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RNA Interference as a Tool to Reduce the Risk of Rejection in Cell-Based Therapies

Constanca Figueiredo and Rainer Blasczyk

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Abstract

Remarkable progress in the experimental and clinical applications of cell-based therapies has identified stem cells and their derived products as potential candidates for regenerative therapies for many disorders. The use of autologous stem cells as source for regenerative therapeutic products is strongly limited by their low availability. Therefore, the future applications of *in vitro* pharmed therapeutic cell products will most likely occur in an allogeneic manner. However, the high variability of the human leukocyte antigen (HLA) represents a major obstacle to the application of off-the-shelf products. We have developed a strategy to decrease the immunogenicity of *in vitro* generated cell products by silencing HLA expression using RNAi. HLA expression was permanently silenced in CD34+ hematopoietic stem and progenitor cells and induced the pluripotent stem cells to generate HLA-universal cells sources, which were then used for the differentiation of low immunogenic cell products. In this chapter, we will provide an overview about an RNAi-based strategy to reduce the immunogenicity of cell-based therapies, and in particular in the generation of HLA-universal platelets and tissues.

Keywords: HLA, immunogenicity, transplant rejection, blood pharming

1. Introduction

The high variability of the human leukocyte antigen (HLA) constitutes a major hurdle in allogeneic transplantation and to the application of off-the-shelf cell products in regenerative medicine. Recently, remarkable progresses in the field of stem cell biology, cell pharming, and tissue engineering have made feasible the differentiation of cells and tissues that might serve as a bridging strategy or even an alternative to the very scarce donated tissues and organs. However, HLA incompatibility may pose a threat to the applicability of such *in vitro* generated cell products by increasing the risk of immune rejection after the transplantation.[1] To

overcome this major hurdle in the fields of transplantation, cell and tissue engineering, we have developed an RNA interference (RNAi)-based approach to reduce the immunogenicity of cells and tissues, allowing their application in an universal manner. A lentiviral vector encoding for specific short hairpins RNA (shRNA), targeting HLA transcripts were used to achieve a permanent silencing of HLA expression. As HLA residual expression is crucial to prevent the natural killer (NK) cell activity, RNAi appears as a superior tool in comparison to gene editing technologies that cause a complete gene deletion such as the transcription activator-like effector nuclease (TALEN) or clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 systems.[2-4] By decreasing the immunogenicity of cells and tissues, the need for immunosuppressive regimens might be reduced after HLA-mismatched transplantation. Furthermore, as HLA plays a pivotal role in the recognition of virus-infected cells and cancer cells, the combination of conditional promoter systems allows the re-expression of HLA and constitutes a safety mechanism. The generation of low immunogenic cells and tissues would bring enormous benefits to the patients and open novel horizons in the field of transplantation and regenerative medicine.

2. The HLA system

Evolution conferred highly refined mechanisms to all animals from sponges to mammals to distinguish self from non-self, and thereby allowing an immune response against potential pathogens. In humans, a tight interplay between adaptive and innate immune systems allows their defense against virtually all pathogens and cancer cells.[5, 6] Nevertheless, those sophisticated surveillance mechanisms pose a major hurdle to allogeneic transplantation. The alloimmune responses are mainly based on the recognition of mismatched major histocompatibility complex (MHC) antigens by antibodies and T-cells. In humans, the MHC is known as HLA and it comprises a group of linked genes. MHC class I and II regulate the immune response through the presentation of peptides to T-cells. Allogeneic MHC in the graft's antigen-presenting cells (APCs) is recognized after transplantation through a direct pathway. Afterwards, own patient APCs process and present the allogeneic antigens to T-cells by an indirect alloantigen recognition pathway.[7, 8] HLA loci are encoded on the short arm of human chromosome 6. Based on their structure, HLA molecules are grouped into class I and class II (Figure 1). HLA class I classical genes comprise the A, B, and C loci, and are expressed in the majority of cells. HLA class II genes include DR, DQ, and DP and are constitutively expressed only in professional APCs.[9] The non-self recognition mediated by the engagement of the T-cell receptor with the donor HLA is the basis for the allogeneic immune response.

2.1. HLA incompatibility increases the risk of rejection

Despite the progresses in the field of transplantation, graft rejection remains the major concern regarding the application of off-the-shelf products. HLA comprises the most polymorphic loci of the entire human genome. The probability to find a complete HLA-matched donor for a specific patient is very low and, therefore, in most of the cases, patients will be treated with partially HLA-mismatched tissues and organs. In addition, even in fully HLA donor/recipient

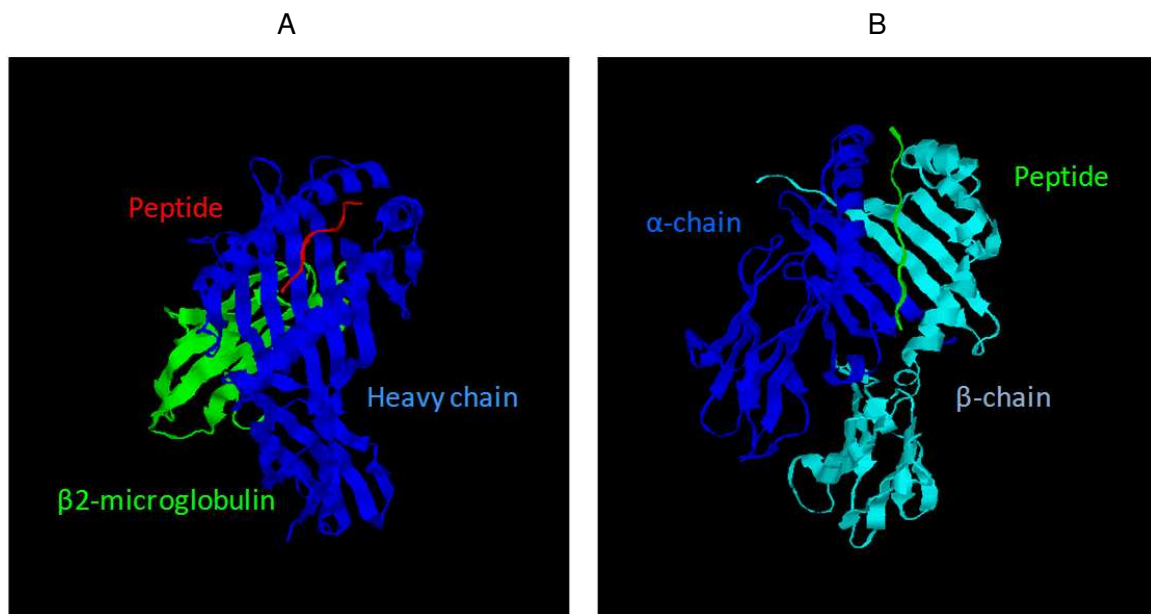


Figure 1. Structure representation of (A) HLA class I (HLA-B) and (B) HLA class II (HLA-DQ) molecules. The structures were designed using the software <http://www.mh-hannover.de/institute/transfusion/histocheck/>.

pairs, disparities at the minor histocompatibility antigens (mHAg) derived from other polymorphic proteins and presented at the HLA complexes are capable of triggering antigen-specific immune responses that cause graft rejection. Improved use of post-transplant immunosuppression to prevent acute and chronic rejection allowed allogeneic transplantation as a widespread, successful therapy.[10-13] However, graft rejection remains a major concern in the field of transplantation. The registry of the International Society for Heart and Lung Transplantation (ISHLT) reported that within the first year after lung transplantation, up to 55% patients need to be treated for acute rejection and only 50% are alive after five years.[14] Also, the number of HLA mismatches between a donor/recipient pair is associated with stronger immunosuppressive regimens. In particular, the number of HLA-DR mismatches and the number of HLA-A and -B mismatches as well as rejection treatment showed significant associations with the dose of maintenance steroids. Although immunosuppression allows the acceptance of the allogeneic graft, it has severe side effects that may contribute to death even with a functioning graft.[15] The occurrence of post-transplant complications related to the immunosuppressive therapy such as cancer, opportunistic infection, toxicity, and hip fractures have indicated the necessity to develop alternative strategies to allow the therapeutical use of off-the-shelf HLA and mHAg-mismatched cell-based products.[16] Furthermore, due to the shortage of organs and tissues for transplantation, there is a high demand regarding the development of *in vitro* pharmed cell products, and engineered tissues or organs. Progresses in the fields of stem cell biology and tissue engineering have demonstrated the feasibility to generate *in vitro* potential alternative cell-based products that might serve as an alternative or overcome the need for using donated tissues. Nevertheless, the future use of such products will occur in an allogeneic manner, and therefore, it will be required that those products will be able to escape an allogeneic immune response.

2.2. RNAi-mediated HLA targeting as a strategy to decrease the cell immunogenicity

Rejection of allogeneic grafts is based on the recognition of the HLA complexes by the specific pre-formed complement-binding anti-HLA antibodies or by the engagement of the T-cell receptor, which leads to T-cell activation and the initiation of the immune response.[17, 18] RNA interference is an invaluable technique in cell biology and regenerative strategies to silence the target gene expression. Our studies have focused on the downregulation of HLA class I and class II expression on the graft cells. So far, several strategies have been developed to induce the acceptance of the allogeneic graft. Similar to the immunosuppression, those strategies involve the modulation of immune responses and aim the induction of tolerance to the graft. In our studies, we genetically modify the graft to silence its HLA expression to prevent the recognition of the allogeneic graft as non-self by the recipient's immune system. In this approach, we do not induce tolerance toward the allogeneic graft, but we generate a condition of immunological blindness in which the recipient's immune system is not able to recognize the allogeneic cells (due to the missing HLA) but is fully capable of defending the patient against common clinical conditions associated with the use of immunosuppressive drugs such as opportunistic infections and leukemia. To prevent the recognition of the grafted cells as off the shelf, we have downregulated the expression of HLA class I and class II antigens using RNAi. We have constructed lentiviral vectors encoding for short-hairpin RNA sequences targeting β 2-microglobulin (sh β 2m) or the alpha-chain of HLA-DR (shDRA) to silence the expression of HLA class I and class II antigens, respectively. Our studies demonstrated the feasibility to stably downregulate HLA class I and II expression in several cell lines (e.g., B-LCL, MonoMac-6, HeLa) as well as in primary cells (e.g., endothelial cells, CD34+ progenitor, induced pluripotent stem cells). Cell transduction for the delivery of shRNAs targeting specific HLA transcripts resulted in a decrease by up to 90% of β 2m or HLA-DRA transcript levels and HLA class I expression. *In vitro* assays have shown that HLA class I-silenced cells were protected against the antibody-mediated complement-dependent cytotoxicity. Furthermore, in T-cell cytotoxicity assays, significantly lower cell lysis rates were observed when HLA-silenced cells were used as targets in comparison to fully HLA-expressing cells. In addition, HLA-silenced cells demonstrated to induce significantly lower T-cell proliferation, pro-inflammatory cytokine secretion, and degranulation. The residual HLA class I expression showed to be sufficient to prevent NK cell cytotoxicity. Altogether, HLA-silenced cells showed a protective effect against the humoral and cellular allogeneic immune response.[2, 3, 19]

2.3. MHC-silenced cells survive after fully HLA-incompatible transplantation

Despite the widespread use of immunosuppressive regimens to prevent graft rejection, their therapeutic window is very narrow. Immunosuppressive drugs frequently cause adverse effects including thrombocytopenia, leukopenia, hypercholesterolemia, stomatitis, nephrotoxicity, and diarrhea, and they lead to an increased risk for infections and cancer.[20, 21] Silencing HLA expression using RNAi may represent an alternative to immunosuppression; hence it has the potential to offer many benefits for the patients. In addition, silencing HLA expression may allow the future application of HLA-mismatched off-the-shelf products in a universal manner independently of the genetic background of the donor and recipient. In an

allogeneic transplantation rat model, we have confirmed the improved capacity of MHC-silenced cells to survive in allogeneic environment upon transplantation and even in the absence of immunosuppression. A lentiviral vector encoding for a shRNA sequence targeting rat MHC class I (RT1-A) and the sequence for firefly luciferase as a reporter gene was used to silence MHC class I Lewis rat-derived fibroblasts. In contrast to nonmodified fibroblasts, MHC class I-silenced fibroblasts were able to survive after subcutaneous transplantation in a complete MHC-mismatched setting. MHC class I-silenced fibroblasts were able to engraft and were detectable during the entire monitoring period (8 weeks). Nonmodified cells were rejected in all animals.[22] This study showed the superior performance of MHC-silenced cells after MHC-incompatible transplantation.

2.4. Generation of HLA-silenced platelets

Since the 1950s, blood transfusion therapy has become routine clinical practice; however, the concept of blood pharming – ex vivo production of mature blood cells – is quite new. In humans, platelet production is sustained by a well-regulated process known as thrombopoiesis. In the bone marrow, CD34⁺ progenitor cells differentiate into polyploid megakaryocytes (the precursor of platelets). Megakaryocytes lack the expression of CD34, but express several glycoproteins essential for the platelet function.[23, 24] In general, platelet numbers in blood range from 150×10^9 to 400×10^9 per liter, and an estimated 1×10^{11} platelets are produced each day in the adult human. Thrombocytopenia and severe thrombocytopenia characterized as platelet counts less than 50×10^9 and 10×10^9 per liter, respectively, increase the risk of spontaneous bleeding and represent a threat for the patient's life.²⁵ Platelet transfusion has been widely used to prevent and treat life-threatening thrombocytopenia; however, preparation of a unit of concentrated platelets for transfusion requires at least 4–6 units of whole blood, thereby significantly increasing the risk of blood-borne infections and adverse immunologic reactions. Furthermore, platelet transfusion refractoriness – lack of adequate post-transfusion platelet counts – remains a major complication often observed in patients receiving multiple transfusions. This condition is frequently caused by the development of antibodies specific to HLA. Currently, platelet transfusion relies on volunteer blood donation; however, the demand for blood products in particular of platelets often exceeds their availability.[25]

The potential of multipotent progenitor and stem cells in regenerative medicine has been recognized.[26, 27] Platelet transfusion refractoriness due to the presence of anti-HLA antibodies constitutes a life-threatening risk for many patients suffering from hematological disorders, and hence require multiple platelet transfusions. Thus, it would be highly desirable to produce HLA-deficient platelets to facilitate the management of severe alloimmunized thrombocytopenic patients. In our studies, we have combined the concept of blood pharming with RNAi as a strategy to downregulate HLA gene expression. The ultimate goal of this approach is the large-scale production of platelets *in vitro* that may be used as an alternative to the conventional donated blood platelets. In addition, we aim for the production of genetically modified platelets with the capacity to survive even under platelet transfusion refractoriness.

In our studies, CD34⁺ hematopoietic progenitor cells and induced pluripotent stem cells (iPSCs) were used to produce HLA-silenced platelets *in vitro*. CD34⁺ cells or iPSCs were transduced with a lentiviral vector encoding for the shRNA sequence targeting $\beta 2m$ which induces HLA class I silencing. We have demonstrated the feasibility to generate HLA-universal CD34⁺ cells and iPSCs which might be used for the differentiation of HLA-silenced cell-products. In our studies, we have differentiated HLA-silenced megakaryocytes and platelets from both cell sources. In our previous studies, we have demonstrated the possibility to generate HLA-silenced platelets with comparable function to blood-derived platelets. However, in contrast to blood-derived platelets, *in vitro* generated HLA-silenced platelets were able to escape antibody-mediated complement-dependent cytotoxicity as well as cellular-dependent cytotoxicity. Also, in a platelet transfusion refractoriness mouse model, HLA-silenced platelets showed the capacity to survive and were even detectable 10 days after transfusion. [28] The limited availability of CD34⁺ cells derived from G-CSF mobilized donors is a major obstacle to the large-scale production of *in vitro* pharmed platelets. The breakthrough Nobel Prize-winning research by Yamanaka and colleagues to induce pluripotency in somatic cells has reshaped the field of stem cell research. Human iPSCs can be used for studying embryogenesis, disease modeling, drug testing, and regenerative medicine. [29] In contrast to CD34⁺ cells, iPSCs represent an unlimited cell source for the *in vitro* production of a variety of cell-based products including platelets. Therefore, we have recently established an efficient protocol for the differentiation of megakaryocytes and platelets from iPSCs (Figure 2). First, we have generated an HLA-universal iPSC line. Then, the lentiviral vector containing the shRNA targeting $\beta 2$ -microglobulin was used to silence HLA expression on iPSCs. A significant and durable reduction of HLA expression was observed even after passaging. Nevertheless, the HLA-silenced iPSC line showed comparable expression of pluripotency markers (such as SSEA-4 and Tra-1-60) as the original HLA class I-expressing iPSC line (Figure 3). The data indicate that silencing HLA expression does not affect the pluripotency potential of iPSCs.

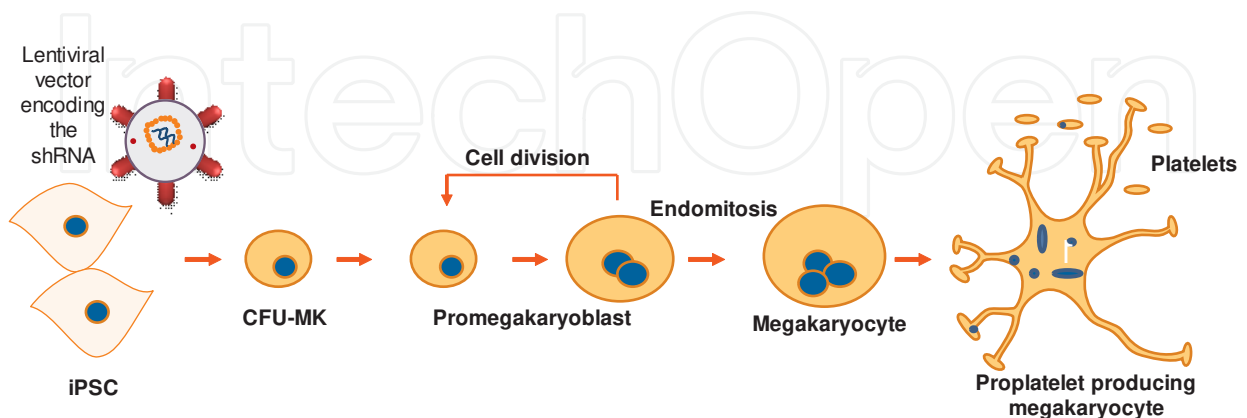


Figure 2. Schematic representation of the differentiation of HLA-silenced platelets from iPSCs. A lentiviral vector encoding for an HLA-specific shRNA is used to transduce the iPSCs. Afterward, HLA-silenced iPSCs will be differentiated using a cytokine cocktail containing thrombopoietin (TPO) until the release of platelets.

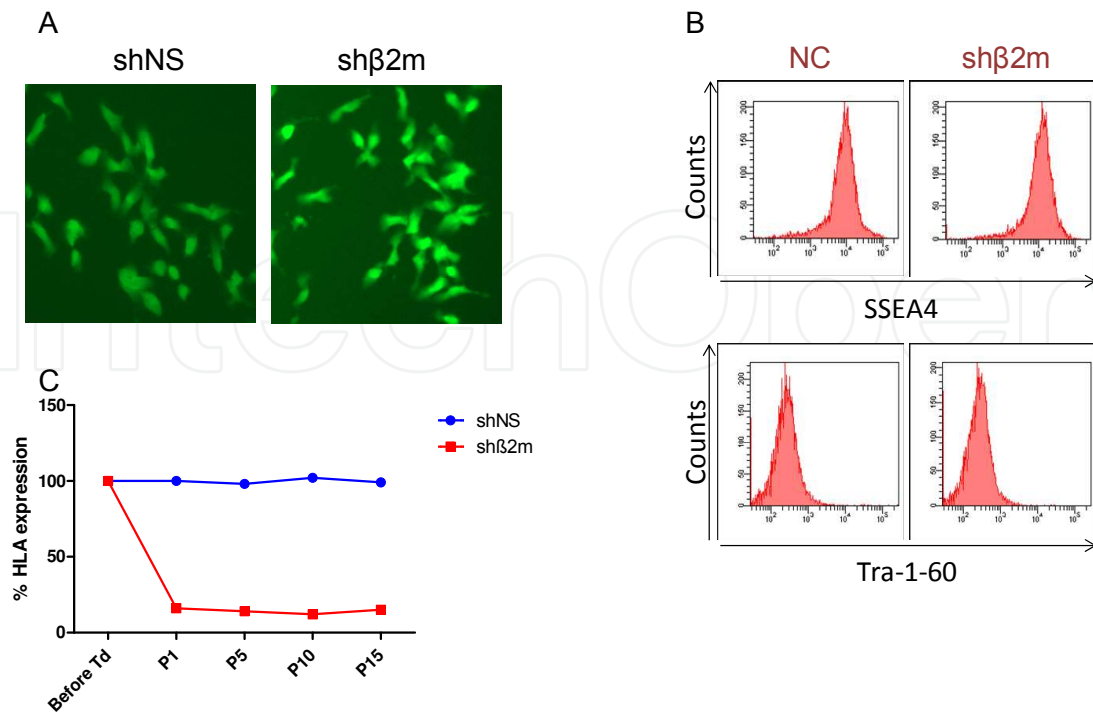


Figure 3. Generation of an HLA-universal iPSC line. (A) iPSCs were adapted to monolayer culture conditions and transduced with a lentiviral vector encoding for a nonspecific shRNA (shNS) or a β 2-microglobulin-specific shRNA to silence the expression of HLA class I; (B) expression of SSEA4 and Tra-1-60 on nonmodified and sh β 2m-expressing iPSCs; (C) expression of HLA class I of a HLA-silenced shNS-expressing iPSC lines. Cells were stained with an HLA class I antibody (W6/32) and the expression of HLA was measured by flow cytometry after different number of passages. Mean fluorescence intensities (MFI) detected on HLA-silenced iPSCs were normalized to the MFI measured on shNS-expressing iPSCs at the same passage.

In addition, the future application of iPSCs will occur most likely in an allogeneic manner to facilitate their availability during the time of need and standardization of the protocols. Hence, the use of HLA-silenced iPSCs may facilitate the application of HLA-mismatched iPSC-derived cell products. For megakaryocyte differentiation, HLA-silenced iPSCs were cultured in monolayer in the presence of vascular endothelial growth factor (VEGF) and BMP-4 for mesoderm induction and afterward in the presence of TPO. During ontogeny, definitive hematopoietic cells are generated de novo from a specialized subset of endothelium, known as hemogenic endothelium. Endothelial-to-hematopoietic transition during embryogenesis provides the first long-term hematopoietic stem and progenitor cells in the embryo.[30] In our differentiation cultures of iPSCs into megakaryocytes, structures resembling the hemogenic endothelium were observed (Figure 4). In suspension, megakaryocytes could be detected by an increase in DNA content higher than 4n and the expression of typical megakaryocytic markers such as CD41 and CD42a. In addition, the megakaryocytes showed the capability to build pro-platelets. Moreover, the shRNA-mediated silencing effect was maintained during the entire differentiation. Importantly, iPSC-derived megakaryocytes and pro-platelets showed a significant reduction of β 2-microglobulin and HLA class I antigens in comparison to those differentiated from iPSC expressing a nonspecific shRNA (control) (Figure 5). Differentiation rates of iPSC into megakaryocytes by up to 82% were observed (Figure 6).

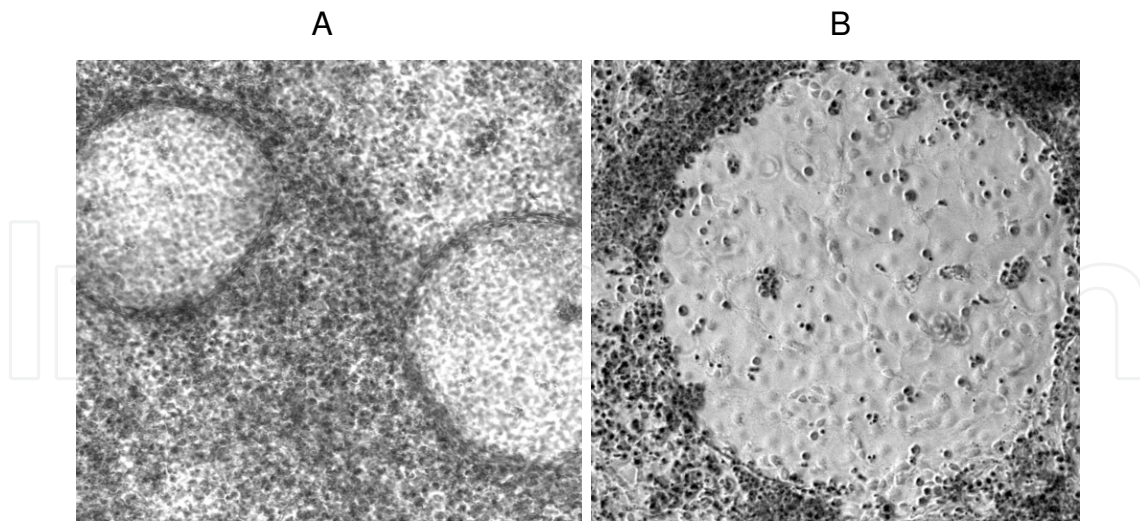


Figure 4. Formation of hemogenic-like endothelium during the differentiation of iPSCs into megakaryocytes. The photos display an island of hemogenic-like endothelium at a magnification of (A) 10x; (B) 20x.

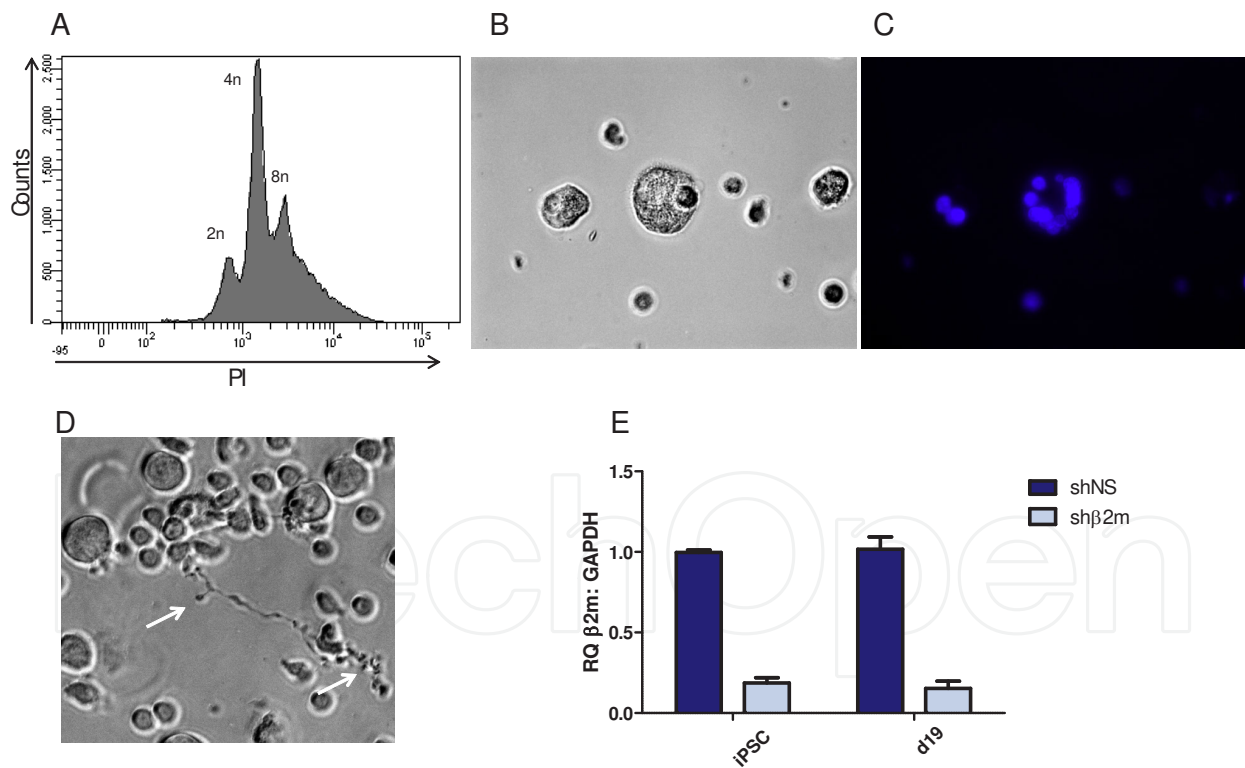


Figure 5. Differentiation of HLA-universal megakaryocytes from HLA-silenced iPSC. (A) The histogram represents the flow cytometric analysis of HLA-silenced iPSC-derived megakaryocytes after staining with propidium iodide; (B) light microscopic and (C) fluorescence microscopic analysis of an iPSC-derived megakaryocyte after staining with 4',6-Diamidin-2-phenylindol (DAPI, blue); (D) light microscopic analysis of pro-platelets indicated with white arrows; (E) real-time PCR analysis of $\beta 2$ -microglobulin levels in megakaryocytes derived from iPSCs transduced with a lentiviral vector encoding the shRNA targeting $\beta 2$ -microglobulin (sh $\beta 2m$) or a nonspecific shRNA (shNS) as a control.

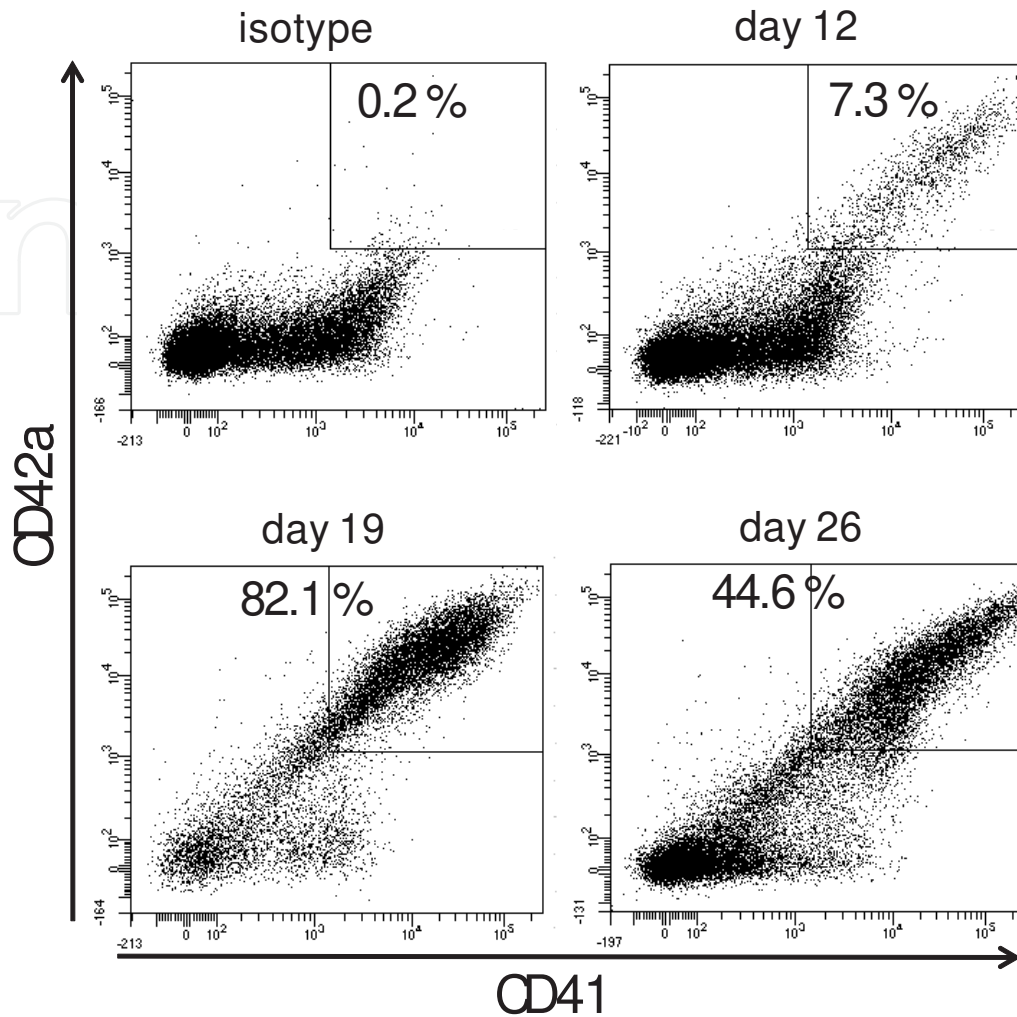


Figure 6. Phenotypic analysis of HLA-universal megakaryocytes derived from iPSCs. The expression of the megakaryocyte markers CD41 and CD42a was measured by flow cytometry in the cells harvested from the iPSC differentiation cultures at different time points. The gates in the dotplots indicate megakaryocytes characterized by the double expression of CD41 and CD42a.

The complement-dependent cytotoxic (CDC) crossmatch is an informative assay that detects alloantibodies in pre- and post-transplant patients, which may guide the most appropriate clinical management of transplant patients.[31] The capacity of *in vitro* generated HLA-silenced megakaryocytes and platelets to escape antibody-mediated complement-dependent cytotoxicity was evaluated in CDC tests. HLA-silenced megakaryocytes incubated with specific HLA antibodies and complement showed comparable cell lyses rates to the megakaryocytes incubated with nonspecific HLA antibodies. In contrast, significantly higher cell lysis rates were observed when HLA-expressing megakaryocytes were incubated with specific anti-HLA antibodies (Figure 7). These data suggest that HLA-universal iPSC-derived megakaryocytes are protected from anti-HLA antibody-mediated complement-dependent cytotoxicity and have the potential to survive under refractoriness conditions.

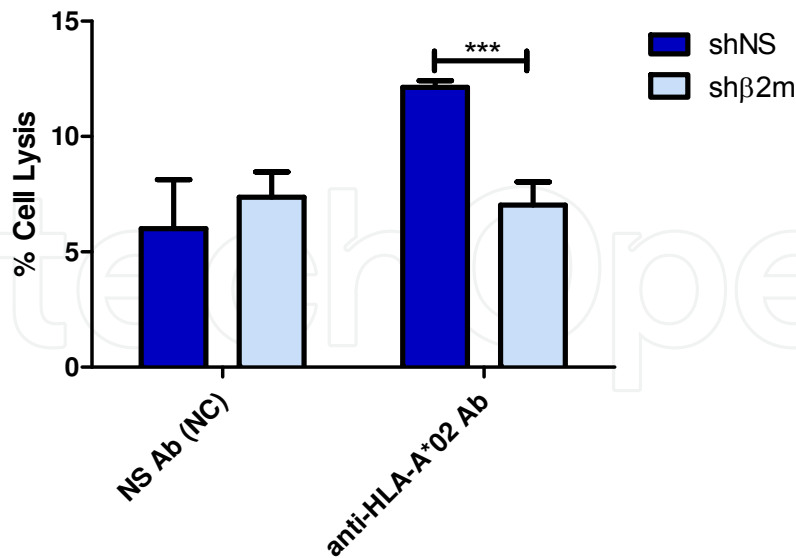


Figure 7. HLA-silenced megakaryocytes are protected from antibody-mediated complement-dependent cytotoxicity. HLA-expressing (shNS) or HLA-silenced (shβ2m) iPSC-derived megakaryocytes were incubated with a nonspecific antibody (NC) or an HLA-specific antibody and complement. Cell lysis was detected by flow cytometric analysis upon staining with propidium iodide. The bar graph represents means and standard deviations of three independent experiments. Statistical significance was calculated using Student's *t*-test (* $p < 0.05$, ** $p < 0.01$; *** $p < 0.001$).

2.5. Generation of HLA-silenced tissues

Due to the high variability of the HLA loci and the shortage of organs and tissues for transplantation, it is very difficult to find a complete HLA-matched graft for a given recipient. The number of HLA mismatches between the graft and the recipient are associated with a higher risk for graft rejection and even morbidity and mortality due to immunosuppression-related side effects. Thus, it would be desirable to engineer the grafts in order to decrease their immunogenicity by silencing HLA expression. Worldwide, the demand for organs and tissues for transplantation is very high and it is not possible to satisfy all needs. This discrepancy is even accentuated in the Middle East and countries in the East such as India and China. According to the World Health Organization (WHO), there are over 10 million people in the world who are blind in one or both eyes due to corneal injury or disease and up to 45 million people could benefit from corneal transplants. However, according to data from eye banks and health agencies, less than 150,000 corneal transplants are done annually worldwide due to the shortage of human cadaver corneas. Furthermore, during the first five years after penetrating keratoplasty, rejection is responsible for 28–35% of total corneal graft loss. High risk-corneal recipients even showed increased rejection rates (30–56%).[32, 33]

The cornea presents a simple anatomical structure, in which the endothelium is easily accessible to the shRNA-encoding viral vector containing supernatant (Figure 8). The integrity of the endothelial cell layer is crucial for the transparency of the cornea and it is the major target for rejection. Therefore, silencing HLA expression on the corneal endothelium may improve cornea survival in high-risk patients after allogeneic keratoplasty.

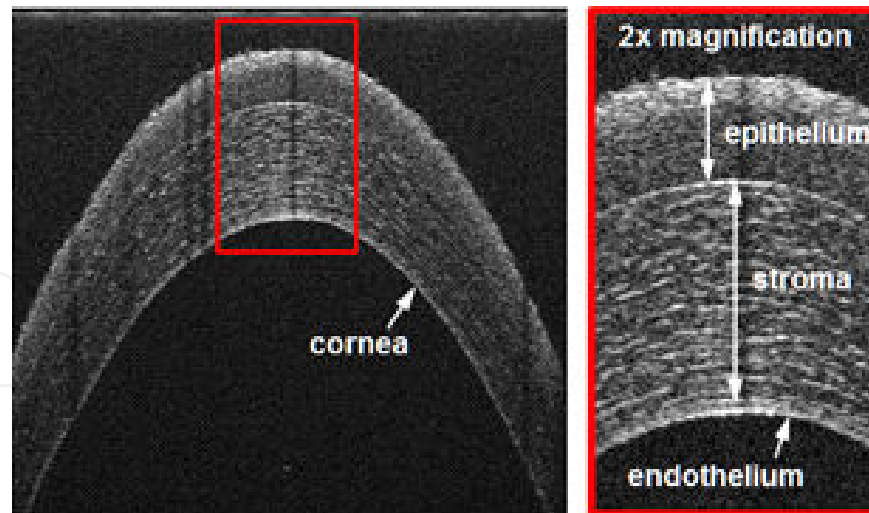


Figure 8. Anatomy of the cornea. Optical coherence tomography of a mouse cornea showing the three main layers.

We have silenced HLA expression in human and mice corneas. The tissue was transduced for 8 h with the lentiviral vector encoding for the MHC-specific shRNAs as described above. We were able to silence the MHC expression on the cornea endothelium, which is the major target during graft rejection. In this study, we demonstrated the feasibility to generate HLA universal tissues in their original 3D structure (Figure 9). Silencing HLA expression on tissues is expected to significantly improve graft survival rates in high-risk keratoplasty patients.

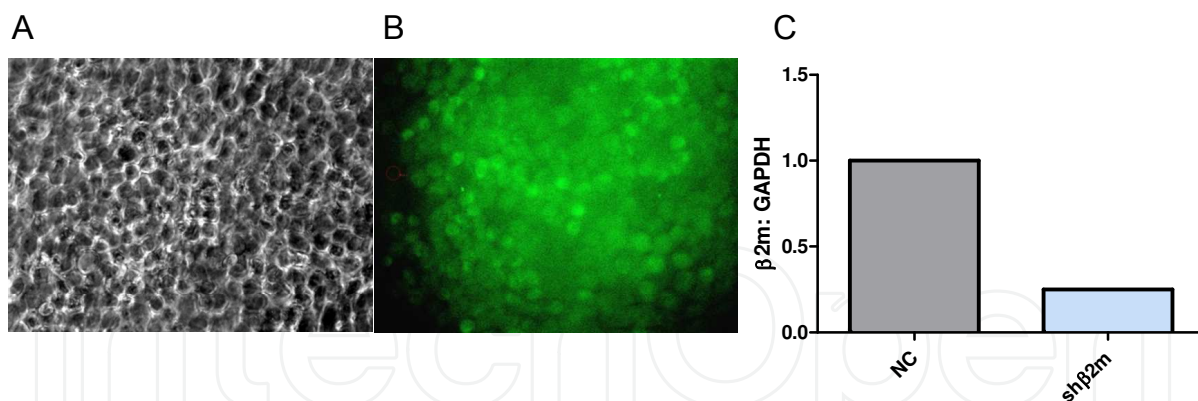


Figure 9. Generation of MHC-silenced corneas. A mouse cornea was explanted and transduced with a lentiviral vector encoding the sh $\beta 2m$ to cause a downregulation of MHC class I antigens and the expression of green fluorescence protein (GFP) sequences. (A) Light microscopic and (B) fluorescence microscopic analyses of a transduced mouse cornea.

2.6. Conclusion

Recently, gene regulation or editing strategies have emerged as powerful tools to improve the design and efficacy of personalized cell-based therapies. In the field of histocompatibility and transplantation, RNAi seems to be a favorite approach to reduce the immunogenicity of

allogeneic and *in vitro* generated cells and tissues. The lentiviral vector-mediated delivery of shRNAs targeting HLA transcripts prevents the activation of cellular and humoral allogeneic immune responses that cause the rejection of the foreign cells. However, this RNAi-based strategy permits the residual expression of HLA class I antigens which are crucial to inhibit NK cell cytotoxicity. With the establishment of iPSCs, the concept of cell pharming came one step closer to reality as iPSCs may serve as unlimited cell sources for different cell products such as platelets. The combination of RNAi-mediated HLA silencing and the capacity to generate platelets *in vitro* may represent a novel therapeutic approach for the management of alloimmunized thrombocytopenic patients with an increased risk to develop refractoriness to platelet transfusion. Furthermore, our results also indicate the feasibility to reduce MHC expression in the 3D original structure of tissues. Abrogating the histocompatibility barrier between donors and recipients may improve therapeutic efficacy, reduce the adverse events associated with strong immunosuppressive regimens, and improve transplant patient life's quality. In conclusion, RNAi-mediated silencing of HLA expression may open new avenues in tissue engineering and transplantation.

Author details

Constanca Figueiredo* and Rainer Blasczyk

*Address all correspondence to: figueiredo.constanca@mh-hannover.de

Institute for Transfusion Medicine, Hannover Medical School, Hannover, Germany

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