Cluster assembled nanostructured TiO_2 film mediates efficient and safe retroviral gene transduction in primary adult human melanocyte for ex-vivo gene therapy

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ABSTRACT

Primary human melanocytes represent the precursor cells to melanoma and also specific targets of inherited and acquired pigmentation disorders [1-3]. We have developed a new gene transfer technology on primary adult human melanocytes by means of retroviral infection in absence of any toxic polycations currently employed to improve infection efficency (i.e. polybrene) that, as we show experimentally, induce apoptosis and DNA damage. We have cultured primary human melanocytes on a biocompatible nanostructured TiO_2 film, obtained by the deposition of a supersonic beam of titania clusters [4-6], coated by retroviral vectors expressing GFP. By means of a "reverse infection " mechanism we achieved 80% of infection in absence of any toxic effect. We expect that such technology will allow efficient and safe genetic manipulation of primary cells for ex-vivo gene therapy.

Keywords: nanostructured TiO_2 films, retroviral gene transduction, adult human melanocytes, ex-vivo gene therapy

1 INTRODUCTION

In recent years different concerns have been made regarding safety issues of gene therapy for the treatment of human diseases [7] after the report of leukemia cases caused by insertional mutagenesis upon retroviral transduction for the correction of SCID disease. Despite this, retroviral gene therapy is still considered a very promising approach for the treatment of specific human diseases [8] through the development of new vectors and methods that may improve safety and efficacy.

One of the most attractive tissue for ex-vivo gene therapy, is represented by the skin.

In particular human melanocytes, which are responsible for the synthesis of melanin, can be affected by different inherited or acquired disorders, resulting in hypopigmentation or hyperpigmentation of the skin [9]. Moreover melanocytes malfunctioning may represent the first step to the development of malignant melanoma [10].

Current methods for efficient retroviral gene delivery, in primary cells, require repeated cycles of infection in presence of cationic polymers (i.e. polybrene). To improve retroviral infection and stable gene tranduction in primary adult melanocytes different approaches have recently been proposed: the retroviral transduction of melanocytes in coculture with keratinocytes [11] and the use of lentivirus [12]; other methods employing adenoviral transduction or r-AAV, do not permit stable correction of the genetic lesion and therefore are not suitable for long term expression.

Despite the improvement in efficiency of these retroviral transduction systems, all these protocols require the presence of Polybrene to enhance infection efficiency; polybrene is known to exert cellular toxicity [13] but no systematic molecular analysis of it's effects have been shown yet. We have tested polybrene effects on human adult primary melanocytes and observed a high degree of toxicity with extensive cell death.

Furthermore we show that polybrene induce a DNA damage response and growth arrest in human adult primary melanocytes. Consequently, cells that undergo retroviral transduction with specific genes not only will display phenotypic behavior due to the overexpression or downregulation of the gene, but surviving cells will have to rescue cellular toxicity of polybrene.

We have recently characterized a new nanostructured TiO_2 (ns-TiO_2) material [4] as a biocompatible substrate for cell culturing [6] which possesses the property of adsorbing biomolecules in a hydrophilic environment, while preserving their biological activity [14-16]. At this purpose we have tested the ns-TiO_2 film for the capability to mediate efficient and safe gene transduction in primary human adult melanocytes by means of retroviral particles adsorption on the surface, in the absence of any polycations, followed by plating of cells. We achieved 80% of infection using a GFP based retroviral vector in absence of any toxic effects. We speculate that the high efficiency of infection might be related to the interaction of retroviral vectors and cells with the nanoparticle-assembled substrates. Our approach allows a safe and efficient gene transduction either for ex-vivo gene therapy on adult primary melanocytes or for in vitro biology of melanoma research.

2 RESULTS

2.1 Polybrene induces DNA damage and apoptosis on primary adult human melanocytes.

To characterize polybrene effects on primary adult melanocytes we treated cell with different amount of polybrene (from 1ug/ml to 16ug/ml) for 48 hours and processed them for morphological analysis by bright field microscopy. As we reported in fig.1 panel A, cells treated with polybrene at 2ug/ml present a lower degree of confluency compared to control cells. This effect is more evident with increasing polybrene concentrations. At 8 and 16ug/ml cells detach from plates and become evident as cellular debris. To verify if this effect was due to apoptosis we processed cells for FACS analysis by PI staining. As shown in panel B we observed a 20% of apoptosis at low polybrene concentration (2ug/ml); the apoptotic effect correlates with the increasing of polybrene dose, reaching 50% at 16ug/ml. We then decide to analyze some component of the DNA damage response to verify if polybrene could affect this pathway. We performed immunostaining analysis of primary melanocytes with antip-ATM, anti p53 and anti γ-H2AX to assess the presence of double strand breaks. We treated cells with 4 ug/ml that correspond to the dose that is commonly used for primary cells infection.

Panel C shows immunofluorescence staining of cells treated with polybrene displaying a strong up-regulation of p-ATM, an increase of double strand breaks by γ-H2AX staining and induction of p53. All these data strongly indicate that primary melanocytes treated with polybrene activate pathways of DNA damage that may be responsible for the observed apoptotic effect. We therefore hypothesized that cells infected with classical infection protocols that include polybrene could be characterized by genetic alterations if some selective pressure is applied to recover infected cells that must overcome/repair the damage and escape apoptosis.

2.2 Ns-TiO_2 substrate mediates efficient and safe retroviral infection on adult primay melanocytes.

We have recently characterized the biocompatibility of a ns-TiO_2 substrate produced by SCBD for cell culturing [6]; this biomaterial possesses unique properties at "nanoscale" in term of granularity and porosity which mimic those of typical ECM structures. Moreover titanium dioxide nanoparticles can form complexes with a variety of chemical groups and immobilize functional peptides and macromolecules that could be employed to functionalize the surface [14-16]. Therefore we set up a reverse infection method by adsorbing on ns-TiO_2 coated coverslips retroviral particles, incubating at 4°C to allow viral binding, and subsequently plating cells. We used two different viral preparations; a PEG concentrated retroviral sample (PEG-virus) that we obtained by PEG addition and low speed centrifugation of the viral supernatant, followed by resuspension in 10 times reduced volume of PBS 1X buffer, and the supernatant collected from Phoenix transfection, without any further treatment (supernatant-virus). To compare the efficiency of our ns-TiO_2 substrate with other cell culture substrates we performed the reverse infection experiment on different biomaterials; in particular we tested tissue culture grade plastic wells, and 0.1% gelatin coated glass coverslips.

We coated with 100 ul of viral samples all the substrates; after 4 hour of incubation at 4°C we performed PBS-tween 0,1% washing to remove viral excess and we plated cells; results were analyzed at 72-96 hour after transduction.

Fig.2 panel A shows the morphological analysis by bright field microscopy: no differences are evident among mock infected and infected cells with PEG-virus after 72h from infection; then we analyzed the percentage of infection by GFP positivity after 96 hours from infection. Fig.2 panel B shows statistical analysis of infected cells by ImageJ software on at least 1000 cells/sample on two independent experiments. Our results show that ns-TiO_2 mediated infection is more efficient with both viral samples (around 25% for supernatant-virus and 80% for PEG- virus) compared to the two others substrates (gelatin respectively 8% and 30%, plastic respectively 3,4% and 25%). Our results show that in case of the not concentrated supernatant-virus sample the ns-TiO2 substrate is three to six time more efficient in retroviral transduction efficiency, compared to respectively gelatin and plastic surface while in the case of concentrated virus is around 3 times: the ns-TiO_2 mediated infection with concentrated virus is probably reaching the maximum efficiency of transduction, that in the case of retroviruses is tightly regulated by the capability of these cells to cycle.

CONCLUSIONS

We have developed a new safe and efficient technology for viral transduction in adult human melanocytes. By means of retroviral coating of a biocompatible ns-TiO_2 film we achieved 80% of genetic transduction of a GFP reporter gene in absence of polycations. To strength our findings we further demonstrated that polycations like polybrene exert strong toxicity on primary melanocytes causing DNA damage and apoptosis: the use of these reagents should be omitted to avoid rescue of the toxic effect and subsequently genetic alterations of primary cells.

Our ns-TiO_2 film mediates high efficiency retroviral infection: this high efficiency might be related to the interaction of retroviral vectors and cells with the nanoparticle-assembled substrates (paper in preparation). We propose that our technology based on reverse infection on ns-TiO_2 film will allow efficient and safe genetic manipulation of primary human melanocytes for ex-vivo gene therapy.

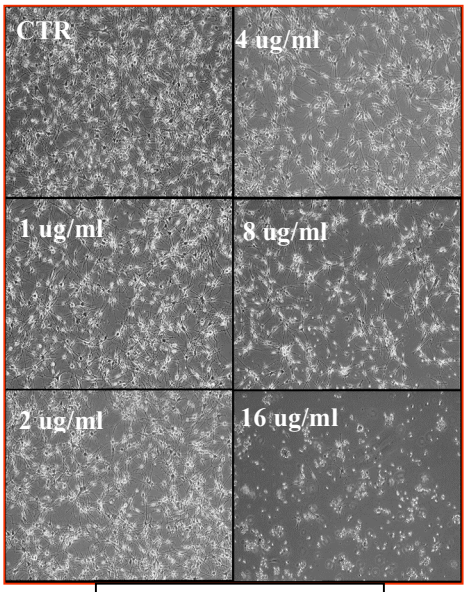

FIGURE 1 Panel A

Bright field analysis of polybrene effects

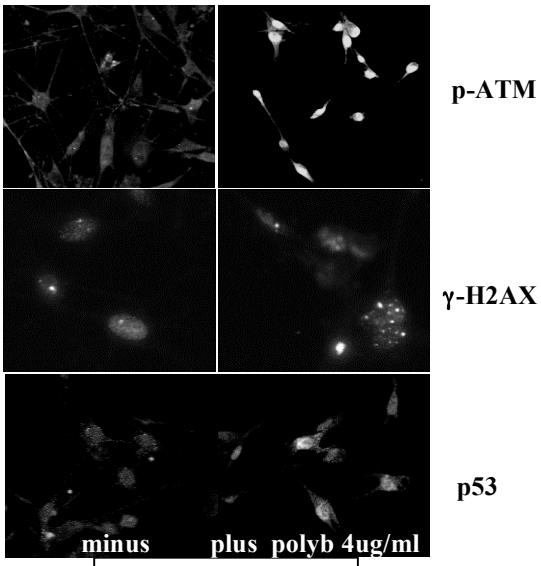

FIGURE 1 Panel C

Immunofluorescence analysis of DNA damage mediators in melanocytes after polybrene treatment

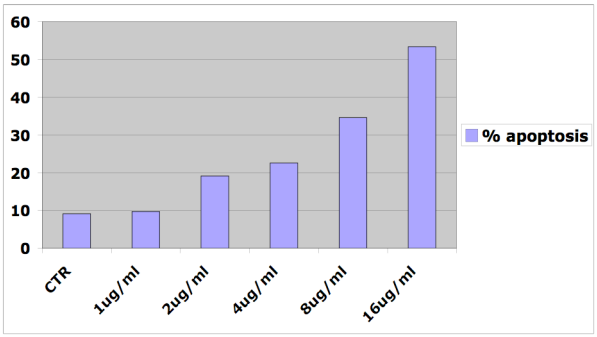

FIGURE 1 Panel B

FACS analysis of apoptosis after polybrene treatment

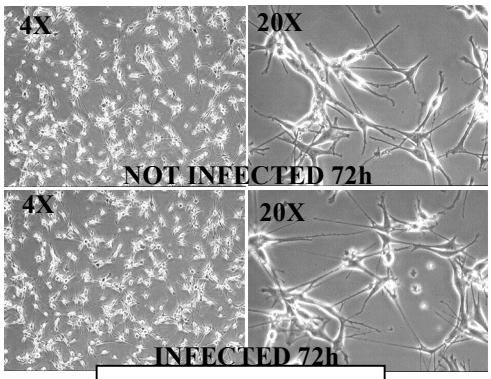

FIGURE 2 Panel A

Bright field analysis of mock infected and infected melanocytes at 72 hours

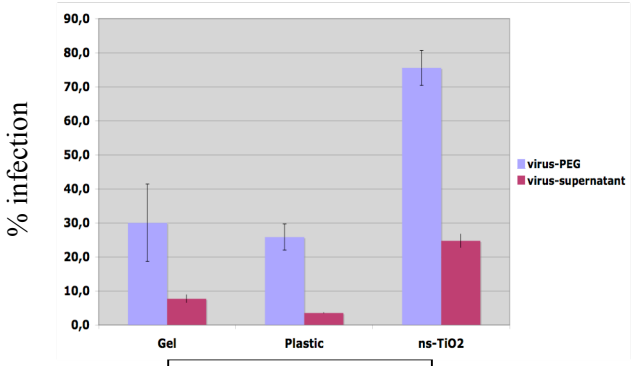

FIGURE 2 Panel B

% of infection of primary melanocytes

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