 contributes to phagocytosis of xenogenic cells by macrophages, which suggests that genetic modification of the donor CD47 will improve interaction with the SIRPα receptor of the recipient and will prevent transplant rejection, which is mediated by macrophages. Due to a lack of the cross reaction between CD47 and SIRPα in other species and the low degree of similarity in amino acid sequences between the human and porcine CD47 sequences, a lack of the cross reaction between porcine CD47 and human SIRPα is also a significant mechanism leading to phagocytosis of porcine cells by human macrophages. For this reason a detailed investigation of reactivity between human and porcine factors should facilitate understanding of mechanisms of macrophage xenoreactivity and as a consequence -overcoming acute cell rejection [12].

Thrombomodulin (TM) is an integral membrane protein that participates in the inhibition of the coagulation process. Miwa *et al*. demonstrated that pig aortic endothelial cells transfected with hTM significantly suppressed prothrombinase activity and delayed human plasma clotting [13]. Iwase *et al*. indicated that platelet aggregation induced by GTKO/CD46/TM pig aortic endothelial cells was significantly lower than that induced by WT or GTKO [14].

The imbalance between pro and anticoagulant activities in pig-to-primate model may be overcome by a addition of the expression of human anti-coagulation genes (TM, CD39 and/or CD47) in pig organism.

MATERIALS AND METHODS :

Genetic constructs

The biological material used in the experiments comprised human peripheral blood and embryonic fibroblasts of the domestic pig (*Sus scrofa*). Sequences coding factors CD39, CD47 and thrombomodulin were prepared based on total RNA isolated from human peripheral blood lymphocytes using guanidinium thiocyanate (GTC) method. Single-stranded cDNA was synthesized using a Stratagene AccuScript High Fidelity RT-PCR System kit. In order to amplify sequences coding factors CD47 (972 bp), CD39 (1554 bp) and TM (1728 bp) PCR was run with the following primers: CD47-F*Kpn*I and CD47-R*Xba*I for CD47, CD39-F*Eco*RI and CD39-R*Xba*I for CD39, and TM-F*Eco*RI and TM-R*Xba*I for the TM factor. The reaction was run under the following conditions: 94^0C for 240 s; 94^0C for 60 s; 54 - 62^0C for 60 s; 72^0C for 90-180 s; 300 - 1200 s; the number of cycles, 35. The PCR products were hydrolyzed with respective restriction enzymes and they were cloned in the pTracer EF/Bsd A vectors (5987 bp).

Transfection

In order to perform lipofection non-transgenic cell lines were produced from porcine embryonic fibro-

blasts. Cells were cultured on DMEM with an addition of 10% FBS and 1% respective antibiotic at a temperature of 37 ^{0}C at 5% CO2. Transfection was performed on cells with min. 80% viability and 50% confluence. Prior to transfection fresh medium containing no FBS or antibiotic was supplemented and it was incubated for 2 h at 37 ^{0}C. The lipofection mixture containing 1 ml DMEM, 16 µl lipofectamine and 10 - 20 µg plasmid was introduced to culture vessels. Cell lines with no plasmid added constituted the negative control. After 16 h incubation at 37 ^{0}C the medium containing the lipofection medium was removed and a medium containing serum and the antibiotic was added. After 72 h a selection medium was applied (DMEM, 10% FBS, 1% antibiotic, blasticidin). After 4 days of selection the control lines were removed from culture and the other lines were cultured with the use of a medium containing no blasticidin. After a culture with 70 - 80% confluence was obtained, integration was analyzed using PCR and expression was verified using flow cytometry.

Integration analysis

Integration of transgenes introduced to porcine embryonic fibroblast cells was analyzed using PCR. The reaction was run using two pairs of primers for each transgene. F primers for each transgene were located in the EF-1α region, while R primers were found in the coding factors CD47, CD39 or TM, depending on the construct. The above selection of primers facilitated amplification of unique sequences, which are not naturally found in the pig genome. The second reaction, whose product differs in size from the first one, was run to confirm the obtained result and eliminate false positive results. The positive control for the reaction comprised the gene construct, which in a given was used in transfection.

Expression analysis

Transgene expression was analyzed with the use of a flow cytometer. For this purpose cell cultures with confluence of 70 - 80% were washed with 3 ml Hanks' Balanced Salt solution and tripsinized (0.25% tripsin, 0.02% EDTA). Fibroblasts were collected by centrifugation. Cells were suspended and incubated in a PBS solution containing respective antibodies obtained from BioLegend: (CD47 - FITC anti-human CD47 Antibody, CD39 - FITC anti-human CD39 Antibody, CD141 - PE anti-human CD141 (Thrombomodulin Antibody) and 5% FBS, at a temperature of 4°C for 45 min. Combination of antibody with the corresponding antigen led to the induction of a fluorescent pigment and the appearance of a light signal of a specific intensity. In the analyses a non-transgenic line of porcine embryonic fibroblasts was also used as a negative control. Human genes were detected using a FACSAria™ III cytometer. Results were compared with those recorded for the control of non-transgenic porcine fibroblasts.

Cytogenetic analysis

Cytogenetic analysis of embryonic fibroblasts included preparation of chromosome preparations, GTG band staining and analysis of karyotype. In order to obtain chromosome preparations 50 µl demecolcine at 10 µg/ml were added to a culture dish. After 5-hour incubation cells were washed with HBSS and tripsinized (0.25% tripsin, 0.02% EDTA). The cell precipitate was supplemented dropwise with 5 ml 75 mM KCl solution, incubated at 37°C for 30 min and centrifuged. The precipitate was fixed three times by adding dropwise a cold mixture of methanol and acetic acid (3:1) and transferred onto glasses. The number and scatter of metaphase plates were assessed by examination under a contrast phase light microscope. In order to determine the number of chromosomes, preparations of metaphase chromosomes were stained by the GTG technique (*G bands by Trypsin using Giemsa*). Chromosome staining was performed on fresh chromosome preparations (4-10 days). Preparations were digested at 37°C with a 0.25% tripsin solution in the Sörensen buffer at pH 6.8 (1/15 molar KH_2PO_4 and 1/15 molar $Na_2HPO4 \cdot 2 H_2O$). In the next stage preparations were rinsed in HBSS and Parker's solution (1:1), followed by another rinsing in HBSS and stained with 3.5% Giemsa solution in Sörensen's buffer. Incubation time in individual buffers was determined experimentally. After staining preparations were rinsed under running water followed by rinsing in distilled water.

Karyotypes of the examined cell lines were arranged based on available standards of idiograms and pig karyotypes [15]. Preparations were examined under an immersion fluorescent microscope and photographic documentation was processed using the *MetaSystems 2004, IKAROS Version 5.0 software.*

RESULTS:

Transfection of porcine embryonic fibroblast lines was performed using the following gene construct: pCD39-GFPBsd, pCD47-GFPBsd and pTM-GFPBsd. Lines, which survived selection were examined to detect the expression of a reporter marker - *green fluorescent protein* (GFP). Green fluorescence of fibroblasts under UV light indicates GFP activity, and thus the presence in cells of the gene construct, which component is the gene coding the GFPBsd fusion protein. Evaluation of fibroblast morphology showed a change in the shape of transfected cells from spindle-shaped to more irregular, at a simultaneous in-

creased presence of the cytoplasm in relation to the nucleus. Transfection was performed on 32 porcine embryonic fibroblast cell lines (Table 1). Analysis of integration for gene constructs showed integration of transgenes with genomic DNA in all analyzed porcine embryonic fibroblast cell lines.

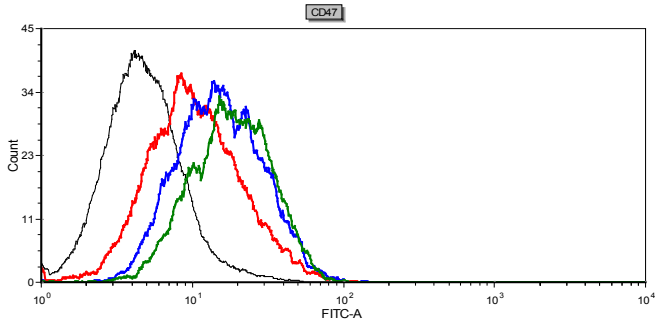
Cytometric analysis of embryonic fibroblast lines, in which integration of specific factors was confirmed, showed expression of CD47, CD39 and TM. Results suggest that gene constructs were integrated with the genome of modified cells in transcription active sites and the formed product, as it could have been expected, is transported to the outer cell membranes (Figures 1A-C).

Cytogenetic analysis of chromosomes in embryonic fibroblasts, in which integration and expression of specific factors was confirmed, showed a lack of chromosomal aberrations. All the analyzed seven cell lines had an appropriate karyotype. The appropriate karyotype image based on line Lip5-39 is presented in Figure 2.

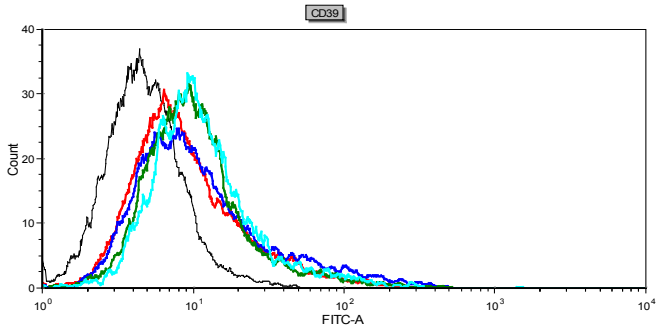
	Genetic construct	No. of transfected lines	No. of selection survived lines	No. of lines with confirmed integration
1	pCD47-GFP/Bsd	12	5 (41%)	5
2	pCD39-GFP/Bsd	10	8 (80%)	8
3	pTM-GFP/Bsd	10	8 (80%)	8
	Total	32	21 (65%)	21

Table 1. Transfection of porcine embryonic fibroblast cell lines with three genetic constructs.

A/



B/



C/

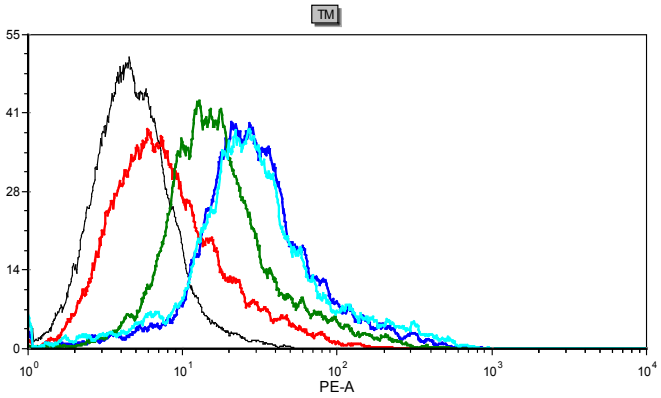


Figure 1. Analysis of transgenes' expression by flow cytometry. Analysis was conducted on porcine embryonic fibroblast cell lines following transfection with gene construct pCD47-GFPBsd (A), pCD39-GFPBsd (B) or pTM-GFPBsd (C) repectively. Cells were labelled with the anti-CD47 (A), anti-CD39 (B) or anti-TM (C) antibody conjugated with FITC fluorochrome (A and B) or PE fluorochrome (C). Axis x defines fluorescence intensity, axis y defines the number of non-labelled cells (black line), labelled non-transgenic 001-K (red line).

A/ transgenic line Lip7-47 (blue line), transgenic line Lip7-47' (dark green line). Analysis showed an increase in the level of receptor CD47 in transgenic lines in comparison with non-transgenic control. For line Lip7-47 expression of receptor CD47 increased by 60% (2.5-fold increase), for line Lip7-47' with expression of CD47 it was 66.9% (3-fold increase in the level of receptor CD47). B/ transgenic line Lip1-39 (dark blue line), transgenic line Lip2-39 (dark green line) and transgenic line Lip5-39 (light blue line). Analysis showed an increase in the level of receptor CD39 in transgenic lines in comparison with non-transgenic control. For line Lip1-39 expression of receptor CD39 increased by 60% (2.5-fold increase), for line Lip2-39 with expression of CD39 it was 66.9% (3-fold increase in the level of receptor CD30) and for Lip5-39 with expression of CD39 it was 66.9% (3-fold increase in the level of receptor CD39). C/ transgenic line Lip1-TM (dark blue line), transgenic line Lip2-TM (dark green line) and transgenic line Lip3-TM (light blue line). Analysis showed an increase in the level of the TM receptor in transgenic lines in comparison with non-transgenic control. For line Lip1-TM expression of the TM receptor increased by 60% (2.5-fold increase), for line Lip2-TM with TM expression it was 66.9% (3-fold increase in the level of the TM receptor) and for Lip3-TM with TM expression it was 66.9% (3-fold increase in the level of the TM receptor).

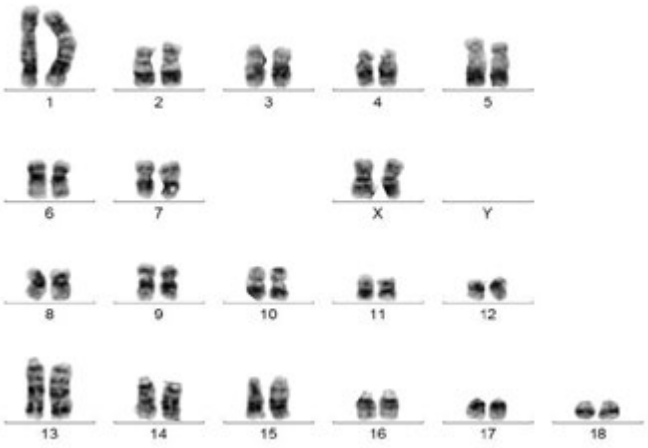


Figure 2. The karyotype of pig embryonic fibroblasts following the transfection of gene construct pCD39-GFPBsd (line Lip5-39) - GTG band staining.

DISCUSSION

Failure or complete damage to internal organs is a problem, which researchers have been trying to solve for many years. Transplantology is a huge success of medicine - transplantation of organs, tissues or cells.

Successful transplantation depends on several factors, such as tissue compatibility, appropriate selection of the donor and recipient, application of a good transplant preservation liquid and proper immunosuppression therapy. Moreover, it is also essential to properly diagnose and treat patients with transplant rejection and prevent and treat complications. Work on the above mentioned aspects are highly advanced and what is even more important - they are effective. At present teams of surgeons have gained considerable experience in this respect; however, a crucial problem has appeared - a shortage of organs for transplantations, which in Poland is particularly evident and may be easily estimated based on a review of data supplied by Poltransplant. Great hopes are connected with transplantation of genetically modified cells, tissues and organs collected from animals, i.e. xenotransplantation [16]. Advances in medicine and genetic engineering make it possible to control processes connected with xenotransplant rejection, e.g. thanks to genetic modification of cells from donor animals and pharmacological protection of organs. The use of pigs as donors of cells, tissues or organs for transplantations is limited by immune barriers leading to transplant rejection. Control of hyperacute rejection through genetic modifications consisting in the limitation of antibody activity against antigen Gal or expression of complement system regulatory proteins encourage further search for strategies aiming at elimination of another barrier, i.e. acute transplant rejection [17]. Introduction of genetic modifications providing expression of factors engaged in the protection against the action of macrophages and NK cells may contribute to eradication of these barriers. Polytransgenic animals may be used by crossing animals with single modifications or co-transfection with several gene constructs of a selected cell line, which will be the source of nuclei for somatic cloning. The above strategies are likely to provide animals - resources of organs for medical purposes.

Three different gene constructs were prepared, containing sequences coding factors CD39, CD47 and TM. Due to the potential risk of lower expression levels of sequences located farther from the promoter, it was decided not to prepare one construct containing sequences of all the three factors. Respective regulatory regions are significant elements in the structure of a gene construct, being a pre-requisite for a satisfactory transgene expression. The type of gene construct is of tremendous importance, determining high expression levels of transgenes introduced to animal cells. In the case of gene constructs containing cDNA, the level of expression depends on the number of inserted copies, with a too low or too high number of copies being potential causes of too low expression or methylation leading to transgene silencing. Long DNA fragments change the structure of the site in which they are inserted and may disturb its stability, leading to its transformation into the transcriptionally inactive site [18]. Complexity and diversity of mechanisms responsible for regulation of expression practically prevent complete control over production of recombinant proteins. However, introduction of natural regulatory elements or artificially produced structures into gene constructs in many cases makes it possible to limit transgene expression to a specific type of tissue and provides adequately high expression [19].

Described in this paper experiments are elements of a large, interdisciplinary project, aiming at production of animals by somatic cloning based on transgenic cell lines. Thus gene constructs containing sequences coding individual factors were introduced to porcine embryonic fibroblast cell lines. Selected monotransgenic cell lines will be sources of nuclei for somatic cloning in order to produce genetically modified animals, and next their crossing.

Lipofection was applied to introduce DNA to cells. Selection was made on the basis of an experiment of the research group, according to which it is a simple and efficient procedure facilitating introduction of DNA with no limitation to its length. Liposomes spontaneously interacting with DNA facilitate maintenance of stability in the introduced constructs and protect against the action of nucleolytic enzymes. Positively charged complexes formed as a result of the action of lipids and DNA penetrate into cells through endocytosis [20]. In this study lipofection was used to transfect 32 porcine embryonic fibroblast lines, of which integration of introduced DNA was confirmed in 21 lines. Effectiveness of the method was approx. 65% which confirms it a simple and effective transfection method. Studies previously conducted by our team comprised cell transformation not only by lipofection, but also precipitation with strontium chloride and electroporation, showing no significant differences in efficiency [21].

Obtaining a stabile transgenic cell line constitutes the first stage in the production of transgenic animals by somatic cloning. The method assumes an increase in the efficiency of transgenesis conducted using microinjection. One of the problems in cloning is connected with the long time required for the selection of transformants. Transformation may be conducted with the application of several methods. However, the low frequency, with which cells survive selection and achievement of a stabile transgene introduction con-

siderably limit their applicability. Moreover, the survival of selection alone does not guarantee the production of transgenic animals, since the mechanism of transgene integration in DNA of transfected cells is very complicated and frequently leads to reduced genetic stability of cells [22,23]. Still in comparison to the production of transgenic animals by microinjection, somatic cloning is more effective, which results from the possibility to confirm transgenesis on the molecular and cytogenetic level prior to studies on animals. Extensive studies on the cellular level make it possible to broaden our knowledge on the expression of foreign DNA sequences in mammalian cells, which may provide more insight into gene structure and functions [24]. There is no information in available literature how the long culture time may affect further application of transgenic cells. Although it results from studies conducted by our team [25] that transgenic cells collected from animals and cells of derived lines do not lose the transgene in successive generations or divisions, it is still difficult to determine in what way the long-term culture of somatic cells affects transgene expression and whether any change in functions occurs in cells.

In these studies it was shown that macrophages considerably contribute to transplant rejection. After transplantation they are activated in the recipient organism, which leads to a strong response targeting xenoantigens and activation of T lymphocytes. There are reports that macrophages considerably contribute to porcine cell rejection [26,27] and pancreatic islets both in rodents and in primates [28]. Although a reduced macrophage activity has a positive effect on the prevention of acute cell rejection, their rapid regeneration indicates the need to limit their activity over a long period in order to extend the survival time of the transplant [29]. For this reason it is necessary to develop a strategy aiming at the silencing of macrophage activity against xenotransplant cells in order to permanently maintain the transplanted organ in the recipient organism**.** Endocellular nucleotides play a significant role in many processes connected with thrombosis, such as activation of platelets, activation of the endothelium and shrinkage of blood vessels. CD39 converts ATP and ADP to AMP, which is next broken down to adenosine exhibiting anti-thrombotic properties. Modifications connected with factor CD39 constitutes a significant element in efforts to prevent vascular diseases and complications observed during attempts at transplantation of organs from pigs to primates.

Thrombomodulin is a transmembrane glycoprotein, which expression occurs on the surface of vascular endothelial cells, platelets and monocytes. It plays a significant role in the initiation of anti-thrombotic pathways by activation of C protein via thrombin, as a consequence blocking the production of procoagulation proteases. In order to assess interactions between clotting factors and blood vessels porcine blood vessels were isolated and the capacity of thrombomodulin to activate human as well as porcine C protein was evaluated in the thrombin-dependent pathway. Studies showed considerable incompatibilities between porcine thrombomodulin and human thrombin and the C protein, while activation capacity of thrombomodulin was observed to be 1% in relation to that in the organisms of pigs. The above mentioned incompatibilities indicate the need to accurately determine and develop factors involved in the deregulation of hemostasis between the human and the pig, since it constitute a significant problem for xenotransplantation [30]. A team headed by Yazaki *et al*. transfected the human thrombomodulin gene to porcine fibroblasts. Thanks to the application of nuclear transfer embryos were produced, which were transferred to surrogate mothers and as a consequence transgenic animals were obtained. Immunohistochemical staining of organs from F1 animals showed expression of human thrombomodulin in liver and kidney endothelial cells. Aortal endothelial cells showed expression at a level sufficient for the activation of the C protein. Appropriate assessment of transgenes connected with coagulation control and the production of genetically modified GalT-KO pigs showing expression of human thrombomodulin and the complement system regulatory proteins constitute the next step in research in this field [31].

CONCLUSIONS:

Only animals with multiple genetic modifications, obtained by crossing individuals with single modifications or through co-transfection with several gene constructs may become a reserve of organs for medical purposes. Overcoming or minimization of immune, microbiological and physiological barriers will make xenotransplantation a powerful tool in modern medicine saving people's health and lives. Thus it is crucial to win the public support, ethical approval and funding for research and medical projects aiming at the production of genetically modified animals.

At present production of transgenic animals is a combined work of an interdisciplinary team of experts in molecular biology, reproduction biology, biotechnology, virusology and surgery. While this research we focused on problems connected with the multi-center project on "Production of transgenic pigs as donors of tissues and organs for transplantation in humans and their biotechnological, physiological and medical characteristics", conducted in the years 2009 - 2012,

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directing our research also to areas being central to the interests of the other teams.

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