

Efficient delivery of transgenes to human hair follicle progenitor cells using topical lipoplex

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The topical delivery of transgenes to hair follicles has potential for treating disorders of the skin and hair. Here we show that the topical administration of liposome–DNA mixtures (lipoplex) to mouse skin and to human skin xenografts resulted in efficient *in vivo* transfection of hair follicle cells. Transfection depended on liposome composition, and occurred only at the onset of a new growing stage of the hair cycle. Manipulating the hair follicle cycle with depilation and retinoic acid treatment resulted in nearly 50% transfection efficiency—defined as the proportion of transfected, newly growing follicles within the xenograft. Transgenes administered in this fashion are selectively expressed in hair progenitor cells and therefore have the potential to affect the characteristics of the follicle. These findings form a foundation for the future use of topical lipoplex applications to alter hair follicle phenotype and treat diseases of the hair and skin.

Keywords: gene therapy, hair follicle, liposomes, xenograft, progenitor cells, alopecia

The topical delivery of transgenes to hair follicles is an attractive approach for treating disorders of the skin and hair. The hair follicle contains epithelial stem cells, which cyclically regenerate the lower follicle^{1,2}, and in times of wounding, repopulate the epidermis as well³. At the onset of each new growing stage (called anagen), stem cells in the bulge area of the hair follicle proliferate and give rise to progenitor (matrix) cells that subsequently generate the hair shaft and its surrounding layers⁴. The properties of the matrix cells are established at anagen onset and determine the characteristics of the new hair. For example, the number of matrix cells correlates with the size of the new hair⁵, and the pigmentation of the hair depends on the presence of melanin in the matrix cells⁶. Therefore, gene-based therapies targeted to follicle progenitor cells at anagen onset could alter the phenotype of the new hair follicle, and its associated hair.

The work of Li and Hoffman on mouse skin suggested the feasibility of using topical liposomes for delivering DNA to the hair follicle⁷; however, the applicability of these findings to human skin was not clear because of the marked differences between mouse and human skin and hair. In particular, human scalp hair follicles are much larger and are generally in the growing stage, whereas mouse follicles are small and are predominantly in the resting stage (telogen) of the hair cycle⁸. These differences, combined with the lack of adequate models for testing this approach on human skin, may explain the absence of reports utilizing topical liposomes for delivery of DNA to human hair follicle cells *in vivo*.

Here we show efficient transfection of human hair progenitor cells after topical application of lipoplexes. Using a novel human scalp xenograft model, we define parameters important for transfection, including liposome composition, timing of liposome application to the onset of a new hair cycle, and pretreatment with depilation and retinoic acid. Application of lipoplex during anagen onset results in selective transfection of human hair follicle matrix cells. Depilation of the hair and application of retinoic acid to the grafts before liposome application markedly increases the transfection efficiency (defined as the proportion of transfected follicles at anagen onset) to $48 \pm 10\%$.

These results provide a foundation for subsequent treatment of alopecias and other skin disorders with a topical gene therapy approach.

Results and discussion

***In vitro* transfection efficiencies of lipoplexes.** Lipoplexes are used routinely to transfect cells with plasmid DNA *in vitro*. Multiple variables affect transfection efficiency, including the ratio of liposomes to DNA, the absolute concentration of liposomes and DNA, and the liposome composition⁹. Because our goal was to transfect keratinocytes within the hair follicle *in vivo*, we screened nine commercially available cationic liposome preparations for their ability to transfect freshly isolated human hair follicles placed in explant culture with a reporter plasmid (pSV- β -galactosidase; pSV- β -gal). Transfected cells were identified by their blue color (Fig. 1). The majority of transfected cells were keratinocytes in the outer root sheath, although cells in other parts of the hair follicle, including the inner root sheath and matrix, were also transfected.

We calculated the number of transfected cells per follicle and the relative transfection efficiencies in comparison to pFx-1 (Invitrogen, Carlsbad, CA), the liposome preparation that resulted in the highest number of transfected cells per follicle (Fig. 2A). Although both concentrations of liposome and DNA and ratios of liposome to DNA affected transfection efficiency, the greatest differences were dependent upon the composition of the liposome preparations. pFx-1, composed of a 1:1 mixture of two cationic lipids, tris(2-(N^2 -(N^2 , N^5 -bis(3-aminopropyl)-L-ornithyl)- N^3), N^3 -dioctadecyl-L-glutaminamide- N -yl)-ethylamine, tridecahydrotrifluoroacetate, and 2-(dimethylamino)-ethyl methylphosphonic acid, 1-heptadecyloctadecyl ester, hydrotrifluoroacetate, transfected an average of 58 ± 11 keratinocytes per follicle. Of the tested liposomes, pFx-1 is the largest, with five $-NH_3^+$ groups per molecule. The other lipid preparations are also cationic, either single agents or mixtures, and three are combined with DOPE (L-dioleoyl phosphatidylethanolamine). Of the latter, pFx-6 resulted in the second highest transfection efficiency, and its efficiency was only minimally altered by changes in DNA:lipid ratios.

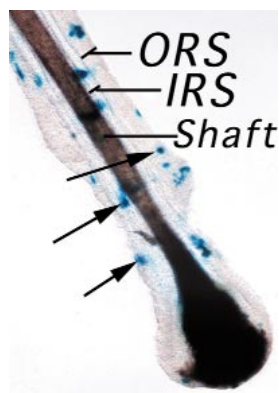


Figure 1. In vitro human hair follicle transfection assay. Transfected hair follicle cells 48 h after lipoplex application and X-gal staining. Cells expressing the *lacZ* gene appear blue (arrows). Transfected cells are mostly outer root sheath (ORS) keratinocytes. IRS, inner root sheath.

In vivo transfection of mouse hair follicles is hair cycle dependent. In vitro studies suggest that proliferating cells express plasmid DNA more efficiently than quiescent cells¹⁰. Because hair follicles only proliferate during anagen, we designed experiments to examine the influence of the stage of the hair cycle on the expression of topically applied plasmid. Mouse hair follicles cycle synchronously during the first three months of life¹¹. Between postnatal days 45 and 65, the hair follicles on the back of the mouse are in the telogen stage of the hair cycle. Hair follicle cycling can be studied during this time period because several stimuli, including depilation or application of proliferative agents such as phorbol ester or retinoic acid, cause resting hair follicles to enter anagen in a predictable and synchronous manner^{11–13}. Thus, by applying lipoplex (composed of pF_x-1 and pCMV- β -gal) to the back skin of mice at different time points (0, 1, 2, 3, 4, 6, 7, 8, and 10 days) after depilation, and collecting the skin 24–48 h later, we tested the concept that hair follicle cells express transfected plasmids only at specific times during the hair cycle. We detected expression of β -gal in the hair follicles of mice transfected only on the first, second, or third day after depilation. Transfections after three days did not result in β -gal activity. At least two mice per time point were used, and transfected matrix cells were noted in the skin of 12 out of 12 mice treated on days 1, 2, or 3 after depilation from four different experiments. No β -gal activity was detected in hair follicles treated with plasmid without the *lacZ* insert, or with reporter plasmid in phosphate-buffered saline (PBS).

During the first three days after depilation, hair follicles are in the earliest phases of the anagen stage¹². The majority of β -gal-expressing cells were matrix keratinocytes located at the leading edge of the epithelial downgrowth at anagen onset (Fig. 3A). No activity was evident in the epidermis or dermis. Rarely, cells within the stem cell-rich bulge area were also transfected (Fig. 3B). No cells within follicles in full-blown anagen were observed to express β -gal. β -gal activity was detected for up to four days after the last transfection. No time points after four days were studied because the plasmid vectors were likely to remain episomal and therefore express the reporter transiently^{14,15}.

Our results may explain the variability of the findings of other investigators¹⁴, who noted successful transfection in only one third of their experiments. These investigators transfected mouse skin at four weeks of age. During this time, follicles in the same mouse may be in telogen, anagen onset, or full-blown anagen, and this may account for the variation in their results. By synchronously inducing anagen we have avoided this problem, and we conclude that the timing of lipoplex application to hair follicles just entering the anagen stage is a critical parameter for successful in vivo transfection.

At anagen onset, progenitor cells within the hair follicle are proliferating and they are accessible, probably because the follicle lacks an inner root sheath, which normally prevents ingress of material from the environment later in anagen. Even transient expression of transgenes at this time alters hair follicle cycling¹⁶, and long-lasting changes in the phenotype of the hair could be achieved by altering

the characteristics of the hair progenitor cells that are re-forming at this time. Our findings should pave the way for straightforward topical transfection experiments geared toward evaluating the in vivo effects of candidate genes on hair follicle growth and differentiation.

In vivo transfection of mouse hair follicle cells depends on liposome composition. To assess whether liposome composition also affects topical in vivo transfection, we tested the nine lipid mixtures topically on mouse skin. The composition of liposome formulations is an important determinant of in vivo transfection efficiency following intravenous administration of lipoplexes¹⁷. We applied the lipoplexes containing pCMV- β -gal to the back skin of mice at the onset of anagen, which was induced by chemical depilation. The pF_x-1-DNA mixture transfected $73 \pm 12\%$ of hair follicles, whereas six of the other eight liposomes did not transfect any cells (Fig. 2B). One possible explanation for the superiority of the pF_x-1 liposome combination may be related to its large polyvalent structure, which should compact DNA¹⁸. We therefore also evaluated lipofectamine, which possesses similar compacting properties¹⁸. Lipofectamine resulted in transfection of $52 \pm 22\%$ of the hair follicles in the in vivo assay, suggesting that liposome composition favoring DNA compaction yields efficient topical transfection. Other liposome properties besides compaction also require further definition, since the pF_x-2 lipoplex transfected $49 \pm 33\%$ of follicles, despite having a smaller structure.

Shi and colleagues¹⁹ and Fan and colleagues²⁰ recently reported that topically applied DNA encoding for a protein can induce a systemic immune response to the protein. Although the work of the Fan group²⁰ suggested that normal hair follicles were necessary for this

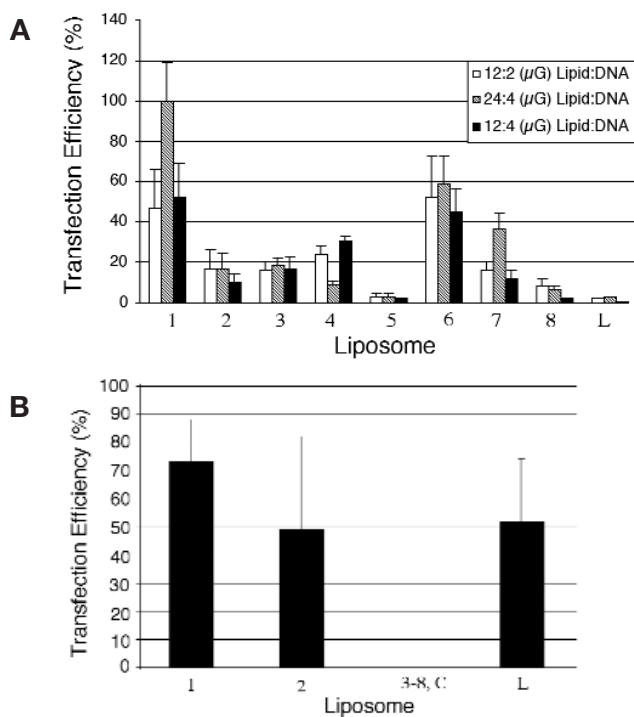


Figure 2. Transfection efficiency of different lipoplex combinations in human hair follicles in vitro (A) and in mouse hair follicles in vivo (B). (A) Freshly isolated human hair follicles were transfected with pSV- β -gal using nine liposome preparations, each under three different conditions. The number of cells stained blue (as in Fig. 1) per follicle was calculated. Transfection efficiencies are expressed relative to 24 μ g liposome 1, which resulted in the highest transfection efficiency, and was assigned 100%. (B) The same nine lipid mixtures were tested for their in vivo transfection ability at concentrations of 50 μ g lipid:10 μ g pCMV- β -gal. Transfection efficiency was calculated as the percentage of total follicles possessing β -gal-expressing cells. 1 to 8 refer to liposome mixtures pF_x-1 to 8. L, Lipofectamine; C, controls (50 μ g pF_x-1 alone and 10 μ g pCMV- β -gal in PBS).

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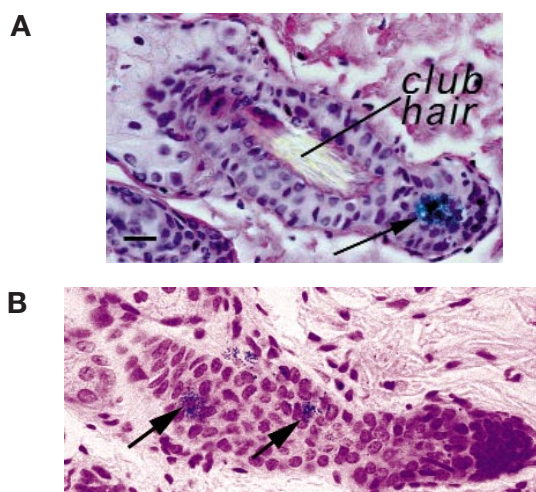


Figure 3. In vivo transfection of mouse hair follicles after depilation and topical application of lipoplex (10 μ g, pCMV- β -gal 50 μ g pF χ -1). Transfected hair precursor cells in the matrix area (A, arrow) and in the stem cell-rich bulge area (B, arrows) of the hair follicle are evident as blue cells. Both follicles are entering anagen. Scale bar = 25 μ m

topical immunization, they did not detect expression of plasmid DNA in follicles, despite using at least 10 times more DNA than required by our protocol. Our results suggest that plasmid DNA, introduced topically as a lipoplex, is selectively taken up and expressed by hair progenitor cells in the matrix. The lack of expression in matrix cells after application of naked DNA suggests that expression by other cells (e.g. dendritic antigen-presenting cells) may have led to immunization in these studies.

Transfection of human hair follicles with lipoplex. Although the ability to topically transfect mouse hair follicles may lead to valuable assays for studying hair follicle growth and differentiation, ultimately, for this technology to be useful in the treatment of human disease, its effectiveness in human skin must be demonstrated. To address whether human hair follicles can be transfected with DNA using topically applied liposomes, we developed a human scalp xenograft model based on the ability of human skin to survive on immunodeficient mice^{21,22}. We grafted human fetal or adult scalp tissue onto immunodeficient mice. The human hair follicles remain viable for many (>12) months, generating abundant normal-appearing human hair (Fig. 4A). Histological analysis (data not shown) reveals that this model parallels the growth of hair in the normal human scalp, in which the majority of follicles are in anagen, while approximately 10% are in telogen, and a small percentage (5–10%) are in transitional phases from anagen to telogen (catagen) and from telogen to full-blown anagen (anagen onset). Furthermore, each graft possesses a large number of follicles (up to 500) that are in different stages of the hair cycle, allowing for detection of reporter expression in a subset of follicles as well as alterations in ratios of growing to resting follicles.

Using the xenograft model, we tested whether human follicle cells can be transfected in vivo with lipoplex. Taking into account our findings in mouse skin, we pretreated xenografts with depilation and/or retinoic acid to increase follicles in anagen onset and then applied lipoplex containing pF χ -1 and pCMV- β -gal. In all experiments—in which more than 2,400 follicles were examined—we found transfected cells only in follicles at anagen onset (Table 1). No telogen, catagen, or full-blown anagen follicles were transfected. Cells positive for β -gal were located at the

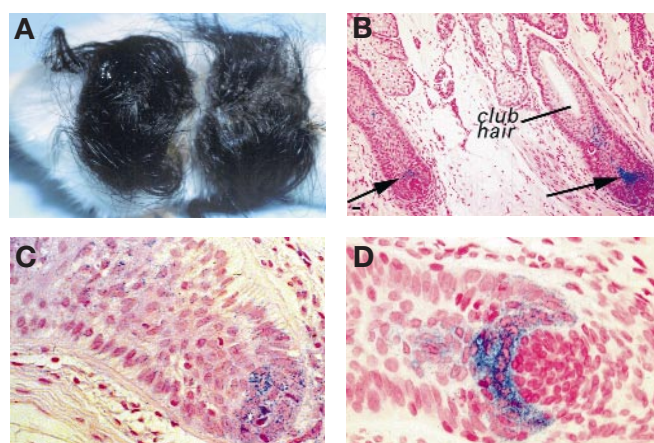


Figure 4. Human scalp xenograft model. (A) Human fetal scalp had been grafted to two sites on the back of an immunodeficient mouse four months before. The grafts possess large numbers of normal-appearing human hairs. (B) After pretreatment with depilation and retinoic acid, and application of lipoplex, two adjacent hair follicles, just entering anagen, possess transfected (blue, arrows) hair precursor cells. The club hair, which remains from the previous hair cycle, denotes the lowermost portion of the resting follicle. (C) Higher magnification views of transfected follicles from adult (C, \times 400) and fetal (D, \times 600) hair follicles showing transfected (blue, arrows) hair precursor cells in the matrix. Scale bar = 25 μ m.

leading edge of the epithelial downgrowth, in the matrix keratinocytes destined to become hair shaft (Fig. 4B–D). Transfected cells were predominantly keratinocytes. No other cells within the hair follicle, sebaceous gland, or epidermis expressed the reporter gene. The dermis and subcutaneous fat were also negative for reporter expression. These results show that the early anagen follicle in human scalp readily accepts and expresses plasmid DNA after topical introduction with liposomes.

Both depilation, and depilation with retinoic acid pretreatment, increased the percentage of follicles in anagen onset as well as the efficiency of transfection (Table 1). Although retinoic acid treatment alone did not increase the percentage of anagen onset follicles, it did increase the efficiency of transfection. Interestingly, the transfection efficiencies of either treatment alone were approximately half that of the combined treatments. One explanation for this effect includes the possibility that each treatment enhances the transfectability of different subpopulations of follicles within the grafts. Alternatively, the effects could be additive on all of the follicles. Further studies are needed to determine the basis of the effect of retinoic acid on transfection efficiency.

The transfection of anagen-onset hair follicles was not a rare event (for instance, see Fig. 4B, which shows two adjacent transfected follicles). The transfection efficiency, calculated as the percentage of positive follicles within the population of follicles in anagen onset,

Table 1. Transfection efficiency in fetal human scalp xenografts treated with topical lipoplex^a

	Hair follicles in anagen onset (%) ^b	Hair follicles in anagen onset transfected (%)	Percentage of total hair follicles transfected
1. Control (no pretreatment)	8 \pm 1 (126)	6 \pm 9	0.6 \pm 0.8
2. Depilated	13 \pm 1 (637)	23 \pm 15	3 \pm 2
3. RA	6 \pm 0 (374)	28 \pm 1	2 \pm 0
4. Depilated + RA	20 \pm 8 (1255)	48 \pm 10	10 \pm 6

^aLipoplex was applied three times every other day for one week after pretreatments 1–4 as indicated. Conditions 1 and 3 represent results from two experiments using two grafts for each condition.

Conditions 2 and 4 represent results of three experiments using two grafts for each condition. Hair follicles were examined histologically by serial sectioning of the xenografts. RA, Retinoic acid.

^bTotal number of hair follicles examined is given in parentheses.

was $48 \pm 10\%$ in xenografts treated with both depilation and retinoic acid. This represented $10 \pm 6\%$ of the total number of follicles within the xenograft. This high-efficiency selective gene targeting of the hair follicle allows, for the first time, the testing of functional genes that can alter the properties and products of the follicle.

Because our protocols target hair progenitor cells as they regenerate at anagen onset, genes introduced at this time, even if expressed transiently, could alter the phenotype (e.g. size, color) of the new hair by altering the characteristics of the progenitor cells. Our demonstration of targeting and gene expression in follicles in human skin has direct implications for therapeutics. Disorders that are candidates for treatment with this approach include common baldness (androgenetic alopecia) and alopecia areata, an autoimmune disorder affecting over 1% of the population²³. In both of these conditions, the preponderance of affected hair follicles are in telogen or anagen onset^{24,25}; thus the majority of follicles should be susceptible to transfection using topical lipoplex. Our human scalp xenograft model provides a system for testing the effects of novel gene-based therapies on human hair growth.

Experimental protocol

Lipoplex. Liposomes were purchased from Invitrogen (pF_x-1 to -8), and from Gibco-BRL (lipofectamine). Plasmid DNA encoding for the *lacZ* reporter gene under the control of the SV40 early promoter (pSV-gal; Promega, Madison, WI) or the CMV promoter (pCMV-β-gal), was purified using Qiagen Maxi Plasmid kit (Clontech, Palo Alto, CA). Each lipoplex was prepared in serum-free OPTI-MEM (Gibco-BRL, Grand Island, NY) media according to the manufacturer's protocol.

Transfection of human hair follicles in vitro. Follicles were isolated from adult human scalp (obtained from cosmetic surgery procedures by the Cooperative Human Tissue Network, CHTN) treated with Dispase (Sigma, 5 mg ml⁻¹ in Dulbecco's modified Eagle Medium, DMEM) overnight at 4°C. Plucked anagen hair follicles were placed in 24-well plates (10 follicles per plate) and transfected immediately with 0.5 ml lipoplex at 37°C in 5% CO₂/air for 24 h. Lipoplex contained pSV-β-gal DNA and nine different lipid reagents (pF_x-1 to -8 and lipofectamine) at different ratios and concentrations (see Fig. 2A). Lipoplex solution was then replaced with DMEM supplemented with 4 mM L-glutamine, 20% fetal bovine serum, 0.5 μg ml⁻¹ hydrocortisone, 10 ng ml⁻¹ epidermal growth factor (100 U/ml), 0.1 mg ml⁻¹ penicillin/streptomycin, and cultured at 37°C for another 24 h. The follicles were then stained with X-gal reagent (5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 2 mM MgCl₂).

In vivo mouse hair follicle transfection. Under general anesthesia, dorsal skin of 50-day-old Balb/c mice (Charles River, Wilmington, MA) was clipped of hair and treated with a depilatory cream (Nee; Premier Consumer Products, Inc., Englewood, NJ) for 5 min. At different time points after depilation, 50 μl of lipoplex containing 50 μg of lipid and 10 μg of pCMV-β-gal was applied topically to 1 cm² of dorsal skin in 5 μl aliquots using a micropipette over 90 min. DNA amounts were similar to previously published studies¹⁴, although we utilized lower DNA:lipid ratios, which worked better in vitro. Control skin was treated with 10 μg naked plasmid or 50 μg of lipid alone. Transfected skin was harvested 24 or 48 h after transfection, and stained for β-gal activity. The University of Pennsylvania Animal Care and Ethics Committee approved all experimental procedures.

Xenograft model. Four-week-old CB-17 Icr-*scid/scid* male mice (Charles River, Wilmington, MA) were maintained under pathogen-free conditions. Human fetal scalp (20-week gestation from Advanced Bioscience Resources, Alameda, CA), and human adult scalp from cosmetic surgery (CHTN) was grafted within 24 h of harvest. Grafting surgery was performed in a laminar-flow hood using sterile procedures. Mice were anesthetized with ketamine-xylazine mixture, after which hair on the dorsum was clipped. Pieces of skin measuring 1 × 1 cm were grafted to a bed of similar size that had been prepared by removing mouse skin down to the fascia. Human skin grafts (usually two per mouse) were held in place with 6-0 nonabsorbable monofilament suture. The transplants were coated with petrolatum and covered with Tegaderm (3M Health Care Ltd. (St. Paul, MN)), and sterile dressing. Bandages were removed after two to three weeks, and grafts were allowed to heal for an additional two to three weeks before proceeding with the experiments.

Transfection of human xenografts in vivo. Before transfection, xenografts were depilated. Some grafts were also treated with 0.05% retinoic acid cream (Retin-A, Johnson & Johnson, Raritan, NJ) every other day for one week. On the day of transfection, mice were anesthetized and the xenografts were prehydrated with PBS for 15 min. After 75 μg of pF_x-1 lipid and 30 μg of pCMV-β-gal were mixed in OPTI-MEM, the mixture was pipetted topically in 5 μl aliquots (75 μl total) to the grafted skin every other day for three days. Higher doses of DNA and liposomes were used in human versus mouse transfections because of the thicker stratum corneum, which could potentially absorb the lipoplex and prevent adequate delivery to the follicle. Skin was harvested 48 h after transfection and processed for β-gal activity.

Histochemical assay of β-galactosidase activity. Mouse and human tissue samples were fixed in freshly prepared 2% formaldehyde/0.2% glutaraldehyde in PBS at 4°C for 2–4 h, then washed in three changes of PBS at room temperature for 1 h. Fixed tissue was incubated at 37°C overnight in 1 mg ml⁻¹ X-gal in PBS. Then tissue was washed with PBS, fixed in formalin, and embedded in paraffin. Sections of 5 μm were counterstained with nuclear fast red.

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