POTENTIAL USE OF RNAi ON HUMAN SKIN

Christine Collin-Djangoné[1], Florent Sahuc[2], Béatrice Bertino[2], Anne de Brugerolle[2], Jean-Eric Baudouin[1], Peggy Sextius[1], Séverine Teluob[1], Rachid Boulgana[1], Dmitry Samarsky[3,4], Peggy Tailor[3], Peter Welch[3] and Yann Mahé[1]

[1]L'Oréal Recherche – Centre Charles Zviak – 90, rue Général Roguet – F-92583 CLICHY Cedex; [2]SkinEthic Laboratories – 45, rue St Philippe – F-06000 NICE; [3]Invitrogen, 5791 Van Allen Way, Carlsbad, CA 92008, USA.
[4]: Present address = RXi Pharmaceuticals, 60 Prescott Street, Worcester, MA 01605 - USA.

ABSTRACT

As a first step to develop either new application routes, vehicles and devices for siRNA delivery in skin and hair for dermatological interventions, we first designed highly efficient Stealth™ RNA targeting human tyrosinase as prototype tools. Their efficacy was evaluated in human primary melanocytes by qPCR and Western blotting. In addition, melanin production was quantified in a co-culture of melanocytes and keratinocytes. Melanogenesis inhibition was thus shown to be efficiently achieved with 3 distinct and potent Stealth™ siRNA with optimum IC_{50} in the 10-100 pM range. Transfected human skin melanocytes were then seeded with keratinocytes to reconstruct a pigmented epidermis according to SkinEthic procedure which was cultured for up to 39 days *in vitro*. By silencing tyrosinase in melanocytes, we achieved the reconstruction of an artificial epidermis showing a long term lasting (>30 days) inhibition of melanogenesis, in contrast with melanocytes treated with a scrambled Stealth™ siRNA. As expected, this observation clearly identifies the lack of redundancy in the melanogenesis machinery to rescue the silencing of tyrosinase. Since skin pigmentation can easily be monitored by colorimetric analysis, the use of validated Stealth™ siRNA against tyrosinase on reconstructed pigmented epidermis is a precious tool for the evaluation of functional skin delivery vehicles and devices, which remains the major challenge towards the use of RNAi for dermatological intervention (either cosmeticalor therapeutical).

INTRODUCTION

RNAi is a recent promising technology allowing to silence any undesirable gene expression and, consequently presenting a dramatic shortcut in long design and selection of specific pharmacological agent against undesirable biological functions. The aim of this study was to establish the potential of RNAi technology for skin applications. As prerequisites, we focused on:

1 • An easy to follow skin phenotypic target (skin pigmentation)

2 • Use of an accessible, reproducible and industrially reliable *in vitro* skin model allowing multiple assays and high-throughput screening without safety issues (SkinEthic model)

3 • Selection of potent and validated siRNA molecules (Stealth™ RNA)

We thus decided to silence the expression of Human Tyrosinase, the key enzyme of skin and hair pigmentation.

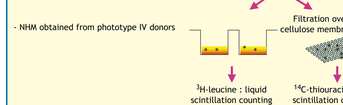
CONCLUSION

After selecting only the few very potent and appropriated siRNA candidates in the gene reporting assay (IC_{50} less than 100 pm) we could achieve a biological interference in primary cell culture models as well as in the more complex reconstructed pigmented epidermis model (SkinEthic). Knocking down Tyrosinase by transfecting melanocytes with 10 nM Stealth™ siRNA produced indeed a visible and a long term pigmentation interference effect (lasting more than 30 days) in 3D-reconstructed SkinEthic epidermis. This knock down of Tyrosinase expression was indeed transient since reconstructed epidermis started recovering pigmentation at day 39 and thus did not irreversibly affected melanocytes differentiation. The use of a pigmented reconstructed epidermis (SkinEthic) is thus becoming a precious tool allowing to study the action of different topical applications and vectors as well as to select, evaluate and design new dermatological interventions, tools and targets. It allows us to envision modifying skin biology exquisitely by the specific use of siRNA designed against any choosen key skin targets, giving promising insights onto the potency of RNAi applications *in vivo* for both therapeutic and esthetic interventions on skin and hair.

RESULTS

Figure 1: Selection of potent siRNA molecules

Invitrogen proprietary RNAi design algorithm was used to select a panel of 50 Stealth™ siRNA compounds to target the tyrosinase gene and their efficacy was determined in A549 cells by using the pScreen-IT vector system *(Invitrogen)*.



A/ Description of pScreen-iT™-Tyrosinase reporter vector

The tyrosinase Ultimate™ ORF Clone (accession number BC027179) was inserted, using an LR recombination reaction, in frame with the lacZ reporter gene downstream the CMV promoter (pSCREEN-IT™/lacZ-DEST).

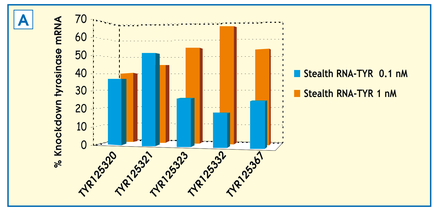
B/ Identification of a subset of the most potent RNAi molecules

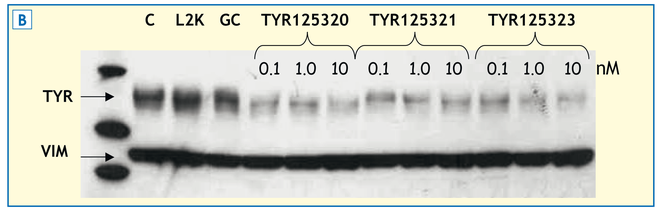
A549 cells were cotransfected in triplicate with 10 ng of the pScreen-iT™-Tyrosinase reporter vector, and 1.0, 0.25, 0.063 or 0.016 nM Stealth™ RNAi duplex against tyrosinase and 20 ng luciferase plasmid as transfection control using Lipofectamine 2000 (L2K, *Invitrogen*).
One positive control Stealth™ duplex targeting LacZ and three negative control Stealth™ duplexes (High, Medium and Low GC content) were used. β-Galactosidase and luciferase activity were measured at 24 hours and β-Galactosidase activity was normalized to Luciferase activity from the same transfection well. Percent inhibition for each Stealth™ duplex is expressed relative to the average of the three controls (H, M, L) from the same transfection plate.

Duplex ID	1 nM	0.25 nM	0.063 nM	0.016 nM
125320	97,37%	94,34%	89,47%	72,07%
125321	97,98%	95,64%	89,34%	66,68%
125323	97,93%	95,22%	90,24%	57,92%
125324	91,33%	79,88%	61,47%	18,40%
125326	96,86%	88,97%	76,45%	38,34%
125327	97,84%	90,76%	81,12%	38,57%
125332	98,13%	95,73%	91,11%	62,56%
125333	97,59%	87,22%	68,77%	33,87%
125335	97,95%	94,92%	83,40%	56,25%
125343	96,05%	87,42%	62,32%	29,97%
125345	93,45%	90,50%	78,36%	46,80%
125349	96,42%	88,24%	70,64%	31,15%
125352	96,88%	90,69%	80,33%	35,04%
125355	97,42%	94,84%	88,34%	54,29%
125357	96,38%	92,46%	88,24%	53,92%
125360	97,65%	95,08%	91,59%	60,64%
125362	98,25%	96,54%	92,58%	53,46%
125363	98,46%	95,60%	89,21%	38,85%
125367	98,77%	94,81%	84,23%	48,74%
125368	94,60%	76,71%	52,94%	30,18%

20 Stealth™ siRNA duplexes that inhibited the target by >90% at 1 nM were thus identified and preselected. Decreasing Stealth™ siRNA duplexes concentration to 16 pM allows the identification of the most potent Stealth™ siRNA molecules. For example, TYR125320 yielded 72% inhibition of tyrosinase at 16 pM. The five most potent molecules at 16 pM concentration (yellow lines) were then selected for further organotypic studies.

Figure 2: Tyrosinase activity extinction in Normal Human Melanocytes (NHM)

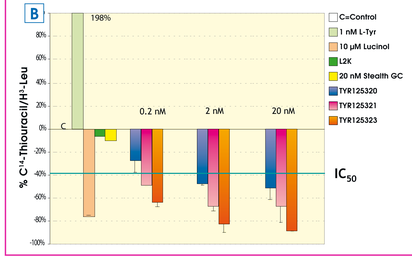




A/ NHM were transfected with 2.0 μg/ml Lipofectamine™ 2000 and 0.1 or 1.0 nM of the five most potent Tyrosinase Stealth™ duplexes or negative control (Universal Medium GC Stealth™ siRNA duplex.) PolyA+ RNA was purified from cell lysates using FastTrack® MAG Maxi mRNA kit *(Invitrogen)* and reverse transcribed using SuperScript™ III *(Invitrogen)*. qPCR was performed on duplicate samples from each RT reaction using Platinum® Taq DNA Polymerase Kit *(Invitrogen)*, and LUX ™ (Light Upon Extension) primers (one FAM labeled primer and one unlabeled) specific for target gene in 384 well plate. Cyclophilin was used as a control house-keeping gene. The knockdown efficiency of tyrosinase mRNA expression is 40 to 66% at 1.0 nM and 19 to 51% at 0.1 nM compared to the corresponding negative control GC Stealth™ siRNA duplex.

B/ The effect of three most efficient Tyrosinase Stealth™ siRNA duplexes was analyzed on tyrosinase protein expression by western blotting. The same amount of proteins was loaded on 4-12% acrylamide gel. After electrophoresis and transfer onto nylon membrane, the blot was hybridized with an anti-tyrosinase antibody (clone T311, 1:500, *Novocastra*) and an anti-vimentin antibody (clone VIM3B4, 1:100 000, *Chemicon*). A strong decrease in tyrosinase protein expression was observed from 0.1 to 10 nM of Stealth™ siRNA compared to the three controls lacking efficient RNAi activity against tyrosinase (C = control, L2K = Lipofectamine 2000, GC = Medium GC Stealth™ RNAi).

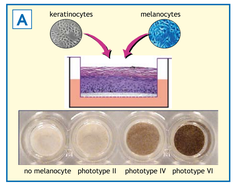
Figure 3 : Inhibition of melanin synthesis by Tyrosinase Stealth™ siRNA

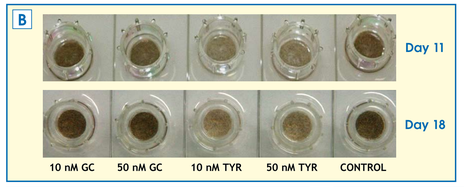


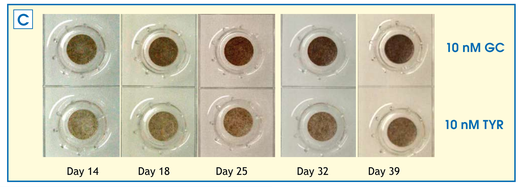
A/ Experimental design to assess melanin synthesis: transfected NHM and Normal Human Keratinocytes (NHK) were mixed and seeded onto 96 well plate and cultured for three days in the presence of ^{14}C-thiouracil and ^{3}H-leucine was added on the last day of incubation. After washing, the cells were lyzed. 1/20 aliquot was evaluated for total protein synthesis (^{3}H-leucine) and the remainder was filtered over DEAE cellulose membrane to evaluate melanin synthesis (^{14}C-thiouracil).

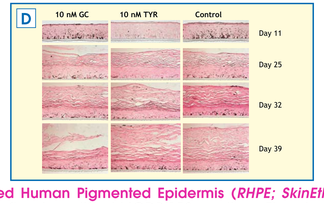
B/ The inhibition of melanin synthesis by the three tyrosinase Stealth™ siRNA duplexes was quantified by measuring the percentage of ^{14}C-thiouracil / ^{3}H-leucine. The validation of the model was performed using 1 mM L-Tyrosine or 10 μM Lucinol respectively as a potent inducer or inhibitor of melanin synthesis. No effect on melanin synthesis was observed with L2K and 20 nM Stealth™ GC while, by contrast, a strong inhibition of melanin synthesis is obtained with an IC_{50} in the range of 2 nM for the three active Stealth™ tyrosinase siRNA duplexes.

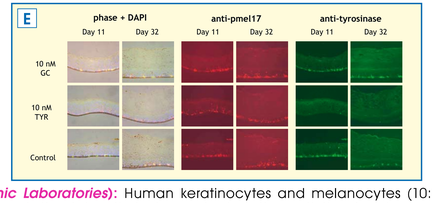
Figure 4 : Reconstruction of a phenotypically silenced epidermis











A/ Description of the Reconstructed Human Pigmented Epidermis (*RHPE; SkinEthic Laboratories*): Human keratinocytes and melanocytes (10:1) were grown on air-lifted NUNC polycarbonate culture inserts in defined modified MCDB 153 medium. The pigmentation intensity is dependent on the activity of melanocytes derived from the pigmented skin with the corresponding phototype II, IV and VI.

B/ Melanocytes (NHM) derived from the high pigmented phototype VI skin were used for the reconstruction of a phenotypically silenced epidermis. NHM were transfected with control Medium GC Stealth™ siRNA (GC 10 and 50 nM) and Stealth™ TYR125320 (TYR 10 and 50 nM) then used the next day for epidermal reconstruction. As expected, no decrease in pigmentation was observed with 10 nM and 50 nM GC compared with the control (not transfected) at days 11 and 18. On the opposite, a striking reduction of pigmentation was obtained with 10 and 50 nM TYR125320 at days 11 and 18. The same inhibition of pigmentation was reached at 10 and 50 nM TYR125320 whereas only a weak reduction of pigmentation was observed at 1 nM (data not shown) indicating that the optimal activity for this visual endpoint lies in the 10 nM range of TYR125320.

C/ Kinetic studies of the effect of 10 nM TYR125320: a strong reduction in pigmentation (compared with the control 10 nM GC) was observed from day 14 until day 39 following transfection indicating both a quite long lasting but reversible effect of the selected TYR siRNA in this organotypic model.

D/ Specific detection of melanin was performed by Masson-Fontana staining (melanin is brown-colored) on reconstructed epidermis. From day 11 to day 32, a strong decrease of melanin content was observed at 10 nM TYR125320 compared to the 10 nM GC and equivalent to the Control. At day 39, no visible difference was observed between 10 nM TYR125320 and the two controls indicating that melanin synthesis is no longer inhibited by TYR125320. This result suggests that the normal melanocyte differentiation machinery was not irreversibly altered by 10 nM TYR125320 treatment as expected.

E/ Freeze-dried cryostated sections of human pigmented reconstructed epidermis were labeled with antibodies against Pmel17 (clone NK1 beteb, 1:20, *Monosan*) and tyrosinase (clone T311, 1:20; *Novocastra*) followed by an incubation with Alexa-Fluor 568 and Alexa-Fluor 488 secondary antibodies (*Molecular Probes*) respectively. Nuclei were counterstained with DAPI. The melanosomal glycoprotein Pmel17 is homogeneously expressed in the melanocytes located in the basal layer of the RHPE while specific extinction of tyrosinase is observed at day 11 until day 32 post-transfection.

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