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Topical Colchicine Selection of Keratinocytes Transduced with the Multidrug Resistance Gene (MDR1) Can Sustain and Enhance Transgene Expression in vivo

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Key Words
Keratinocytes · MDR · Retroviral vector · Selection

Abstract
In gene therapy, a clinically relevant therapeutic effect requires long-term expression of the desired gene at a level sufficient to correct or at least alleviate the underlying gene defect. One approach to achieve persistent as well as high-level transgene expression in a significant percentage of target cells would be to select cells expressing both the desired transgene and a linked selectable gene – such as the human multi-drug resistance (MDR1) gene – in a bicistronic vector. Because of its accessibility, the skin is a very attractive target tissue to select genetically modified cells, allowing topical application of a selecting agent, thus minimizing potential toxic side effects. Among the potential selecting drugs, agents that block cell division, such as colchicine, are of particular interest because the use of anti-mitotic drugs takes advantage of the rapid keratinocyte (KC) turnover in the epidermis and the need for continued proliferation to substitute the KC lost due to selection. Before assessing the therapeutic benefit of such an approach, several key questions need to be answered in preclinical models: (1) Does topical colchicine application achieve the desired in vivo effect by blocking KC mitosis without eliciting unwanted toxic side effects? (2) Are MDR-transduced (MDR+) human KC still able to proliferate and differentiate when treated with colchicine? (3) Can MDR+ KC be enriched by topical selection? (4) Does topical selection result in persistent transgene expression by selecting KC stem cells expressing MDR? To answer these questions and to test the feasibility of such an approach both an in vitro skin equivalent and an in vivo human skin graft model were developed in which MDR+

Abbreviations used in this paper

BrdU	bromodeoxyuridine
FACS	flow-activated cell sorting
KC	keratinocytes
MDR	multidrug resistance
PCR	polymerase chain reaction

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KC were treated with different dosages of colchicine. Quantitative and qualitative analyses of MDR expression in human KC showed that topical colchicine treatment selects high-level transgene expression in a high percentage of KC. Moreover, determination of transgene copy numbers demonstrated that MDR+ KC progenitor cells were enriched by topical selection resulting in long-term expression of the transgene in the skin. Thus, in summary, these models demonstrate that topical selection of MDR+ KC is a safe approach to efficiently enhance long-term gene expression in the skin and holds future promise for clinical gene therapy applications.

Introduction

In gene therapy, a clinically relevant therapeutic effect usually requires both highly efficient gene transfer to the target tissue and a sufficient level of expression of the inserted gene. Although retroviral vectors are able to stably integrate into the host genome, gene expression is often lost or diminished in vivo over time [Palmer et al., 1991; Strauss, 1994; Fenjves et al., 1996; Lange et al., 1997]. Possible reasons include a failure to efficiently target long-lived progenitor cells (stem cells) and gene silencing of the integrated retroviral vector due to methylation or histone acetylation [Chen et al., 1997; Bestor, 2000]. Therefore, to ensure a clinically meaningful, lasting therapeutic effect, strategies need to be developed which focus on maintaining gene expression in a high percentage of target cells. One approach would be to target cells transduced with a selectable gene linked to the desired transgene in a bicistronic vector. When treating with a cytotoxic drug, transduced cells protected by the selectable gene would have a selective advantage, enriching transduced progenitor cells and cells with increased transgene expression. Attempts to select hematopoietic cells transduced with a selectable gene often have failed due to the high toxicity of systemic chemotherapy or the need of higher initial transgene expression levels in the treated cells [Allay et al., 1995, 1998; Havenga et al., 1999; Machiels et al., 1999; Davis et al., 2000]. As a result, the feasibility of in vivo selection of genetically modified cells has been questioned [Brenner, 1999].

The skin is a very compelling organ for gene therapy approaches [Taichman, 1994; Khavari, 1997; Pfützner and Vogel, 2000]. KC, the primary cell type in the epidermis, are easily obtained and readily expanded from small skin biopsy specimens allowing retroviral vector transduction with high efficiency. Genetically modified KC can be formed into artificial skin and then be grafted to establish a functional, durable epidermis [Kolodka et al., 1998; Pfützner et al., 2002]. Previously, the potential of skin gene therapy to treat both skin and systemic diseases has been shown [Gerrard et al., 1993; Fenjves et al., 1994; Choate et al., 1996; White et al., 1998; Robbins et al., 2001]. However, although gene expression from retroviral vectors has been detected for prolonged periods in grafted KC [Deng et al., 1997; Kolodka et al., 1998; Levy et al., 1998; White et al., 1998], in vivo transgene expression was frequently low or declined over time when assessed quantitatively [Morgan et al., 1987; Gerrard et al., 1993; Stockschlader et al., 1994; Fenjves et al., 1996; Choate et al., 1997; White et al., 1998]. These results highlight the need for a strategy to efficiently augment the number of KC expressing the gene of interest.

One approach would be the topical application of an agent targeting KC containing a selectable marker gene, thereby minimizing possible systemic side effects. Since the human epidermis is a tissue with high proliferative activity and continuous renewal [Halprin, 1972; Gelfant, 1982], good candidate drugs for topical selection would be anti-mitotic compounds that block cell division. When topically applied, only KC transduced with the selectable gene – including KC stem cells – will still be able to divide and proliferate while non-transduced KC should be blocked in mitosis and replaced by the transduced KC. Thus, topical selection should also increase the percentages of genetically modified KC that express a desired transgene linked to the selectable gene.

To test this hypothesis, we transduced human KC with the multidrug resistance (MDR1) gene. The human MDR1 gene possesses several desirable characteristics for topical selection of KC. First, the MDR gene is expressed in several human tissues associated with secretory or barrier functions, but not in KC [Thiebaut et al., 1987; Cordon-Cardo et al., 1990]. Second, it encodes a transmembrane glycoprotein that functions as an ATP-dependent efflux transporter for many different cytotoxic compounds, allowing greater flexibility in choosing an appropriate selecting drug [Endicott and Ling, 1989; Gottesman and Pastan, 1993; Higgins, 1993]. Third, since the MDR gene encodes a human protein, unlike many other selectable marker genes, it would not be expected to elicit a host immune response. To assess the feasibility of MDR selection for the skin, both an in vitro skin equivalent and an in vivo grafting model were developed in order to answer several key questions: (1) Does topical colchicine application achieve the desired in vivo effect by blocking

KC mitosis without eliciting unwanted toxic side effects? (2) Are MDR-transduced (MDR+) human KC still able to proliferate and differentiate when treated with colchicine? (3) Can MDR+ KC be enriched by topical selection? (4) Does topical selection result in persistent transgene expression by targeting KC stem cells expressing MDR? In the following, we will summarize and discuss the results of our in vitro and in vivo experiments, which have been published in detail previously [Pfützner et al., 1999; Pfützner et al., 2002].

Material and Methods

Tissue Preparation

Human KC were obtained from newborn foreskin and cultured in monolayers as described [Pfützner et al., 1999]. Transduction was performed by incubation of first-passage KC with the human MDR-expressing pHaMDR1/A-retroviral vector [Pastan et al., 1988]. Raft cultures were generated by seeding transduced KC on de-epidermized, acellular dermis (basement membrane side up), prepared as described [Pfützner et al., 1999]. For selection, different concentrations (10 or 50 ng/ml) of colchicine were added every other day to selected raft cultures, starting 2 days after seeding of KC. To assess the proliferative capacity of raft culture KC, bromodeoxyuridine (BrdU; 10 μM) was incubated with raft organ cultures on day 7 for 4 h. Raft cultures consisting of MDR-transduced human KC and fibroblasts were constructed by established methods [Parenteau et al., 1992; Garlick and Taichman, 1994] and maintained at the air/liquid interface for 3 days until grafting.

Animals

Grafting was performed on 4- to 5-week-old NIH male Swiss nu/nu mice (Taconic Farms). All animals were housed and used in accordance with NIH guidelines. Grafts were placed on the muscle fascia on the back of the mice in correct anatomical orientation and covered with a dressing [Pfützner et al., 2002]. The dressing was changed at 1 week and removed after 2 weeks. Different dosages of colchicine cream (prepared as described [Pfützner et al., 2002]) were topically applied either on mouse skin or grafts of human KC, 2–3 times/week for up to 10 weeks, starting 4–5 weeks after grafting (in two mice topical colchicine selection was initiated 12 weeks after grafting and then continued for another 12 weeks).

Histology

Fixed tissue was paraffin embedded, and either stained with hematoxylin and eosin or used for immunohistochemistry. Unstained paraffin-embedded sections were de-waxed, rehydrated and rinsed in phosphate-buffered saline prior to the immunohistochemical staining procedure. For detection of MDR expression, tissue sections were also incubated in the antigen-unmasking agent Retrieve-All (Signet, Dedham, Mass., USA), for 10–20 min at 92°C. Staining was performed using a Vectastain Universal ABC-AP Kit and Vector Red Alkaline Phosphatase Substrate Kit I according to the protocols provided by the manufacturer (Vector Laboratories, Burlingame, Calif., USA). Sections were incubated with antibodies against either human MDR1 (C219; Signet), proliferating cell nuclear antigen (PC10; Boehringer Mannheim, Indianapolis, Ind., USA), cytokeratin 10 (AXL7125; Accurate Chemical & Scientific Corporation, Westbury, N.Y., USA), filaggrin (BT-576; Biomedical Technologies, Stoughton, Mass., USA), overnight at 4°C, or human involucrin (MED-CLA130; Accurate Chemical & Scientific Corporation), for 30 min at room temperature [Pfützner et al., 1999; Pfützner et al., 2002]. Control samples were stained with corresponding isotype control antibodies. S-phase nuclei incorporating BrdU were detected using an alkaline-phosphatase-conjugated monoclonal anti-BrdU antibody (Boehringer Mannheim) as described [Pfützner et al., 1999]. The number of BrdU-positive nuclei in a basal or immediately suprabasal position per unit length of 500 basal cells was determined by a blinded observer.

Flow Cytometry Analysis

KC cell suspensions obtained either from cell culture monolayers or from epidermal sheets harvested from raft cultures or grafts as described [Pfützner et al., 1999, 2002] were assessed by fluorescence-activated cell sorting (FACS) using a FACScan flow cytometer equipped with CellQuest software (Becton-Dickinson, Mountain View, Calif., USA). Antibodies used for detection of human MDR, proliferating cell nuclear antigen, or HLA-A, -B or -C were MRK16 (Kamiya, Seattle, Wash., USA), PC10 (applied after permeabilization of KC as described [Pfützner et al., 1999]) and anti-human HLA-A, -B or -C conjugated to R-phycoerythrin (PharMingen, San Diego, Calif., USA), respectively [Pfützner et al., 2002]. MRK16 and PC10 incubation was followed by addition of goat anti-mouse secondary antibody conjugated to fluorescein isothiocyanate (Biosource, Camarillo, Calif., USA). All samples were compared to cells stained with appropriate isotype control antibodies. For multicolor analysis, cells were preincubated with saturating concentrations of anti-FcRγII/III (PharMingen). Propidium-iodide-permeable (nonviable) cells were excluded from the analyses.

Quantitative Real-Time Polymerase Chain Reaction Analysis

By using a DNeasy tissue collection kit (Qiagen, Valencia, Calif., USA), DNA samples were obtained either from epidermal sheets of harvested grafts or from first-passage nontransduced and transduced KC serving as a negative and positive control, respectively. Real-time polymerase chain reaction (PCR) was performed on an Applied Biosystems PRISM sequence detector and software version 1.7 (GIBCO/BRL and Applied Biosystems) using specific primers and probes as described [Pfützner et al., 2002].

Results and Discussion

Topical Colchicine Treatment Results in a Dose-Dependent Increase of KC Blocked in Mitosis

A very important issue is to show that topical selection can achieve effective selection without causing unwanted side effects. Compounds that inhibit mitosis, such as colchicine, may have important advantages over other drugs because they have a wide therapeutic window, and doses that are effective for selection might not cause extensive cell death and necrosis in KC not expressing MDR. To test if topical colchicine application can block KC mitosis and to determine the optimal dose range for topical selec-

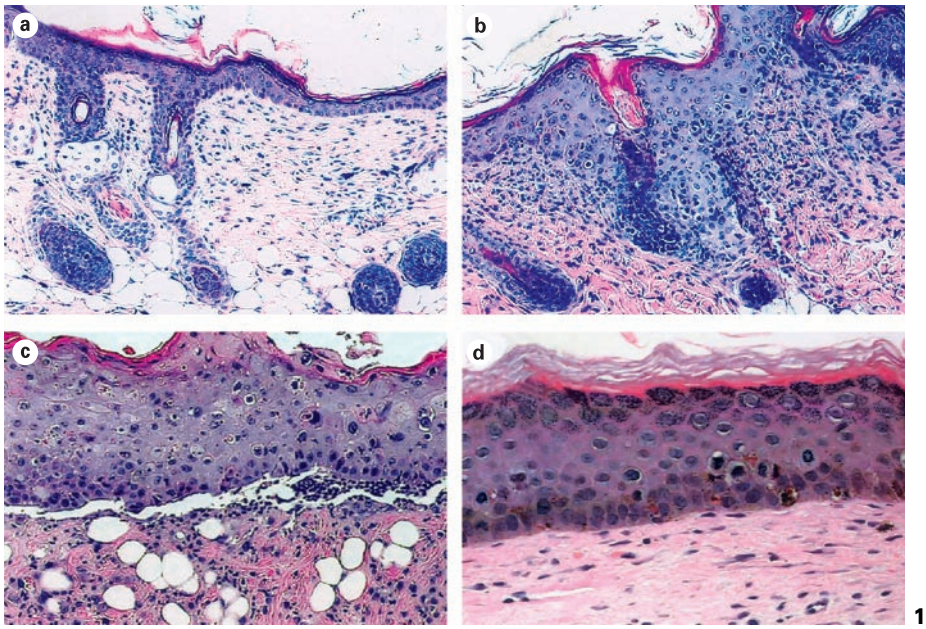

Fig. 1. Topical titration of colchicine treatment demonstrates dose-dependent effects on mouse and human skin. HE-stained sections from nude mouse skin treated for 2 weeks with either vehicle control (**a**, ×20), or 200 (**b**, ×20) and 500 μg/g colchicine (**c**, ×20) and from human skin grafts treated for 2 weeks with 200 μg/g (**d**, ×40) of colchicine cream. Treatment with 200 μg/g resulted in acanthosis and marked increase in KC blocked in mitosis in both mouse skin (450/1,000 basal cells vs. 15/1,000 basal cells in vehicle control) and human skin (66/1,000 basal cells vs. 8/1,000 basal cells). Higher colchicine doses of 500 μg/g led to cellular necrosis and ulceration.

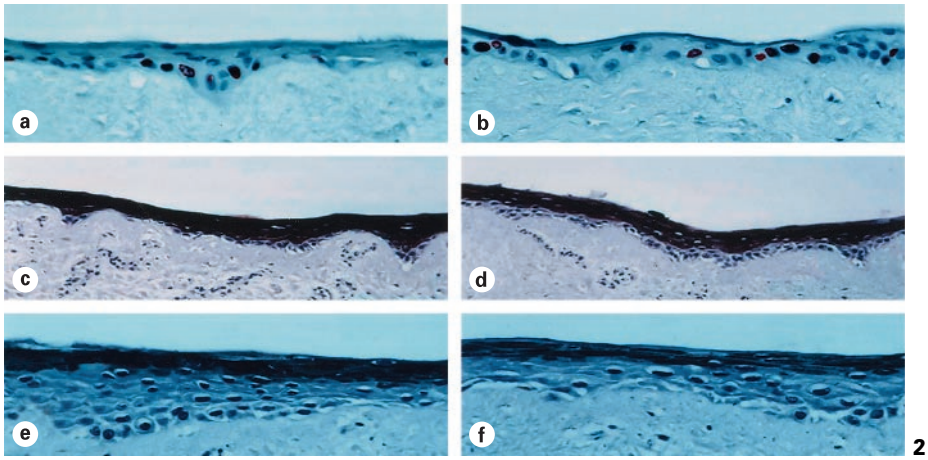

Fig. 2. MDR+ KC are protected against the cytotoxic effects of colchicine, maintaining their proliferative activity and forming a well-differentiated, stratified epidermis when treated with colchicine. Immunohistostaining of BrdU incorporation in MDR+ KC raft cultures harvested on day 7 showed similar percentages of BrdU-positive cells in rafts either not treated (**a**), 25.08 ± 1.24% (n = 3), or treated with 50 ng/ml colchicine (**b**; both ×40), 23.08 ± 1.42%. Expression of the early differentiation marker keratin 10 (**c**, **d**; ×20) and the late differentiation marker filaggrin (**e**, **f**; ×40) in MDR+ KC skin raft cultures on day 11 was comparable in rafts either not treated (**c**, **e**) or treated with 50 ng/ml colchicine (**d**, **f**).

tion of human KC grafted onto nude mice, both nude mouse skin and human skin grafts were treated with different doses of colchicine cream (0–500 μg/g) for a period of 2–4 weeks (3 times/week). Histological analyses showed that topical application of colchicine cream resulted in dose-dependent increases in the number of KC blocked in mitosis with high doses (500 μg/g) leading to abundant cell necrosis and total disruption of the epidermal architecture (fig. 1). Thus, topical treatment with moderate doses of colchicine (100–200 μg/g) allows to block KC division without causing unwanted toxic side effects.

Human KC Transduced with the MDR Gene Are Protected against the Cytotoxic Effects of Drug Selection

The most well-known cytotoxic effect of colchicine is the inhibition of the assembly of the microtubulin spindle, thereby blocking cell division. However, colchicine has additional toxic effects on cells. It can also disrupt the cellular cytoskeleton and interfere with protein synthesis and secretion, cell movement, cellular orientation and stratification [Epstein et al., 1983; Thyberg and Moskalewski, 1985]. Thus, it is critical to determine if colchicine may reduce the proliferative capacity of MDR+ KC or impede their ability to form a differentiated epidermis

and stratify. This is important because the MDR+ KC will be required to replace the targeted KC lacking MDR.

To test the effects of topical colchicine selection on the epidermal integrity, an in vitro skin raft culture, containing MDR+ KC seeded onto acellular dermis, was treated with different concentrations of colchicine. When the proliferative capacity was assessed by BrdU incorporation assay and analysis of proliferating cell nuclear antigen expression, there was no significant decrease in proliferating MDR+ KC in colchicine-treated skin rafts compared to control samples (fig. 2a, b). Hematoxylin-eosin staining performed on skin raft cultures after 11 and 14 days demonstrated that KC transduced with a retroviral control vector and treated with low doses of colchicine (10 ng/ml) did not form an epidermis while MDR+ KC treated with the same or higher doses (10–50 ng/ml) were still able to develop a stratified epidermis similar in structure and integrity to untreated controls [Pfützner et al., 1999]. Furthermore, colchicine treatment of MDR+ KC showed similar expression of early and late differentiation marker genes in the correct epidermal layers compared to untreated control samples (fig. 2c–f). These results demonstrate that KC expressing MDR maintain high proliferative activity and the ability to form a well-differentiated, stratified epidermis during colchicine selection.

MDR+ KC Can Be Enriched by Topical Colchicine Selection both in vitro and in vivo

The next question we asked was if MDR+ KC can be enriched by topical colchicine selection. Initial in vitro studies demonstrated a marked increase in MDR+ KC in skin raft cultures treated with either 10 or 50 ng colchicine/ml when compared to untreated raft cultures. This was quantitatively confirmed by FACS analysis of KC suspensions generated from skin raft cultures by enzymatic treatment [Pfützner et al., 1999]. Application of colchicine to raft cultures transduced with the control vector resulted in the loss of all KC, ruling out the possibility that colchicine treatment might induce or select for endogenous MDR expression.

To test the feasibility of this approach in vivo, a grafting model was utilized in which skin raft cultures composed of human KC seeded onto a collagen gel embedded with fibroblasts were grafted onto immunocompromised mice [Kolodka et al., 1998]. This model allows efficient, long-lasting skin engraftment, with growth factors and adhesion molecules secreted by metabolically active fibroblasts in the skin raft possibly supporting the proliferation of the transplanted KC and anchoring the epidermis to the dermis [Maas-Szabowski and Fusenig, 1996; Parenteau et al., 1996; Fleischmajer et al., 1998]. In grafts that initially contained 50% MDR+ KC, a decrease in MDR+ KC was noticed over time with 34 ± 5 and 24 ± 11% of transduced KC still showing MDR expression 2 and 4 weeks after grafting, respectively. At later time points, the percentage of MDR+ KC continued to decline to 13 ± 5 and 7 ± 4% at 9 and 15 weeks, respectively [Pfützner et al., 2002]. Thus, a significant loss of transgene expressing KC occurred in vivo, as observed by others [Morgan et al., 1987; Gerrard et al., 1993; Stockschlader et al., 1994; Fenjves et al., 1996; Deng et al., 1997].

Our hypothesis is that treatment of the MDR+ grafts with a chemotoxic agent like colchicine should lead to a selective growth advantage of MDR+ KC and thereby prevent the loss of gene expression. Based on the results of colchicine dose titration onto mouse and human skin, we decided to treat MDR+ KC grafts with colchicine dosages of either 100 or 200 μg/g (3 times/week) to determine if topical application of colchicine would select MDR+ KC. Seven weeks after grafting skin raft cultures that contained 50% MDR+ KC, grafts treated with control cream contained only 20 ± 9% MDR+ KC, while colchicine treatment (100 or 200 μg/g colchicine) increased the percentages of MDR+ KC in the grafts to 41 ± 24 and 58 ± 22%, respectively [Pfützner et al., 2002]. Since the higher dose of 200 μg/g resulted in a significant rise in MDR+ KC with large segments of the graft positive for MDR expression by immunohistochemistry, this dose was chosen for longer-term studies.

Continued selection of MDR+ KC grafts with 200 μg/g showed that 9 weeks after grafting (representing approximately 3 epidermal turnovers), colchicine treatment had resulted in an increased percentage of 50 ± 26% (median = 40%) MDR+ KC compared to 13 ± 5% (median = 10%) in the controls [Pfützner et al., 2002]. Fifteen weeks after grafting, only 7 ± 4% KC (median = 8%) of the controls showed MDR expression, with 2 grafts experiencing a complete loss of MDR-positive cells (fig. 3a, b). On the other hand, colchicine treatment resulted in a tenfold-increased MDR expression (44 ± 15%) of the KC (median = 46%). The FACS data were confirmed by immunohistostaining that showed MDR expression in up to 50% of the human KC, while much smaller percentages of MDR+ KC were present in vehicle-treated controls. Thus, MDR+ human KC can be selected in vivo by topical colchicine application leading to a dose-dependent increase in MDR+ KC whereas control grafts show a loss of MDR expression over time. Interestingly, colchicine treatment also selected MDR+ KC that expressed increased amounts of MDR transgene per cell when com-

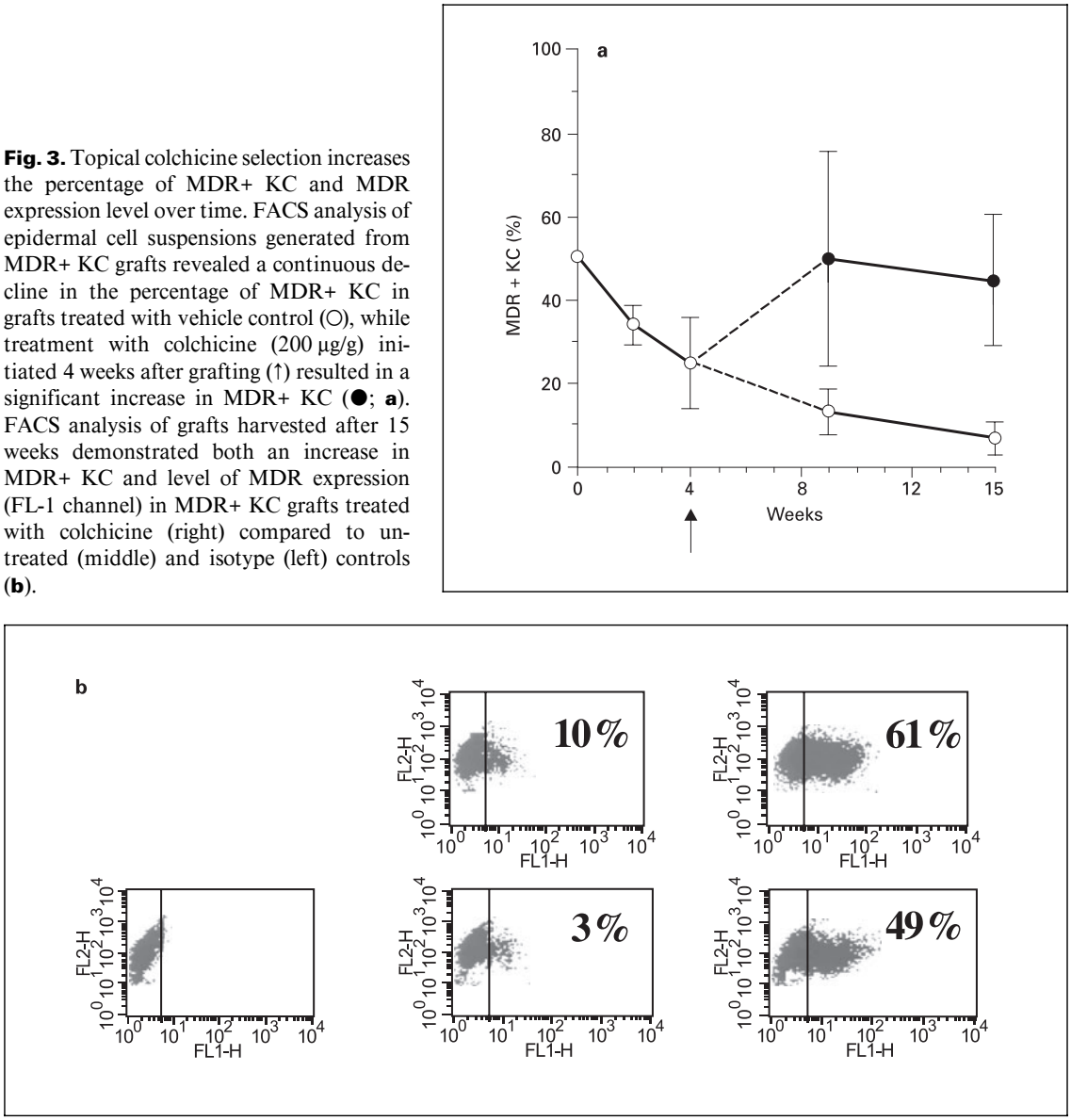

Fig. 3. Topical colchicine selection increases the percentage of MDR+ KC and MDR expression level over time. FACS analysis of epidermal cell suspensions generated from MDR+ KC grafts revealed a continuous decline in the percentage of MDR+ KC in grafts treated with vehicle control (○), while treatment with colchicine (200 μg/g) initiated 4 weeks after grafting (↑) resulted in a significant increase in MDR+ KC (●; **a**). FACS analysis of grafts harvested after 15 weeks demonstrated both an increase in MDR+ KC and level of MDR expression (FL-1 channel) in MDR+ KC grafts treated with colchicine (right) compared to untreated (middle) and isotype (left) controls (**b**).

pared to vehicle-treated KC, as could be demonstrated by FACS analysis (fig. 3b). The mean fluorescence intensity of MDR expression in the FL1 channel (detection of fluorescein isothiocyanate antibody bound to anti-MDR antibody) was 16 ± 3 for colchicine-treated KC compared to 11 ± 2 for vehicle-treated grafts, a 30% increase with colchicine treatment.

We also demonstrated that colchicine treatment could select increased percentages of MDR+ KC even when initiated at much later time points after grafting. Two MDR+ KC grafts treated with vehicle control cream for 12 weeks (a point in time when MDR+ KC in control grafts would be expected to decline from 50% down to approximately 9%) were switched to colchicine treatment (200 μg/g, 2 times/week) for another 12 weeks. Following this selection, FACS analysis revealed elevated numbers of MDR+ KC (35 and 34%, respectively) [Pfützner et al., 2002]. Thus, 24 weeks after grafting (8 epidermal turnover cycles), colchicine selection – initiated when only a low percentage of MDR+ KC is present – is able to substantially increase the number of MDR+ KC, demonstrating that a small number of MDR+ KC can be expanded by topical selection.

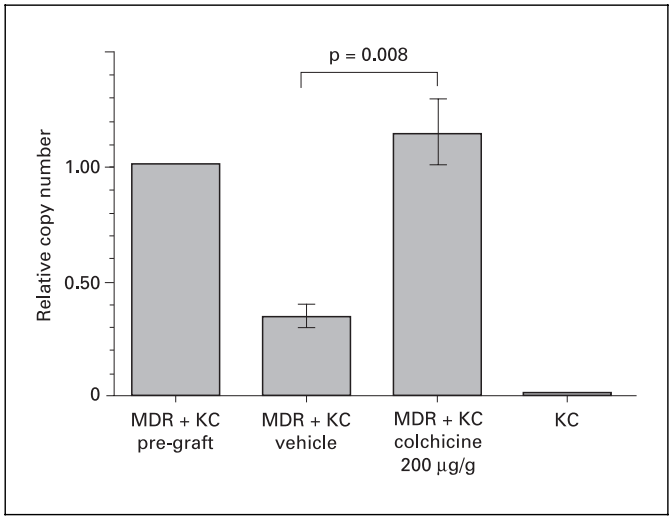

Fig. 4. Topical colchicine selection of MDR+ KC significantly increases transgene copy numbers in grafts. Determination of the relative MDR1 gene copy number in MDR+ KC grafts by quantitative real-time PCR demonstrated significantly increased transgene copy numbers in grafts treated with colchicine after 15 weeks compared to vehicle-control-treated grafts. Normal human KC lacking the retroviral vector transgene (right) served as a negative control.

Topical Colchicine Selection Increases Transgene Copy Numbers in Treated Grafts Suggesting Enrichment for MDR+ KC Progenitor Cells

Since the epidermis represents a highly proliferative tissue showing an epidermal turnover cycle of ~21 days in humans [Halprin, 1972; Gelfant, 1982], KC progenitor cells have to carry the transgene to ensure a sustained high level of gene expression. Immunohistochemistry and FACS analysis of MDR+ KC grafts demonstrated that topical selection resulted in long-term, enhanced transgene expression at 15 weeks or approximately 5 epidermal turnover cycles (time frame of 105 days) while control-vehicle-treated grafts showed significant loss of MDR+ KC (fig. 3a). This suggests that MDR+ KC progenitors have been enriched by colchicine treatment of the grafts. Consequently, the percentage of human KC containing the MDR gene would be increased in colchicine-treated grafts compared to control grafts. Since we cannot experimentally determine if individual KC contain an integrated MDR gene, quantitative real-time PCR allows us to determine how many MDR genes are present in a defined population of KC, indicating if there are differences between both groups of grafts. We therefore prepared genomic DNA from MDR+ KC harvested 15 weeks after grafting and determined the relative copy number of the MDR transgene, normalized to the endogenous human hematopoietic cell kinase gene. Compared to the MDR+ KC skin raft cultures at the time of grafting, the colchicine-treated MDR+ KC grafts contained a relative MDR copy number of 1.14 ± 0.41 while the vehicle-treated grafts had a significantly lower relative MDR copy number of 0.34 ± 0.16 (fig. 4). Thus, KC in colchicine-treated grafts contain 3–4 times more MDR transgenes at 15 weeks than vehicle-treated grafts. When the PCR results are compared to the percentage of MDR+ KC determined by FACS in the different treatment groups (fig. 3a), these data argue that colchicine selection increases the percentages of MDR+ KC, primarily by expanding the population of KC progenitor cells that contain the MDR gene. Only KC progenitor cells expressing the MDR protein are able to divide, proliferate, and repopulate the epidermis in the presence of colchicine, while KC progenitors that lack MDR protein will be blocked in mitosis and eventually undergo apoptosis [Tsukidate et al., 1993; Bonfoco et al., 1995]. Additionally, the low percentage of MDR+ KC in vehicle-treated grafts after 15 weeks indicates that initially only a small percentage of the KC progenitor cells (<7%) may have been transduced with MDR during ex vivo culture.

One tremendous advantage of the skin compared to other tissues is that topical selection allows high local concentrations of the selecting agent in the target tissue while minimizing unwanted systemic effects. Although the percentage of MDR+ KC in untreated grafts declined over time, we could demonstrate that topical colchicine selection can reverse this decline and significantly increase both the percentage of MDR+ KC and the transgene expression level per cell. Of note, this was not a short-term effect but was shown to be persistent for a least 15 weeks, representing 5 epidermal turnovers. Given the problem of persistent in vivo expression of a desired gene at high levels in gene therapy studies [Gottesman et al., 2000; Licht et al., 2002], our approach of topical selection for MDR1 gene expression in KC could pave the way for elevated and durable gene expression in the skin. However, important questions still remain to be answered, for example whether the maximum percentage of MDR+ KC can be increased to >50%, the percentage achieved in this study, by optimizing the colchicine dosing schedule and increasing the duration of treatment. The question of what will happen to the percentage of MDR+ KC after long-term colchicine treatment is discontinued should also be solved in future studies. If colchicine has in fact increased the percentage of KC progenitor cells containing and expressing MDR, then the percentage of MDR+ KC should con-

tinue to be elevated in the absence of colchicine selection. Based on the results presented here, we anticipate that topical transgene selection holds great promise for future applications in clinical skin gene therapy trials that require significant levels of gene expression in a high percentage of KC.

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