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Research Article

Targeted Elimination of Keloid Fibroblasts via Dm-dnk Expression Driven by the Fibronectin Promoter: An In Vitro Experimental Study

Enfeng Wang, Lina Yang*

[1]*Department of Burn Surgery, Western Hainan Central Hospital.*

Abstract **Objective:** This study aimed to design and validate a gene therapy vector utilizing promoter elements of the fibronectin gene to achieve conditional expression of the apoptosis-inducing gene *Dm-dnk* in keloid fibroblasts, so as to evaluate its capacity to selectively eliminate target cells in vitro. **Methods:** The experimental period spanned from January 2020 to December 2024. A total of 46 patients were enrolled, and primary fibroblasts were isolated and cultured from surgically resected keloid tissues and autologous normal skin tissues of each patient. The core procedure involved the construction of recombinant adeno-associated virus (AAV-FN-Dm-dnk), in which the expression of *Dm-dnk* was regulated by the fibronectin (FN) promoter. This viral vector was transfected into keloid-derived fibroblasts and normal skin-derived fibroblasts separately; an empty viral vector group and an untreated cell group were set as experimental controls. Cellular viability, the level of programmed cell death, cell cycle distribution, and the expression of fibrosis-related marker molecules including collagens were systematically detected and compared among all groups. **Results:** The FN promoter was effectively activated in keloid fibroblasts, driving robust expression of *Dm-dnk*. Consequently, the cell survival rate in this group decreased drastically to approximately 34.2%, the total apoptotic rate increased to around 42.7%, and prominent G1-phase cell cycle arrest was observed. Meanwhile, both the gene and protein expression of type I collagen and α-smooth muscle actin were markedly suppressed in keloid fibroblasts. By contrast, no statistically significant alterations were observed in all aforementioned detection indices in normal skin fibroblasts subjected to identical treatment. **Conclusion:** The genetic strategy employing the FN promoter to mediate *Dm-dnk* expression can specifically and efficiently trigger the death of keloid fibroblasts and inhibit their pivotal profibrotic functions in vitro, with negligible adverse effects on normal skin fibroblasts. These findings provide a crucial experimental foundation for the development of transcriptional targeted gene therapy against keloids.

Keywords: keloid; fibroblasts; fibronectin promoter

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Introduction

Keloid is a proliferative disorder characterized by abnormal hyperplasia of cutaneous connective tissue, and its treatment remains a major clinical challenge. The fundamental pathogenesis lies in the presence of hyperfunctional and apoptosis-resistant fibroblasts within keloid lesions. Conventional therapeutic modalities, including surgical excision, intralesional drug injection and

*Correspondence: Lina Yang,Department of Burn Surgery, Western Hainan Central Hospital,E-mail:wnzgsm@126.com

radiotherapy, seldom yield radical curative outcomes, and are frequently accompanied by adverse side effects and high recurrence rates [1]. Accordingly, it is urgently required to develop novel therapeutic strategies that can precisely recognize and eliminate pathogenic fibroblasts while maximally preserving adjacent normal cutaneous tissues.

Gene therapy represents a promising modality for precise therapeutic intervention, and the identification of a genetic switch exclusively activated in diseased cells constitutes the key determinant for its successful application [2]. As a core constituent of the extracellular matrix, fibronectin exhibits markedly elevated biosynthesis during tissue fibrosis and in activated fibroblasts. This feature endows its gene regulatory region with the potential to drive specific transcription of downstream genes within fibrotic lesions [3].

Based on the aforementioned rationale, the present study constructed a targeted regulatory system governed by the fibronectin (FN) promoter to initiate the expression of the *Dm-dnk* gene. This system can trigger an intrinsic apoptotic suicide program specifically in keloid fibroblasts, thereby achieving the precise elimination of pathological cells at the cellular level. Collectively, this work offers a novel molecular recognition-based strategy for the clinical management of keloids.

1 Materials and Methods

1.1 General Clinical Data

A total of 46 patients diagnosed with keloid were enrolled between January 2020 and December 2024. The cohort consisted of 21 males and 25 females, with ages ranging from 18 to 52 years and a median age of 31.4 years. The disease duration varied considerably, ranging from six months to 15 years. All tissue specimens were harvested via routine surgical resection from common anatomical sites including the chest, back, shoulder, and earlobe. To ensure comparability throughout the experiments, paired tissue samples were collected from each patient: keloid lesions and autologous normal skin tissues distant from the keloid lesions, which were subsequently used for primary fibroblast isolation and culture.

1.2 Methods

Fibroblasts were isolated from tissue specimens via enzymatic digestion, followed by primary culture and subculture under standard culture conditions. Cells at passages 3 to 5 were selected for all subsequent experiments. Using genetic engineering techniques, the screened core promoter sequence of the human fibronectin (FN) gene was cloned into an adeno-associated viral vector to enable specific transcription of the downstream effector gene *Dm-dnk*, yielding the recombinant viral plasmid pAAV-FN-Dm-dnk [4]. For control purposes, two additional viral constructs were generated: a control virus driven by the ubiquitously expressed CMV promoter and an empty viral vector lacking the effector gene. After sequence verification confirming the correctness of all plasmids, high-purity recombinant AAV2/8 viral particles were packaged and amplified in HEK293T cells with the assistance of helper plasmids [5]. Once target cells reached an appropriate confluency, different viral preparations were transduced into cells at an identical multiplicity of infection (MOI), and untreated cells were established as the blank control group.

1.3 Observation Indicators

Cell biological effects were detected at 72 h post viral transduction in this study. Cell proliferative viability was quantitatively determined using the CCK-8 assay. The

proportions of early and late apoptotic cells were precisely quantified by flow cytometry after Annexin V/propidium iodide (PI) double staining [6]. Additionally, PI single staining combined with flow cytometry was applied to analyse the cellular distribution across different cell cycle phases (G1, S, G2/M). Real-time quantitative polymerase chain reaction (qRT-PCR) was performed to detect the mRNA transcription levels of collagen type I alpha 1 (COL1A1), α-smooth muscle actin and fibronectin [7]. Western blotting was conducted to verify the protein expression levels of fibrotic markers and exogenous Dm-dnk protein.

1.4 Statistical Analysis

All quantitative experiments were performed with multiple replicate wells and independently repeated no less than three times. All data were presented as mean ± standard deviation (SD). Statistical analyses were carried out using SPSS 26.0 software. For overall comparisons among multiple groups, homogeneity of variance test was conducted first. One-way analysis of variance (ANOVA) followed by the least significant difference (LSD) post-hoc test was adopted for pairwise comparisons when the variance was homogeneous; otherwise, the corresponding nonparametric test was utilised [8]. The independent samples t-test was used for comparisons between two groups. A P value less than 0.05 was considered

2 Results

2.1 Differential effects of the targeted vector on the viability of two types of fibroblasts

Cell viability assays were performed to verify the selectivity of the therapeutic vector. Transduction with AAV-FN-Dm-dnk induced a sharp reduction in cell survival rate in keloid fibroblasts. In contrast, the identical treatment exerted negligible influences on the viability of normal skin fibroblasts. Constitutive overexpression of *Dm-dnk* driven by the ubiquitous strong CMV promoter led to severe cytotoxic damage to both cell populations, whereas the empty viral vector caused no obvious cellular alterations. These findings collectively demonstrated that the FN promoter possessed sufficient transcriptional activity exclusively within pathological keloid fibroblasts to trigger cytotoxic effects.

Table 1 Comparison of relative cell viability among all groups at 72 h after viral treatment (%, mean ± SD)

Experimental group	Keloid fibroblasts	Normal skin fibroblasts
Blank control group	100.0±3.5	100.0±4.1
AAV-FN-empty group	98.2±4.7	96.9±5.2
AAV-FN-*Dm-dnk* group	34.2±6.8*	92.4±7.1
AAV-CMV-*Dm-dnk* group	15.3±3.1*	18.9±4.5*

Note: * indicates statistically significant difference compared with the blank control group within the same cell type ($P < 0.05$).

2.2 Targeted vector induces programmed cell death in keloid fibroblasts

Apoptosis analysis provided direct evidence for the selective cytotoxic effect. Treatment with AAV-FN-Dm-dnk triggered extensive apoptotic events in keloid fibroblasts, accompanied by markedly elevated proportions of both early and late apoptotic cells. By contrast, no remarkable fluctuations in apoptotic levels were observed among all treatment groups in normal skin fibroblasts. Collectively, these results confirmed that FN promoter-driven expression of Dm-dnk specifically activated the apoptotic cascade exclusively within keloid fibroblasts.

Table 2 Analysis of celllaur apoptosis at 72 hours

following viral administration (%, mean ± SD)

Experimental Group	Cell Source	Early apoptosis (Annexin V^+/PI^-)	Late apoptosis (Annexin V^+/PI^+)	Total apoptotic rate
Blank control group	KF	4.2±1.1	1.8±0.7	6.0±1.5
	NSF	3.8±0.9	1.5±0.6	5.3±1.2
AAV-FN-empty group	KF	5.1±1.4	2.2±0.8	7.3±1.8
	NSF	4.0±1.0	1.7±0.5	5.7±1.3
AAV-FN-*Dm-dnk* group	KF	28.5±3.6*	14.2±2.4*	42.7±5.3*
	NSF	4.5±1.2	2.0±0.9	6.5±1.8
AAV-CMV-*Dm-dnk* group	KF	45.3±4.8*	22.1±3.1*	67.4±6.9*
	NSF	48.6±5.2*	20.5±2.8*	69.1±7.5*

Notes: KF: keloid fibroblasts; NSF: normal skin fibroblasts; * $P < 0.05$, compared with the blank control group of the same cell type.

2.3 Cell cycle arrest induced by the targeted vector in target cells

Alterations in cell cycle distribution further elucidated the underlying mechanism of action. In keloid fibroblasts treated with AAV-FN-Dm-dnk, the proportion of cells arrested at the G1 phase was markedly elevated, accompanied by decreased percentages of cells progressing into the S and G2/M phases. These findings indicated that DNA replication and cell division were effectively suppressed. Nevertheless, no evident cell cycle arrest was detected in normal skin fibroblasts subjected to the same intervention.

Table 3 Distribution proportion of cells in each cell cycle phase at 72 h after viral treatment (%, mean ± SD)

Experimental group	Cell source	G1 phase	S phase	G2/M phase
Blank control group	KF	58.3±3.2	28.5±2.4	13.2±1.8
	NSF	62.1±4.0	24.8±2.1	13.1±1.9
AAV-FN-empty group	KF	59.8±3.5	27.9±2.6	12.3±1.7
	NSF	61.5±3.8	25.2±2.3	13.3±1.8
AAV-FN-*Dm-dnk* group	KF	75.6±4.7*	16.2±2.8*	8.2±1.5*
	NSF	63.0±4.2	23.9±2.5	13.1±1.7

Note: * $P < 0.05$, compared with the blank control group of the identical cell type.

2.4 Inhibitory effect of the targeted vector on the fibrotic phenotype of target cells

Molecular detection revealed that this targeted therapeutic strategy exerted an additional inhibitory effect against the fibrotic phenotype. In surviving keloid fibroblasts, the transcriptional levels of COL1A1 and α-SMA, two marker genes responsible for extracellular matrix synthesis and cellular contraction, were significantly downregulated. Such suppressive effects were further validated at the protein level. By contrast, the expression levels of these molecules remained unchanged in normal skin fibroblasts following the same treatment.

Table 4 Alterations in mRNA expression of fibrosis-related genes at 72 h after viral treatment (relative expression level, mean ± SD)

Experimental Group	Cell Source	COL1A1	α-SMA	FN
Blank control group	KF	1.00±0.08	1.00±0.07	1.00±0.09
	NSF	1.00±0.10	1.00±0.11	1.00±0.12
AAV-FN-empty group	KF	1.05±0.11	0.98±0.09	1.03±0.10

Experimental Group	Cell Source	COL1A1	α-SMA	FN
	NSF	0.97±0.09	1.02±0.10	0.99±0.11
AAV-FN-*Dm-dnk* group	KF	0.32±0.06 *	0.41±0.07*	0.38±0.08*
	NSF	0.96±0.13	1.04±0.12	0.95±0.14

Note: All data were normalized to the blank control group of the corresponding cell type (set as 1); * $P < 0.05$ versus the blank control group within the same cell population.

3 Discussion

The present in vitro experimental results demonstrated that regulation of the pro-apoptotic gene Dm-dnk via the fibronectin promoter enables highly specific functional intervention against keloid fibroblasts. Given the abundant expression of fibronectin during tissue repair and within fibrotic lesions, its promoter serves as a promising targeting element for gene therapy [9]. Our experimental data verified that this promoter is robustly activated in pathological fibroblasts to drive the expression of cytotoxic Dm-dnk, whereas its transcriptional activity in quiescent normal skin cells remains too low to elicit detectable cytotoxicity [10]. Such differential transcriptional regulation constitutes the core mechanism underlying the therapeutic selectivity of this system.

Notably, the selective cytotoxicity achieved by this strategy follows a threshold-dependent pattern. The ubiquitous CMV promoter mediated indiscriminate cell death in both keloid and normal fibroblasts, recapitulating the off-target adverse effects that ought to be avoided in clinical practice. By contrast, the FN promoter exhibits distinct transcriptional activity across cell phenotypes: its activity exceeds the minimal threshold required to induce substantial apoptosis and viability loss in keloid fibroblasts, yet falls below this threshold in normal cutaneous cells, thereby safeguarding healthy tissue integrity [11]. Characterizing and refining this transcriptional activity window is critical for elevating the therapeutic index of this regimen in subsequent translational research.

Pronounced G1-phase cell cycle arrest was observed during targeted elimination of keloid fibroblasts. This phenomenon may arise from direct disruption of cell cycle regulatory machinery by the protein product of Dm-dnk, or alternatively represent an intermediate cellular event prior to the execution of apoptotic cascades [12]. Cell cycle arrest not only suppresses the excessive proliferation of pathological fibroblasts but also locks cells in an apoptosis-prone state, further potentiating targeted cell clearance [13). Further exploration of the precise molecular targets modulated by Dm-dnk will facilitate optimization of this therapeutic approach.

Beyond direct apoptotic cell elimination, an important secondary outcome was identified: the expression of key fibrosis-associated genes was markedly downregulated in surviving target cells after treatment. Collectively, this dual-effect strategy exerts therapeutic benefits through two pathways: it directly reduces the population of hyperactive fibroblasts responsible for excessive extracellular matrix deposition, and remodels the phenotype of residual cells by attenuating their collagen synthesis and contractile capacities [14]. Functional inactivation of profibrotic fibroblasts provides additional advantages for ameliorating and even reversing the fibrotic architecture of keloids, whose underlying molecular mechanisms warrant further investigation.

Nevertheless, multiple challenges remain before translating these in vitro findings into clinical applications. The transcriptional performance of the FN promoter within the complex in vivo pathological microenvironment

(characterized by persistent inflammation and hypoxia) requires validation using animal models. Systematic evaluations are also essential to characterize the in vivo delivery efficiency, tissue tropism, immunogenicity and long-term biosafety of the AAV vector system. Furthermore, keloid lesions comprise heterogeneous cell populations; advanced analytical techniques are needed to confirm whether the FN promoter is consistently activated across all disease-driving fibroblast subsets. Future work will focus on establishing mouse models that closely recapitulate human keloid pathology to assess the in vivo efficacy and safety of this system, as well as exploring combinatorial regimens with established conventional therapies [15].

In conclusion, this study proposed and preliminarily validated a novel transcription-targeted gene therapeutic strategy, offering a promising preclinical candidate for the intractable clinical management of keloids.

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Conflict of Interest

None.

Acknowledgements

None.

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