Chapter 4
Recent Application of Intronic MicroRNA Agents in Cosmetics

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Abstract Utilization of gene silencing effectors, such as microRNA (miRNA) and small hairpin RNA (shRNA), provides a powerful new strategy for human skin care *in vivo*, particularly for hyperpigmentation treatment and aging prevention. For example, tyrosinase (Tyr), a melanocytic membrane-bound glycoprotein, is the rate-limiting enzyme critical for melanin (black pigment) biosynthesis in skins and hairs. There are over 54 native microRNA capable of targeting human tyrosinase for skin whitening and lightening. In this study, we have designed a mir-434-5p homologue and used it to successfully demonstrate the feasibility of miRNA-mediated skin whitening *in vitro* and *in vivo*. Under the same experimental condition in trials, Pol-II-directed intronic mir-434-5p expression did not cause any detectable sign of cytotoxicity, whereas siRNAs targeting the same sequence induced certain non-specific mRNA degradation as previously reported. Because the intronic miRNA-mediated gene silencing pathway is tightly regulated by multiple intracellular surveillance systems, including Pol-II transcription, RNA splicing, exosome digestion and NMD processing, the current findings underscore the fact that intronic miRNA agents, such as mir-434-5p homologues, are effective, target-specific and safe to be used for skin whitening without any overt cytotoxic effect. Given that the human skins also express a variety of native miRNAs, we may re-design these miRNAs based on their individual functions for skin care, which will provide significant insights into areas of opportunity for new cosmetic interventions.

Keywords microRNA (miRNA), intronic microRNA (Id-miRNA), mir-434-5p, tyrosinase (Tyr), hyaluronidase (Hyal), RNA interference (RNAi), skin whitening, anti-aging, cosmetics.

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4.1 Introduction

Prevention of hyperpigmentation (i.e. sun-burn) and aging is the key means for having healthy skins. However, many of the skin pigmentation and aging processes are associated with personal gene activities. For example, tyrosinase (Tyr), a melanocytic membrane-bound glycoprotein, is the rate-limiting enzyme critical for melanin (black pigment) biosynthesis in skins and hairs, while hyaluronidase (Hyal) often causes skin wrinkle by degrading subcutaneous hyaluronan (HA), the major anti-aging extracellular matrix in skins. Therefore, a good skin care can be achieved by suppressing these unwanted gene activities.

Among a variety of currently available skin whitening and lightening products, many chemical and naturally extracted agents have been applied to inhibit tyrosinase function, using materials such as hormone-derived inhibitory oligopetides, hydroxytetronic acid derivatives, benzoyl compounds, hydroquinone compositions, alcohol diol and triol analogues, kojic acid derivatives, ascomycete-derived enzymes, and plant extracts. Although these cosmetic agents may work well *in vitro*, only a few of them, such as hydroquinone and its derivatives, are able to induce good hypopigmenting effects in clinical trials [25]. Nevertheless, all hydroquinone derivatives leading to a reactive quinone are putative cytotoxic agents. Thus, the gap between *in-vitro* and *in-vivo* studies suggests that innovative strategies are needed for validating their safety and efficacy.

With the advance of recent RNA interference (RNAi) technologies, novel small RNA agents have been found to provide more potent effects in targeted gene suppression, including the utilization of double-stranded short interfering RNA (e.g. dsRNA/siRNA) [3, 4] and deoxyribonucleotidylated-RNA interfering molecules (e.g. D-RNAi) [12]. Conceivably, these small RNA agents may be used to develop new cosmetic designs and products for skin care. In principle, the RNAi mechanism elicits a post-transcriptional gene silencing (PTGS) phenomenon capable of inhibiting specific gene function with high potency at a few nanomolar dosage, which has been proven to be effective longer and much less toxic than conventional gene-knockout methods using antisense oligonucleotides or small molecule chemical inhibitors [12]. As reported in many previous studies [3, 6, 12, 14], the siRNA-induced gene silencing effects may last over one week, while the D-RNAi effects can even sustain up to one month after one treatment. These siRNA/D-RNAi agents evoke a series of intracellular sequence-specific mRNA degradation and/or translational suppression processes, affecting all highly homologous gene transcripts, namely co-suppression. It has been observed that such co-suppression results from the generation of small RNA products (21–25 nucleotide bases) by the enzymatic activities of RNaseIII endoribonucleases (*Dicer*) and/or RNA-directed RNA polymerases (RdRp) on aberrant RNA templates, which are usually the derivatives of foreign transgenes or viral genomes [3, 6, 12].

4.2 Limitations of SiRNA/ShRNA-Based Gene Silencing Agents

Although the modern RNAi technologies may offer a new avenue for suppressing unwanted gene function in skins, the applications thereof have not been demonstrated to work constantly and safely in higher vertebrates, including fish, avian, mammal and human. For example, almost all of the current siRNA agents are based on a double-stranded RNA (dsRNA) conformation, which has been shown to cause interferon-mediated non-specific RNA degradation in vertebrates [3, 26]. Such an interferon-mediated cytotoxic response reduces the target specificity of siRNA-induced gene silencing effects and often results in global RNA degradation in vertebrate cells. Particularly in mammalian cells, it has been noted that the RNAi effects are disturbed when the siRNA/dsRNA size is longer than 25 base-pairs (bp) [3]. Transfection of siRNA or small hairpin RNA (shRNA) sized less than 25 bp may not completely overcome such a problem, because both [24] and [15] have reported that the high dosage of siRNAs and shRNAs (such as >250 nM in human T cells) is able to cause strong cytotoxic effects similar to those of long dsRNAs. This toxicity is due to their double-stranded RNA conformation, which activates the interferon-mediated non-specific RNA degradation and programmed cell death through the activation of cellular PKR and 2–5A signaling pathways. It is well known that interferon-activated protein kinase PKR can trigger cell apoptosis, while the activation of interferon-induced 2′,5′-oligoadenylate synthetase (2–5A) system leads to extensive cleavage of single-stranded RNAs, such as mRNAs [26]. Both PKR and 2-5A systems contain dsRNA-binding motifs, which possess high affinity to the double-stranded RNA conformation. Further, the most difficult problem is that these small siRNA/shRNA agents are not stable enough to be maintained at an optimal dose *in vivo* due to the abundant RNase activities in higher vertebrates [1].

As the RNAi effects are naturally caused by the production of small RNA products (21–25 nucleotide bases) from a transcriptional template derived from foreign transgenes or viral genomes [6, 12], the recent utilization of Pol-III-directed siRNA/shRNA expression vectors has shown to offer relatively stable RNAi efficacy *in vivo* [27]. Although previous studies [9, 20, 22] using such a vector-based siRNA approach have succeeded in maintaining constant gene silencing effects, their strategies fail to focus the RNAi effects on a targeted cell or tissue population because of the ubiquitous existence of type III RNA polymerase (Pol-III) activity. Pol-III promoters, such as U6 and H1, are activated in almost all cell types, making tissue-specific gene silencing impossible. Moreover, because the leaky read-through activity of Pol-III transcription often occurs on a short DNA template in the absence of pr oper termination, large RNA products longer than desired 25 bp can be synthesized and cause unexpected interferon cytotoxicity [8, 23]. Such a problem can also result from the competitive conflict between the Pol-III promoter and another vector promoter (i.e. *LTR* and *CMV* promoters). Furthermore, it is recently noted that high

siRNA/shRNA concentrations generated by the Pol-III-directed RNAi systems can over-saturate the cellular native microRNA (miRNA) pathway and thus cause global miRNA inhibition and cell death [7]. These disadvantages discourage the use of Pol-III-based RNAi vector systems in health care. In order to improve the delivery stability, targeting specificity and safety aspects of modern RNAi technologies for healthy skin care, a better transduction and maintenance strategy is highly desired.

4.3 Intronic MicroRNA-Mediated RNAi Mechanism

Research based on gene transcript (e.g. mRNA), an assembly of protein-coding exons, is fully described throughout the literature, taking the fate of spliced non-coding introns to be completely digested for granted [21]. Is it true that the intron portion of a gene is destined to be a genetic waste without function or there is a function for it, however, which has not yet been discovered? Recently, this misconception was corrected by the observation of intronic microRNA (miRNA) [13, 29, 30]. Intronic miRNA is a new class of small single-stranded regulatory RNAs derived from the gene introns, which are spliced out of the precursor messenger RNA (pre-mRNA) of the encoding gene and further processed into small mature miRNAs. MiRNA is usually about 18–27 nucleotides (nt) in length and is capable of either directly degrading its messenger RNA (mRNA) target or suppressing the protein translation of its targeted mRNA, depending on the complementarity between the miRNA and its target. In this way, the intronic miRNA is functionally similar to previously described siRNA/shRNA, but differs from them in the requirement of intracellular type II RNA polymerase (Pol-II) transcription and RNA splicing processes for its biogenesis [13]. Also, because introns naturally contain multiple translational stop codons for recognition by the intracellular nonsense-mediated decay (NMD) system [11, 31], most of the unstructured intron sequences can be quickly degraded after RNA splicing to prevent excessive accumulation, which is toxic to the cells. It has been measured that approximately 10–30% of a spliced intron is preserved after the exosome and NMD digestion in cytoplasm with a relatively long half-life, indicating the cellular origin of native intronic miRNAs [2].

Natural intronic miRNA biogenesis relies on the coupled interaction between nascent Pol-II-mediated pre-mRNA transcription and intron splicing/excision (Fig. 4.1), occurring within certain nuclear regions proximal to genomic perichromatin fibrils [5, 14]. In eukaryotes, protein-coding gene transcripts, such as mRNAs, are produced by type-II RNA polymerases (Pol-II). The transcription of a genomic gene generates precursor messenger RNA (pre-mRNA), which contains four major parts including 5′-untranslated region (UTR), protein-coding exon, non-coding intron and 3′-UTR. Broadly speaking, both 5′- and 3′-UTR can be seen as a kind of intron extension. Introns occupy the largest proportion of non-coding sequences in the pre-mRNA. Each intron can be ranged up to 30 or so kilo-bases and is required to be excised out of the pre-mRNA content before mRNA maturation. This process of pre-mRNA excision and intron removal is called RNA splicing, which is executed by intracellular

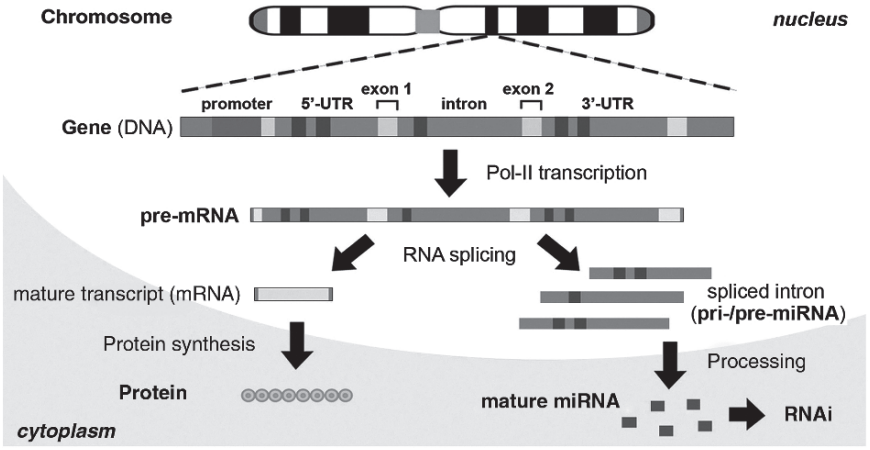


Fig. 4.1 Biogenesis of native intronic microRNA (miRNA). Intronic miRNA is co-transcribed with precursor messenger RNA (pre-mRNA) by Pol-II and cleaved out of the pre-mRNA by RNA splicing, while the ligated exons become a mature messenger RNA (mRNA) for protein synthesis. The spliced intronic miRNA with a high secondary structure (i.e. hairpin and/or stem-loop) is further processed into mature miRNA capable of triggering RNAi-related gene silencing effects

spliceosomes. After RNA splicing, some of the intron-derived RNA fragments are further processed to form microRNA (miRNA) derivative molecules, which can effectively silence their targeted genes, respectively, through an RNA interference (RNAi)-like mechanism, while exons of the pre-mRNA are ligated together to form a mature mRNA for protein synthesis.

4.4 Differences Between miRNA and siRNA Biogenesis Pathways

We have demonstrated that effective mature miRNAs can be generated from the introns of vertebrate genes, of which the biogenetic process is different from those of siRNA and intergenic miRNA [13, 16]. To demonstrate their differences, Fig. 4.2 shows the comparison of native biogenesis and RNAi mechanisms among siRNA, intergenic (exonic) miRNA and intronic miRNA. Presumably, siRNA is formed by two perfectly complementary RNAs transcribed by two reversely positioned promoters from one DNA template, then hybridized and further processing into 20–25 bp duplexes by RNaseIII endoribonucleases, namely *Dicer*. Different from this siRNA model, the biogenesis of intergenic miRNA, e.g. *lin*-4 and *let*-7, involves a long non-coding precursor RNA transcript (pri-miRNA), which is directly transcribed from a Pol-II or Pol-III RNA promoter, whereas intronic miRNA is co-transcribed with its encoding gene by only Pol-II and released after

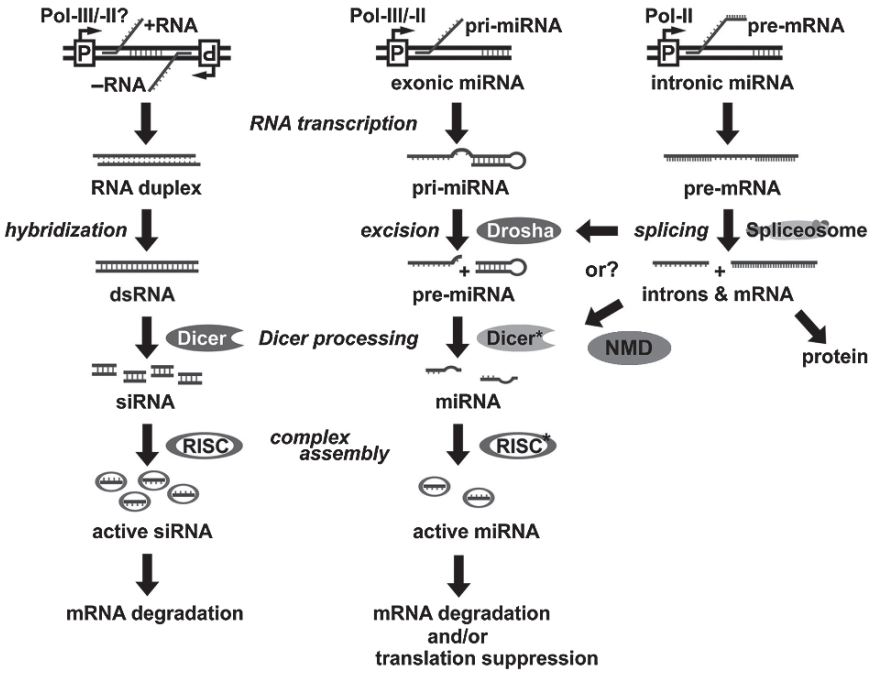


Fig. 4.2 Comparison of biogenesis and RNAi mechanisms among siRNA, intergenic (exonic) miRNA and intronic miRNA. SiRNA is likely formed by two perfectly complementary RNAs transcribed from two different promoters (remains to be determined) and further processing into 19–22 bp duplexes by RNaseIII-familial endonucleases, *Dicer*. The biogenesis of intergenic (exonic) miRNA, e.g. *lin*-4 and *let*-7, involves a long transcript precursor (pri-miRNA), which is probably generated by a Pol-II or Pol-III RNA promoter, whereas intronic miRNA is mainly transcribed by the Pol-II promoter of its encoded gene and co-expressed in the intron region of the gene transcript (pre-mRNA). After pre-mRNA splicing, the spliced intron functions as a pri-miRNA for intronic miRNA generation. In the nucleus, the pri-miRNA is excised by either *Drosha*-like RNases (intergenic miRNA) or spliceosomal components (intronic miRNA) to form a hairpin-like pre-miRNA template and then exported to cytoplasm for further processing by *Dicer** to form mature miRNAs. The *Dicers* for siRNA and miRNA pathways are different. For instance, some exosome and NMD components are likely involved in the process of intronic miRNA maturation. All three small regulatory RNAs are finally incorporated into a RNA-induced silencing complex (RISC), which contains either strand of siRNA or the single-strand of miRNA. The effect of miRNA is considered to be more specific and less adverse than that of siRNA because only one strand is involved. On the other hand, siRNAs primarily trigger mRNA degradation, whereas miRNAs can induce either mRNA degradation or suppression of protein synthesis depending on the sequence complementarity to the target gene transcripts

RNA splicing as a spliced intron. The spliced intron is then served as a pri-miRNA for processing into an intronic precursor miRNA (pre-miRNA) or a multi-pre-miRNA cluster. In the cell nucleus, the pri-miRNA is further excised by either *Drosha*-like RNases (for intergenic miRNA) or spliceosomal components (for intronic miRNA) to form a hairpin-like stem-loop precursor or a cluster of multiple

stem-loop structures, termed pre-miRNA, and then exported to cytoplasm for final processing into mature miRNA by a miRNA-associated *Dicer (Dicer**). Subsequently, all three small regulatory RNAs are incorporated into a RNA-induced silencing complex (RISC), which contains either strand of siRNA or the mature strand of miRNA. The *Dicers* and RISCs for siRNA and miRNA pathways are known to be different [28]. For example, some enzymes of the nonsense-mediated decay (NMD) system may play the role of *Dicer** in intronic miRNA maturation. As a result, the effect of miRNA is generally more specific and less adverse than that of siRNA because only one strand is involved. On the other hand, siRNAs primarily trigger mRNA degradation, whereas miRNAs can induce either mRNA degradation or suppression of protein synthesis, or both, depending on the sequence complementarity to their targeted gene transcripts. Because the intronic miRNA pathway is well coordinated by multiple intracellularly regulatory systems, including Pol-II transcription, RNA splicing and NMD processing, the gene silencing effects of intronic miRNAs are considered to be effective, specific and safe [19].

4.5 Development of miRNA-Based Gene Silencing Agents

Based on the intronic RNA splicing and processing mechanisms (Figs. 3A, B), we designed and developed a Pol-II-mediated recombinant gene expression system containing at least a splicing-competent intron, namely *SpRNAi*, which is able to inhibit the function of a unwanted gene with high complementarity to the intron sequence. The *SpRNAi* is co-transcribed with the precursor mRNA (pre-mRNA) of the recombinant gene by Pol-II RNA polymerases (P) and cleaved out of the pre-mRNA by RNA splicing. Subsequently, the spliced *SpRNAi* was further processed into mature gene silencing agents, such as shRNA and miRNA, capable of triggering RNAi-related gene silencing. After intron removal, the exons of the recombinant gene transcript are linked together to form a mature mRNA molecule for translational synthesis of a marker or functional protein.

As shown in Fig. 4.3A, the essential components of the *SpRNAi* intron include several consensus nucleotide elements, consisting of a 5′-splice site, a branch-point motif (BrP), a poly-pyrimidine tract (PPT), and a 3′-splice site. In addition, a hairpin RNA-like pre-miRNA sequence is inserted inside the *SpRNAi* intron located between the 5′-splice site and the branch-point motif (BrP). This portion of the intron would normally form a lariat structure during RNA splicing and processing. We have observed that spliceosomal U2 and U6 snRNPs, both helicases, are involved in the unwinding and excision of the lariat RNA fragment into pre-miRNA; however, the detailed processing remains to be elucidated. Further, the 3′-end of the *SpRNAi* construct contains a multiple translational stop codon region (T codon) in order to increase the accuracy of intronic RNA splicing and NMD processing. When presented in a cytoplasmic mRNA, this T codon would signal the activation of the nonsense-mediated decay (NMD) pathway to degrade any unstructured RNA accumulation in the cell. However, the highly secondary structured hairpin RNA and

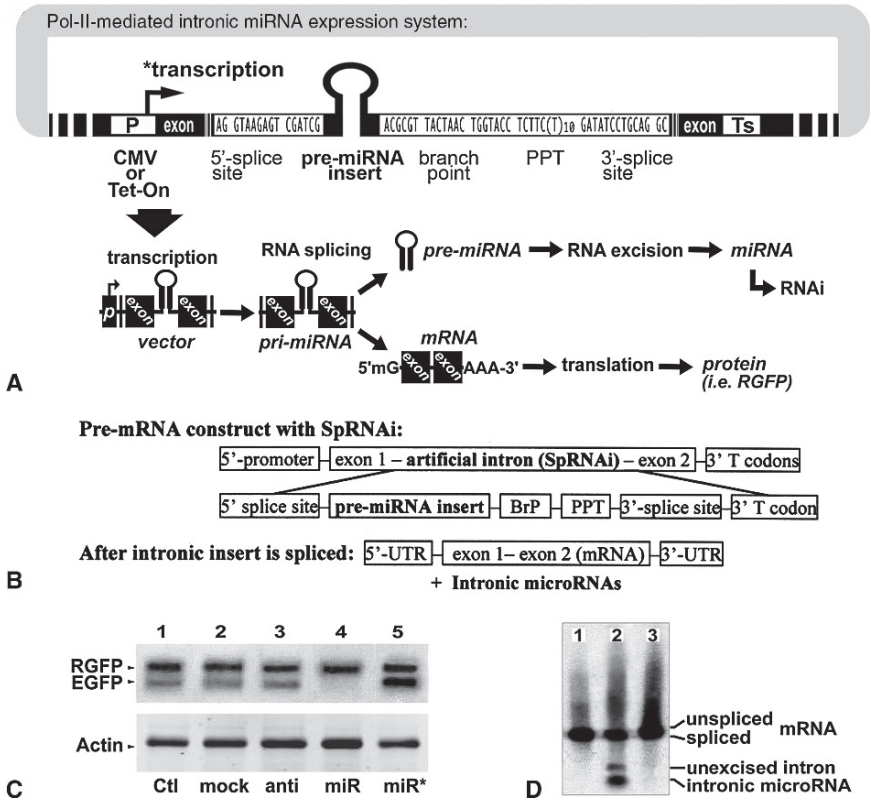


Fig. 4.3 Structural composition of the *SpRNAi*-incorporated recombinant *RGFP* gene (*SpRNAi-RGFP*) in an expression-competent vector (**A**), and the strategy (**B**) of using this composition to generate man-made microRNA, mimicking the biogenesis of the natural intronic miRNA. *In vivo* tests of an *SpRNAi-RGFP* expression composition directed against green EGFP in fish show an over 85% knockdown effect specifically on the targeted EGFP gene expression, as determined by Western blot analysis (**C**). The intron-derived anti-*EGFP* microRNA and its spliced precursor can be observed on a 1% formaldehyde agarose gel electrophoresis after Northern blot analysis (*D*)

pre-miRNA insert will be preserved for further *Dicer* cleavage, so as to form mature siRNA and miRNA, respectively. Moreover, for intracellular expression, we manually incorporate the *SpRNAi* construct in the *DraII* restriction site of a red fluorescent protein (*RGFP*) gene isolated from mutated chromoproteins of the coral reef *Heteractis crispa*, so as to form a recombinant *SpRNAi-RGFP* gene. The cleavage of *RGFP* at its 208th nucleotide site by the restriction enzyme *DraII* generates an AG–GN nucleotide break with three recessing nucleotides in each end, which will form 5′- and 3′-splice sites respectively after the *SpRNAi* insertion. Because this intronic insertion disrupts the structure of a functional RGFP protein, which can be recovered by intron splicing, we can determine the release of intronic shRNA/miRNA and *RGFP*-mRNA maturation through the appearance of red RGFP around the affected

cells. The *RGFP* gene also provides multiple exonic splicing enhancers (ESEs) to increase RNA splicing accuracy and efficiency.

In this intronic miRNA expression system (Fig. 4.3B), we provides a genetic engineering method for using synthetic RNA splicing and processing elements, such as 5′-splice site, branch-point motif (BrP), poly-pyrimidine tract (PPT), and 3′-splice site, to form an artificial *SpRNAi* intron containing at least a desired RNA insert for antisense RNA, small hairpin RNA (shRNA) and/or microRNA (miRNA) production. A DNA synthesizer can chemically produce and link these elements. Alternatively, the linkage of these elements can be achieved by enzymatic restriction and ligation. The intron so obtained can be used directly for transfection into cells of interest or further incorporated into a cellular gene for co-expression along with the gene transcript (i.e. pre-mRNA) by Pol-II. During RNA splicing and mRNA maturation, the desired RNA insert will be excised and released by intracellular spliceosome, exosome and NMD mechanisms and then triggers a desired gene silencing effect on specific gene transcripts with high complementarity to the inserted RNA sequence, while the exons of the recombinant gene transcript are linked together to form mature mRNA for expression of a desirable gene function, such as translation of a reporter or marker protein selected from the group of red/green fluorescent protein (RGFP/EGFP), luciferase, lac-Z, and their derivative homologues. The presence of the reporter/marker protein is useful for locating the production of the inserted shRNA/miRNA molecules in affected cells, facilitating the identification of the desired gene silencing/RNAi effects.

In accordance with the biogenesis of intronic miRNA, mature mRNA formed by the linkage of exons can also be useful in conventional gene therapy to replace impaired or missing gene function, or to increase specific gene expression. Alternatively, this method provides novel compositions and means for inducing cellular production of gene silencing molecules through intronic RNA splicing and processing mechanisms to elicit either antisense-mediated gene knockout or RNA interference (RNAi) effects, which are useful for inhibiting targeted gene function. The intron-derived gene silencing molecules so obtained may include antisense RNA, ribozyme, short temporary RNA (stRNA), double-stranded RNA (dsRNA), small interfering RNA (siRNA), tiny non-coding RNA (tncRNA), short hairpin RNA (shRNA), microRNA (miRNA), and RNAi-associated precursor RNA constructs (pri-/pre-miRNA). The use of these intronic RNA-derived gene silencing agents is a powerful tool for targeting and silencing unwanted genes selected from the group consisting of pathogenic transgenes, viral genes, mutant genes, oncogenes, disease-related small RNA genes and any other types of protein-coding as well as non-coding genes.

Using this novel Pol-II-mediated *SpRNAi-RGFP* expression system, we have successfully generated mature shRNA and miRNA molecules with full gene silencing capacity in human prostate cancer LNCaP, human cervical cancer HeLa and rat neuronal stem HCN-A94–2 cells [17] as well as in zebrafish, chicken and mouse *in vivo* [18]. We have tested different pre-miRNA insert constructs targeting against green *EGFP* and other cellular gene expression in zebrafish and various human cell lines, and have learned that effective gene silencing miRNAs are

derived from the 5′-proximity of the intron sequence between the 5′-splice site and the branching point. As shown in Fig. 4.3C, a strong gene silencing effect occurs only in the transfection of anti-*EGFP* pre-miRNA insert (lane 4), whereas no effect can be detected in those of other inserts indicated by lanes from left to right: 1, blank vector control (Ctl); 2, pre-miRNA insert targeting *HIV-p24* (mock); 3, antisense *EGFP* insert without the hairpin loop structure (anti); and 5, reverse pre-miRNA sequence which is completely complementary to the anti-*EGFP* pre-miRNA (miR*). No effect was detected on off-target genes, such as marker RGFP and house-keeping β-actin, suggesting that such intronic miRNA-mediated RNA interference (RNAi) is highly target-specific. To further confirm the role of RNA splicing in this intronic RNAi effect, we have also tested three different *SpRNAi-RGFP* expression systems as shown in Fig. 4.3D by lanes from left to right: (1) vector expressing intron-free *RGFP* without any pre-miRNA insert; (2) vector expressing *RGFP* with an intronic anti-*EGFP* pre-miRNA insert; and (3) vector similar to the 2 construct but with a defective 5′-splice site in the *SpRNAi* intron. As a result of this, Northern bolt analysis shows that mature miRNA is released only from the spliced intron of the vector 2 construct, which is exactly identical to the *SpRNAi* vector construct with the anti-*EGFP* pre-miRNA insert in the Fig. 4.3C, indicating the requirement of cellular RNA splicing for intronic miRNA biogenesis.

4.6 Optimization of Intronic miRNA Designs

After the above understanding, we have further determined the optimal structural design of the pre-miRNA inserts for inducing maximal gene silencing effects and learned that a strong structural bias exists in the cellular selection of a mature miRNA strand during assembly of the RNAi effector, the RNA-induced gene silencing complex (RISC) [16]. RISC is a protein–RNA complex that directs either target gene transcript degradation or translational repression through the RNAi mechanism. Formation of siRNA duplexes plays a key role in assembly of the siRNA-associated RISC. The two strands of the siRNA duplex are functionally asymmetric, but assembly into the RISC complex is preferential for only one strand. Such preference is determined by the thermodynamic stability of each 5′-end base-pairing in the strand. Based on this siRNA model, the formation of miRNA and its complementary miRNA (miRNA*) duplexes was thought to be an essential step in the assembly of miRNA-associated RISC. If this were true, no functional bias would be observed in the stem-loop structure of a pre-miRNA. Nevertheless, we observed that the stem-loop orientation of the intronic pre-miRNA is involved in the strand selection of a mature miRNA for RISC assembly in zebrafish.

To find the correct miRNA structures for RISC assembly, we have constructed two different intronic pre-miRNA-inserted *SpRNAi-RGFP* expression vectors containing a pair of symmetric pre-miRNA constructs, respectively, namely miRNA*-stemloop-miRNA [1] and miRNA-stemloop-miRNA* [2], as shown in Fig. 4.4A. Both pre-miRNAs contain the same double-stranded stem-arm structure,

which is directed against the *EGFP* nucleotide 280–302 sequence. In definition here, miRNA refers the exactly complete sequence of a mature microRNA, while miRNA* refers the reverse nucleotide sequence complementary to the mature microRNA sequence. After liposomal transfection of these miRNA-expressing *SpRNAi-RGFP* vectors (60 µg each) into two-week-old zebrafish larvae for 24 hours [16], we have isolated the zebrafish small RNAs using *mirVana* miRNA isolation columns (Ambion, Austin, TX) and then precipitated all the potential miRNAs matched to the targeted EGFP region by latex beads containing the target sequence. After sequencing, one effective miRNA identity, *miR-EGFP(280–302)*,

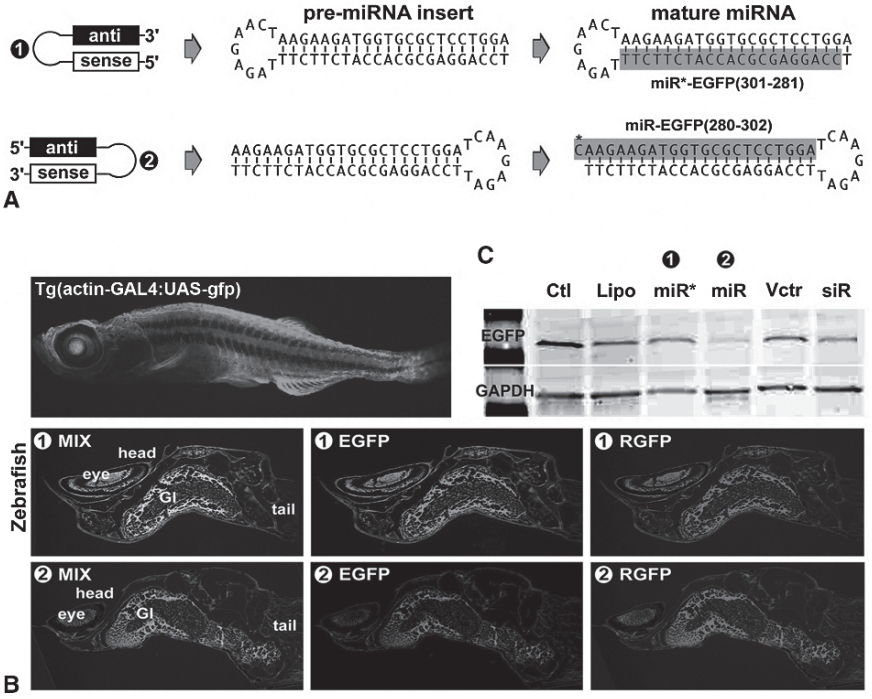


Fig. 4.4 Different designs of intronic RNA inserts in an *SpRNAi-RGFP* construct for effective microRNA biogenesis. Gene silencing of a targeted green fluorescent protein (*EGFP*) expression in Tg(*actin*-GAL4:UAS-gfp) zebrafish demonstrates the asymmetric preference of RISC assembly between the transfection of [1] 5′-miRNA*-stemloop-miRNA-3′ and that of [2] 5′-miRNA-stemloop-miRNA*-3′ hairpin RNA structures, respectively (**A**). *In vivo* gene silencing is only observed in the transfection of the [2] pre-miRNA construct, but not the [1] construct. Since the color combination of EGFP and RGFP displays more red than green (as shown in deep orange), the expression level of target EGFP (green) is significantly reduced in the [2] pre-miRNA transfection, while vector indicator RGFP (red) is evenly present in all vector transfections (**B**). Western blot analysis of the EGFP protein levels confirms the specific silencing result of the [2] pre-miRNA transfection (**C**). No detectable gene silencing is observed in fish with other treatments, such as liposome only (Lipo), empty vector without any insert (Vctr), and siRNA (siR)

is identified to be active in the transfections of the 5′-miRNA-stemloop-miRNA*-3′ construct [2], as shown in Fig. 4.4A (gray-shading sequences). Since the mature miRNA is detected only in the zebrafish transfected by the [2] construct, the miRNA-associated RISC must preferably interact with the construct [2] rather than the [1] pre-miRNA, demonstrating the existence of a structural bias for intronic miRNA–RISC assembly.

In this experiment, we use an *actin*-promoter-driven Tg(UAS:gfp) strain zebrafish, namely Tg(*actin*-GAL4:UAS-gfp), which constitutively express a green fluorescent *EGFP* protein in almost all cell types of the fish body. As shown in Fig. 4.4B, transfection of the *SpRNAi-RGFP* vector in these zebrafish silences the targeted EGFP and co-expresses a red fluorescent marker protein RGFP, serving as a positive indicator for intronic miRNA generation in the affected cells. The gene silencing effect in the gastrointestinal (GI) tract is somehow lower than other tissues, probably due to the high RNase activity in this region. Based on further Western blot analysis (Fig. 4.4C), the indicator RGFP protein expression is detected in both of the fish transfected with either 5′-miRNA*-stemloop-miRNA-3′ [1] or 5′-miRNA-stemloop-miRNA*-3′ [2] pre-miRNA, whereas gene silencing of the target EGFP expression (green) only occurs in the fish transfected with the [2] pre-miRNA construct, confirming the result of Fig. 4.4B. Because thermostability of the 5′-end stem-arm of both pre-miRNA constructs is the same, we conclude that the stem-loop of the intronic pre-miRNA is involved in the strand selection of a mature miRNA sequence during RISC assembly. Given that the cleavage site of *Dicer* in the stem-arm is known to determine the strand selection of mature miRNA [10], the stem-loop of an intronic pre-miRNA may function as a determinant for the recognition of the special cleavage site.

In this early design, because the over sizes of many native pre-miRNA stem-loop structures cannot fit in the *SpRNAi-RGFP* expression vector for efficient expression, we must use a short $tRNA^{met}$ loop (i.e. 5′-(A/U)UCCAAGGGGG-3′) to replace the native pre-miRNA loops. The $tRNA^{met}$ loop has been shown to efficiently facilitate the export of designed miRNAs from nucleus to cytoplasm through the same Ran-GTP and Exportin-5 transporting mechanisms [16]. Later, we use a pair of manually improved pre-mir-302 loops (i.e. 5′-GCTAAGCCAGGC-3′ and 5′-GCCTGGCTTAGC-3′), which provide the same nuclear export efficiency as the native pre-miRNAs but not interfere with the tRNA exportation. The design of these new pre-miRNA loops is based on a mimicking modification of short stem-loops of mir-302s, which are highly expressed in embryonic stem cells but not in other differentiated tissue cells. Thus, the use of these man-made pre-miRNA loops will not interfere with the native miRNA pathway in the adult human body.

For different pre-miRNA generation, because the intronic insertion site of the recombinant *SpRNAi-RGFP* gene is flanked with a *PvuI* and an *MluI* restriction site at its 5′- and 3′-ends, respectively, the primary intronic insert can be easily removed and replaced by various gene-specific pre-miRNA inserts (e.g. anti-*EGFP* and anti-*Tyr* pre-miRNA) possessing matched cohesive ends. By changing the pre-miRNA inserts directed against different gene transcripts, this intronic miRNA generation

system can be served as a powerful tool for inducing targeted gene silencing *in vitro* and *in vivo*. For confirming the correct insert size, the pre-miRNA-inserted *SpRNAi-rGFP* gene (10 ng) can be amplified by a polymerase chain reaction (PCR) with a pair of oligonucleotide primers (i.e. 5′-CTCGAGCATG GTGAGCGGCC TGCTGAA-3′ and 5′-TCTAGAAGTT GGCCTTCTCG GGCAGGT-3′) for 25 cycles at 94 °C, 52 °C and then 70 °C each for one minute. The resulting PCR products are fractionated on a 2% agarose gel, and then extracted and purified by gel extraction kit (Qiagen, Valencia, CA) for sequencing confirmation.

4.7 Evaluation of Natural Anti-tyrosinase miRNA Agents

We adopt the proof-of-principle design of the Pol-II-mediated *SpRNAi-RGFP* expression system and use it for developing new cosmetic products for skin care. In this new approach, we apply skins a non-naturally occurring intron capable of being processed into hairpin-like precursor microRNA (pre-miRNA) molecules by the skin cells and thus inducing specific gene silencing effects on epidermal pigment-related genes and/or aging-causing genes. In this case, the RNA splicing- and processing-generated gene silencing molecule is the hairpin-like pre-miRNA insert located within the intron area of the recombinant gene and is capable of silencing a targeted gene, such as tyrosinase (Tyr), hyaluronidase (Hyal), hyaluronan receptors CD44 and CD168, and other pigmentation-related and/or aging-related genes and oncogenes. Alternatively, such a pre-miRNA insert can also be artificially incorporated into the intron region of a cellular gene in the skin. In general, this kind of intronic insertion technology includes plasmid-like transgene transfection, homologous recombination, transposon delivery, jumping gene integration and retroviral infection.

In the present design, the recombinant *SpRNAi-RGFP* gene expresses an intronic insert construct reminiscent of a hairpin-like pre-mRNA structure. The recombinant gene is consisted of two major different parts: exon and intron. The exon part is ligated after RNA splicing to form a functional mRNA and protein for identification of the intronic RNA release, while the intron part is spliced out of the recombinant gene transcript and further processed into a desired intronic RNA molecule, serving as a gene silencing effector, including antisense RNA, miRNA, shRNA, siRNA, dsRNA and their precursors (i.e. pre-miRNA and piRNA). These desired intronic RNA molecules may comprise a hairpin-like stem-loop structure containing a sequence motif homologous to 5′-GCTAAGCCAG GC-3′ or 5′-GCCTGGCTTA GC-3′, which facilitates not only accurate excision of the desired RNA molecule out of the intron but also nuclear exportation of the desired RNA molecule to the cell cytoplasm. Also, the stem-arms of these intron-derived RNA molecules contain homology or complementarity, or both, to a targeted gene or a coding sequence of the targeted gene transcript. The homologous or complementary sequences of the desired RNA molecules are sized from about 18 to about 27 nucleotide bases. The homology and/or complementarity rate of the desired

intronic RNA molecule to the targeted gene sequence is ranged from about 30–100%, more preferably 35–49%, for a desired hairpin-like intronic RNA and 90–100% for a linear intronic RNA molecule.

In addition, the 5′-end of the non-naturally occurring intron contains a donor splice site homologous to 5′-GTAAGAGK-3′ motifs, while its 3′-end is a acceptor splice site that is homologous to 5′-GWKSCYRCAG-3′ motifs. Moreover, a branch point sequence is located between the 5′- and 3′-splice sites, containing homology to 5′-TACTWAY-3′ motifs. The adenosine "A" nucleotide of the branch-point sequence forms a part of (2′–5′)-linked lariat intron RNA by cellular (2′-5′)-oligoadenylate synthetases and spliceosomes in almost all spliceosomal introns. Furthermore, a poly-pyrimidine tract is closely located between the branch-point and 3′-splice site, containing a high T or C content oligonucleotide sequence homologous to either 5′-(TY)m(C/–)(T)nS(C/–)-3′ or 5′-(TC)nNCTAG(G/–)-3′ motifs. The symbols of "m" and "n" indicate multiple repeats ≥1; most preferably, the m number is equal to 1~3 and the n number is equal to 7~12. The symbol "–" refers an empty nucleotide in the sequence. There are also some linker nucleotide sequences for the connection of all these intron components. In definition, the symbol W refers to an adenine (A) or thymine (T)/uracil (U), the symbol K refers to a guanine (G) or thymine (T)/uracil (U), the symbol S refers to a cytosine (C) or guanine (G), the symbol Y refers to a cytosine (C) or thymine (T)/uracil (U), the symbol R refers to an adenine (A) or guanine (G), and the symbol N refers to an adenine (A), cytosine (C), guanine (G) or thymine (T)/uracil (U)."

Based on the above design, we have tested an optimized *SpRNAi-RGFP* gene construct expressing either anti-*Tyr* or anti-*Hyal* pre-miRNA directed against the unwanted pigmentation-related gene *Tyr* or aging-related gene *Hyal* in mouse skins (Fig. 4.3A). These pre-miRNAs target a highly conserved region (>98% homology) in both human and mouse *Tyr* and *Hyal* genes, respectively. In nature, there are 54 native miRNAs capable of targeting human tyrosinase (*Tyr*; 2082 bp) for pigmentation gene silencing, including mir-1, mir-15a, mir-16, mir-31, mir-101, mir-129, mir-137, mir-143, mir-154, mir-194, mir-195, mir-196b, mir-200b, mir-200c, mir-206, mir-208, mir-214, mir-221, mir-222, mir-292-3p, mir-299-3p, mir-326, mir-328, mir-381, mir-409-5p, mir-434-5p, mir-450, mir-451, mir-452, mir-464, mir-466, mir-488, mir-490, mir-501, mir-522, mir-552, mir-553, mir-570, mir-571, mir-582, mir-600, mir-619, mir-624, mir-625, mir-633, mir-634, mir-690, mir-697, mir-704, mir-714, mir-759, mir-761, mir-768-5p, and mir-804. According to the miRNA-target database of the miRBase:: Sequences program (http://microrna.sanger.ac.uk), all these anti-*Tyr* miRNAs are directed against a region within the first 300 nucleotides of the *Tyr* gene transcript (NCBI accession number NM000372). Moreover, there are 9 native miRNAs capable of targeting hyaluronidase (*Hyal*; 2518 bp; NCBI accession number NM007312) for aging gene silencing, including mir-197, mir-349, mir-434-5p, mir-549, mir-605, mir-618, mir-647, mir-680, mir-702, and mir-763. In these native miRNAs, the mir-434-5p is the only one targets both *Tyr* and *Hyal* genes in human and also it is one of the most efficient miRNAs targeting the least off-target genes other

than *Tyr* and *Hyal*. However, because almost all native miRNAs have several to over fifty targets and they tend to bind with some of the target genes more strongly than others, the use of these native miRNAs is likely not specific and safe enough for the skin care purpose.

To test the feasibility of miRNA-mediated skin whitening, we have utilized the *SpRNAi-RGFP* expression system to express native pre-mir-434-5p in mouse skin. As shown in Fig. 4.5, patched albino (white) skins of melanin-knockdown mice (W-9 black) can be created by a succession of intra-cutaneous (*i.c.*) injections of the pre-mir-434-5p expression vector (50 μg) directed against *tyrosinase (Tyr)* for four days (total 200 μg). The Tyr, a type-I membrane protein and copper-containing enzyme, catalyzes the critical and rate-limiting step of tyrosine hydroxylation in the biosynthesis of melanin (black pigment) in skins and hairs. Starting from about two

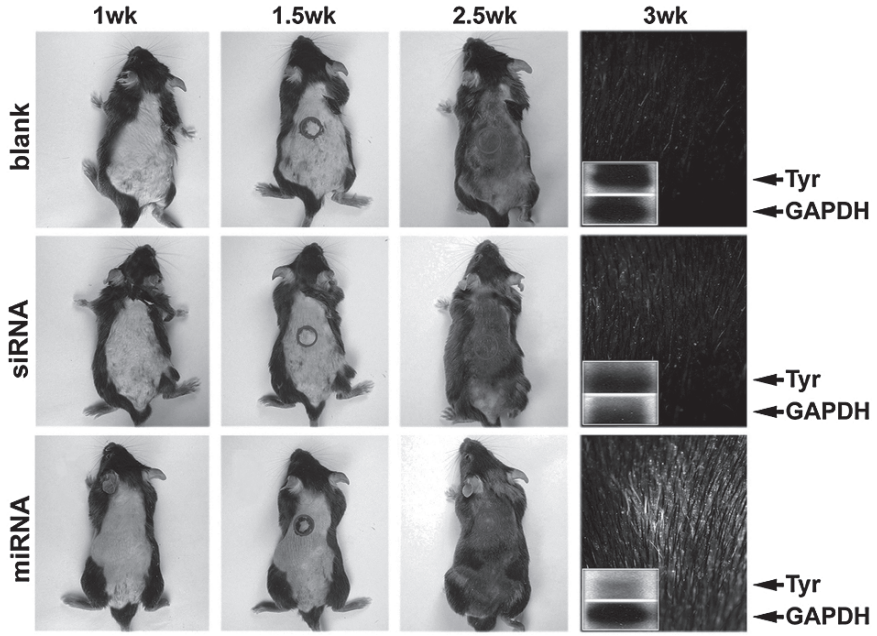


Fig. 4.5 Depigmentational effects of RNAi-mediated tyrosinase (*Tyr*) gene silencing on mouse skins and hairs, indicating the feasibility of targeted gene knockdown in epidermal tissues using subcutaneous transfection of the recombinant *SpRNAi-RGFP* gene vector expressing a native mir-434 pre-miRNA insert. Transfection of this mir-434-5p expression construct induces a strong and specific gene silencing effect on *Tyr* but not house-keeping *GAPDH* expression, whereas that of a U6 promoter-based siRNA expression vector against the same *Tyr* target sequence triggers non-specific RNA degradation of both *Tyr* and *GAPDH* gene transcripts. Because Tyr plays an essential role in melanin (black pigment) production, the successful *Tyr* gene silencing can be observed by a significant loss of the black color in mouse skins and hairs. The circles indicate the location of *i.c.* injections. Small windows show the Northern blotting of *Tyr* mRNA knockdown in local hair follicles, confirming the effectiveness of the mir-434-mediated gene silencing effect

weeks after the first *i.c.* injection, we observe that skin and hair pigments was significantly lost only in the pre-miRNA transfections. On the contrary, the blank control and the Pol-III (U6)-directed siRNA transfections present no significant effect. Northern blot analysis using mRNAs isolated from the hair follicles of the pre-mir-434-5p transfections show a 76.1% ± 5.3% reduction of *Tyr* expression two days post-transfection, whereas mild, non-specific degradation of random gene transcripts is detected in the siRNA-transfected skins (seen from the smearing patterns of both house-keeping control *GAPDH* and targeted *Tyr* mRNAs). Since [7] have reported that high siRNA/shRNA concentrations generated by the Pol-III-directed RNAi systems can over-saturate the cellular microRNA pathway and cause global miRNA dysregulation, this result indicates that the siRNA pathway is incompatible with the native miRNA pathway in skin tissues. Thus, the use of miRNA will likely provide a more effective, compatible and safe means for skin care. However, because the native mir-434-5p also targets five other cellular genes for silencing, including *TRPS1, PITX1, LCOR, LYPLA2* and *Hyal*, the off-target effect of this native pre-mir-434-5p transfection remains to be determined.

4.8 Re-design of mir-434-5p for Skin Whitening Use in Human

In order to improve the target-specificity and safety of anti-*Tyr* miRNA agents, we have re-designed the seed sequence of the mir-434-5p to form a highly matched region binding to either *Tyr* nucleotides 3–25 (namely miR-Tyr) or *Hyal* nucleotides 459–482 (namely miR-Hyal). The pre-miRNA insert sequence for *Tyr* gene silencing (pre-miR-Tyr) is 5′-GTCCGATCGT CGCCCTACTC TATTGCCTAA GCCGCTAAGC CAGGCGGCTT AGGCAATAGA GTAGGGCCGA CGCGTCAT-3′, which will form a hairpin-like RNA after splicing and will be further processed into a mature miR-Tyr microRNA (miRNA) sequence containing or homologous to 5′-GCCCTACTCT ATTGCCTAAG CC-3′. Alternatively, the pre-miRNA insert for *Hyal* gene silencing (pre-miR-Hyal) is 5′-GTCCGATCGT CAGCTAGACA GTCAGGGTTT GAAGCTAAGC CAGGCTTCAA ACCCTGACTG TCTAGCTCGA CGCGTCAT-3′, which will form a different kind of hairpin-like RNA after splicing and will be further processed into a mature miR-Hyal miRNA sequence containing or homologous to 5′-AGCTAGACAG TCAGGGTTTG AA-3′. Although both pre-miR-Tyr and pre-miR-Hyal constructs are re-designed based on the same mir-434-5p backbone and mir-302 stem-loop, the mature miR-Tyr and miR-Hyal so obtained are totally different from each other. As shown in Fig. 4.6, the transfective expressions of miR-Tyr and miR-Hyal in mouse skins specifically knock down the targeted Tyr (reduction >90%) and Hyal genes (reduction >67%), respectively, without any crossover off-target effect. The expression levels of mature miR-Tyr and miR-Hyal microRNAs are directly measured by Northern blot analysis, while the knockdown rates of the targeted Tyr and Hyal gene products (proteins) are determined by Western blot analysis.

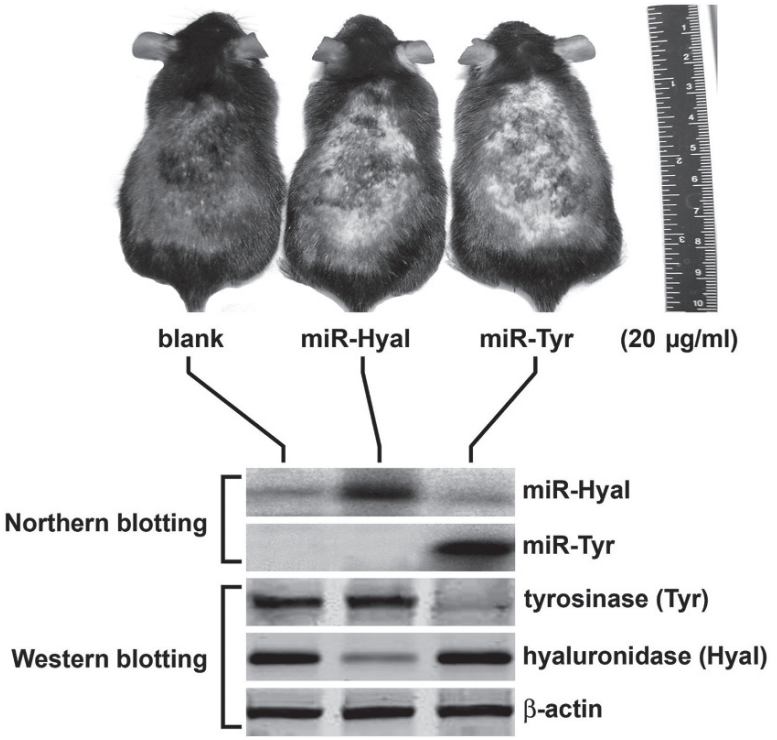


Fig. 4.6 Improvement of *Tyr* gene silencing using a man-made anti-*Tyr* pre-miRNA (miR-Tyr) insert expressed by the recombinant *SpRNAi-RGFP* gene vector in mouse skins, showing a more specific and less off-target gene silencing effect on the targeted tyrosinase (*Tyr*) gene. Neither off-target (hyaluronidase) nor house-keeping (ß-actin) genes are affected by the transfection of this man-made intronic miR-Tyr microRNA

After understanding the optimized gene silencing effects of the re-designed miR-Tyr and miR-Hyal miRNAs in mice, we continue to test their efficacy, target specificity and safety in human skins. For efficient vector transfection into the human epidermal cell layers, a 1 µg/ml *SpRNAi-RGFP* vector solution is made by mixing 100 µg of the purified *SpRNAi-RGFP* vector in 1 ml of autoclaved ddH_2O with 99 ml of 100% DNase-free glycerin (or called glycerol). DNase-free glycerin is used to encapsulate miR-Tyr for deep skin delivery and cell membrane penetration. This forms the major ingredient base for skin whitening and lightening products. Based on this, more other cosmetic ingredients may be added to increase the color, fragrance, effectiveness and/or stability of the final cosmetic products. As shown in Fig. 4.7A, Asian male arm skins treated with 2 ml of this major ingredient base expressing the aforementioned miR-Tyr (right site) versus empty *SpRNAi-RGFP* vector without any miRNA insert (glycerin control, left site) are compared. The result of skin whitening (loss of the black pigment–melanin) by the miR-Tyr treatment can be clearly observed in three days after two single treatments per day, as shown in this figure.

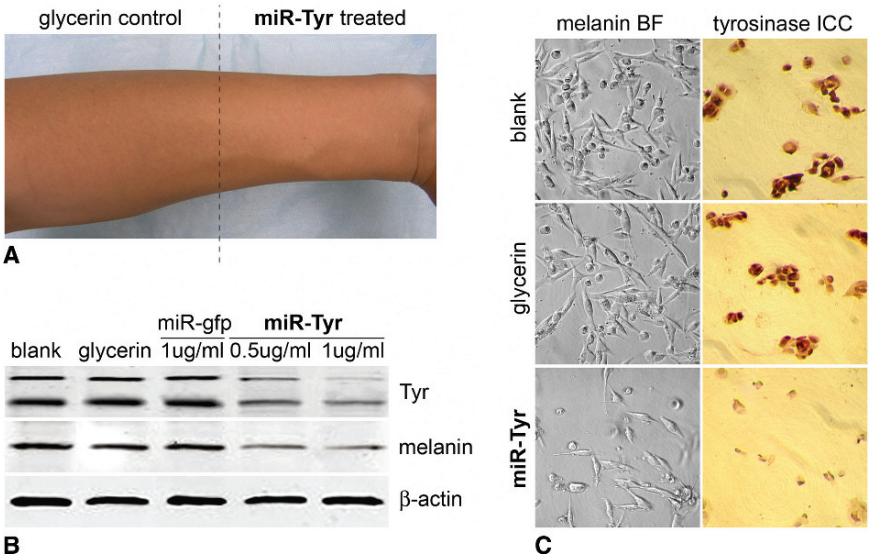


Fig. 4.7 Human trial results of the improved anti-*Tyr* pre-miRNA (miR-Tyr) insert expressed by the recombinant *SpRNAi-RGFP* gene vector, identical to the Fig. 4.6 approach but in the human arm skins (**A**) and primary skin cell cultures (**B** and **C**), showing an over 50% knockdown rate in tyrosinase (Tyr) expression as determined by Western blot analysis

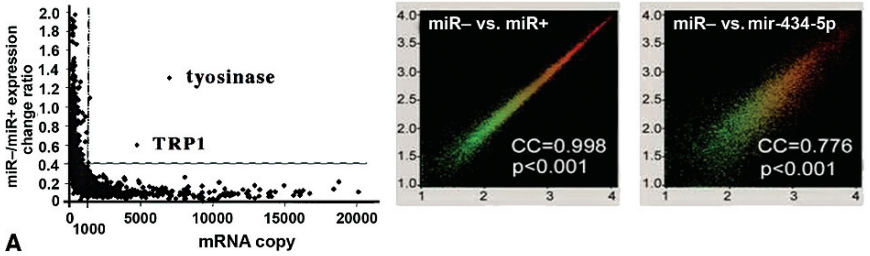
Then, primary skin cell culture is obtained by trypsin-dissociated skin explants from the tested donor with personal consent. The *SpRNAi-RGFP* vector transfection (final 6 μg/ml) in the primary skin culture is performed using a FuGene liposomal reagent (Roche Biochemicals, Indianapolis, IN), as described previously [13, 2006a]. Figure 4.7B shows that Western blot analyses of the loss of the targeted tyrosinase proteins and its substrate melanin are biostatistically significant ($p > 0.001$). The reduction amounts of tyrosinase proteins and its substrate melanin in skins is proportional to the treated concentrations of the miR-Tyr expression vector, indicating the positive correlation between the increase of the miR-Tyr treatment and the loss of the targeted tyrosinase proteins and its substrate melanin. No effect is found in other treatments, such as an empty *SpRNAi-RGFP* vector without any miRNA insert (glycerin) and an *SpRNAi-RGFP* vector expressing an anti-*EGFP* pre-miRNA insert (miR-gfp). At the concentration of 1 μg/ml of the miR-Tyr expression vector transfection, the optimal *Tyr* gene silencing rate is approximately 55–60% for tyrosinases and 30–45% for melanin, while the expression of non-target house-keeping control ß-actin is not affected by the miR-Tyr treatment, indicating the high target-specificity of this man-made microRNA molecule.

Figure 4.7C further shows that the skin melanin levels are significantly reduced as shown in bright-field (BF) photographs of the primary skin cell culture (upper

panels), while melanin (black dots around the cell nuclei) is highly expressed in the normal skin cells without the miR-Tyr treatment (i.e. blank and glycerin only). The miR-Tyr-treated skin cells present very limited melanin accumulation, demonstrating an effective skin-whitening effect *in vivo*. In regard to this loss of skin melanin, the targeted tyrosinase expression is concurrently reduced in the miR-Tyr-treated skin cells, as determined by immunocytochemical (ICC) staining analysis (Fig. 4.7C, lower panels). Therefore, based on these results, the re-designed miR-Tyr microRNA can be used to knock down the tyrosinase expression and successfully blocks melanin production in the human skins *in vivo*.

4.9 Microarray Analyses of Target Specificity and Safety

After establishing the gene silencing efficacy of the miR-Tyr in human skins, we use gene microarray analysis (Human GeneChip U133A&B arrays, Affymetrix, Santa Clara, CA) to assess the changes of approximately 32,668 human gene expression in the above miR-Tyr-transfected versus non-treated primary skin cell cultures, showing a much more target-specific and less off-target gene silencing effect than the use of native mir-434. Total RNAs from each tested cell culture is isolated using RNeasy spin columns (Qiagen, CA). As shown in Fig. 4.8A (left), the result of microarray analysis in non-treated (miR–) versus miR-Tyr-transfected (miR+) primary skin cell cultures shows that there are only two genes altered more than 1.5 fold (>50% change of gene expression), including the targeted tyrosinase (*Tyr*) and its associated *TRP1* gene (Fig. 4.8B), indicating that the miR-Tyr-mediated gene silencing effect is highly specific to the targeted *Tyr*. Furthermore, no gene related to either cytotoxicity or interferon-mediated PKR/2-5A pathways is affected, suggesting that this gene silencing effect is safe for skin care treatments. We have also used Northern blot analysis to compare and assess the gene expression levels of these microarray-identified genes (Fig. 4.8C), confirming the results of Figs. 4.8A, B. In further comparison with the result of the native mir-434-5p transfection (Fig. 4.8A, right), the correlation coefficiency (CC) rate clearly indicates that a high 99.8% population of the 32,668 tested human genes remains to be unchanged in the miR-Tyr-transfected (miR+) cells, while a low 77.6% CC rate is found in the mir-434-5p-transfected cells. This means that the expression patterns of at most only 65 cellular genes are altered by the re-designed miR-Tyr transfection, whereas those of over 7,317 genes may be changed by the native mir-434-5p transfection. Because it is a well-known fact that almost all native microRNAs (miRNAs) target multiple cellular genes due to their mismatched stem-arms, our present study demonstrates that the re-design of these stem-arm regions is required for the safe use of these miRNAs in target-specific gene silencing applications.



Microarray analysis -- miR–/miR-Tyr

Gene	Accession #	change
tyosinase	NM000372	−1.3
tyosinase-associated protein 1 (TRP1)	NM000550	−0.6
endothelin 2 (ET2)	NM001956	−0.3
proliferating cell nuclear antigen (PCNA)	NM182649	−0.3
hUpf 1	NM002911	+0.3
decapping protein (DCP2)	NM152624	+0.3
ribonuclease XRN1	NM019001	+0.4

B

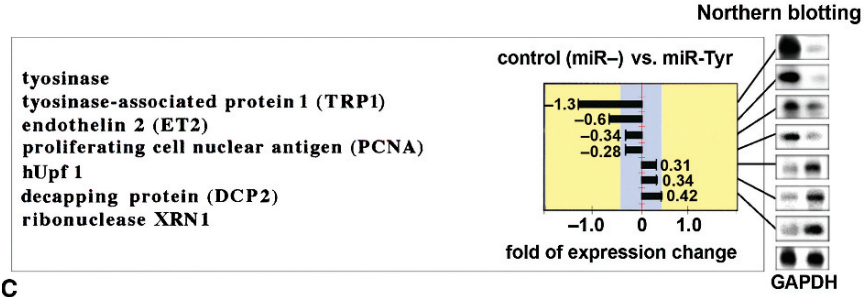


Fig. 4.8 Gene microarray analysis (Affymetrix human GeneChip U133A&B, CA) of altered gene expression in the above human primary skin cell cultures with or without anti-*Tyr* pre-miRNA transfection, showing a much more target-specific and less off-target gene silencing effect than the use of native microRNAs, such as mir-434-5p

4.10 Conclusion

In sum, utilization of intronic hairpin-like microRNA (miRNA) expression provides a powerful new strategy for human skin care *in vivo*, particularly for hyperpigmentation treatment and aging prevention. Under the same treatment in animal trials, Pol-II-directed intronic miRNA expression does not cause any detectable cytotoxicity, whereas Pol-III-directed siRNAs induced non-specific mRNA degradation as previously reported [15, 24]. This underscores the fact that the intronic miRNA agent is effective, target-specific and safe *in vivo*. Because the intronic miRNA-mediated gene silencing pathway is regulated by multiple intracellular surveillance systems, including Pol-II transcription, RNA splicing, exosome digestion and NMD processing, the gene silencing of intronic miRNA is considered to be the most effective, specific and safe approach among all three currently known RNAi

pathways. Advantageously, using this intronic miRNA expression strategy, many cosmetic applications can be designed and developed for skin care, offering more long-term effectiveness, better target-specificity and higher safety in skin gene manipulation, which prevents the unspecific off-target cytotoxicity as commonly seen in the conventional siRNA methods.

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