



# A SKIN SUBSTITUTE TISSUE BIOENGINEERED USING A NON-VIRAL VECTOR TO EXPRESS TIMP-1 INHIBITS METALLOPROTEINASE ACTIVITY

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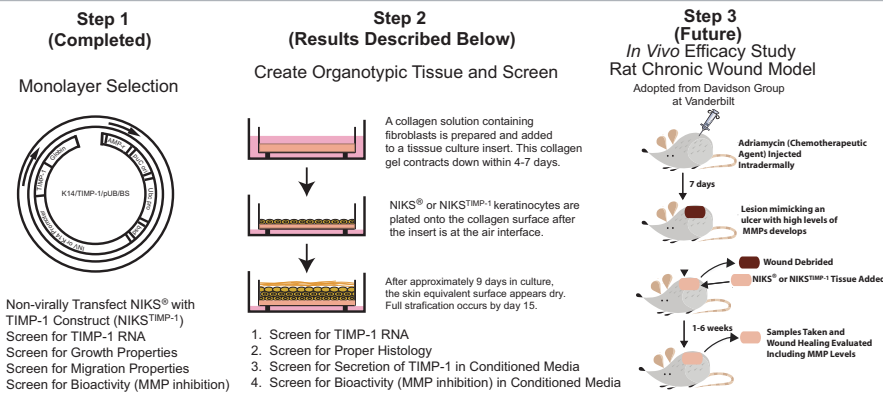
## Abstract

Successful wound closure requires balanced matrix metalloproteinase (MMP) and tissue inhibitor of metalloproteinase (TIMP) activity for proper granulation tissue formation and keratinocyte growth and migration. Chronic cutaneous wounds exhibit a highly proteolytic environment due to an increase in MMP activity and a corresponding decrease in TIMP activity inhibiting the healing process. The goal of this study is to develop a genetically modified therapeutic skin substitute with a non-viral vector that reduces the proteolytic imbalance of chronic wounds by expressing elevated levels of TIMP-1. NIKS<sup>®</sup> keratinocytes<sup>1</sup> stably-transfected with the TIMP-1 transgene were previously described to form a fully-stratified skin substitute tissue (ExpressGraft<sup>™</sup>Shield) that can secrete approximately 4-fold higher levels of TIMP-1 than unmodified tissue (StrataGraft<sup>®</sup><sup>2</sup>). Indirect immunofluorescent analysis of NIKS<sup>TIMP-1</sup> indicated that TIMP-1 was expressed throughout the epidermis. Differentiation marker analysis also confirmed proper formation of stratified tissue. Conditioned medium from ExpressGraft<sup>™</sup>Shield inhibited MMP-2 activity by as much as 48% compared to unmodified tissue in an *in vitro* fluorescent gelatin cleavage assay. A preliminary study has now been conducted to evaluate the effectiveness of NIKS<sup>TIMP-1</sup> tissue-secreted inhibitor activity against a patient chronic wound exudate sample using this same assay. Thus far, testing of chronic wound fluid from a venous ulcer patient has revealed a 50% inhibition of protease activity by NIKS<sup>TIMP-1</sup> conditioned medium compared to StrataGraft<sup>®</sup>. Additional tests for tensile strength indicate that NIKS<sup>TIMP-1</sup> tissue is comparable to clinically-tested StrataGraft<sup>®</sup>. Furthermore, NIKS<sup>TIMP-1</sup> possesses a stable karyotype with a transgene insertion site located on chromosome 3q. In summary, NIKS<sup>TIMP-1</sup> cells produce a fully-stratified skin tissue that can inhibit both MMP-2 and chronic wound protease *in vitro*. We next will test this therapeutic in an *in vivo* chronic wound model and ultimately will translate this technology into the clinic to combat the highly proteolytic environment of chronic cutaneous wounds.

## Objective

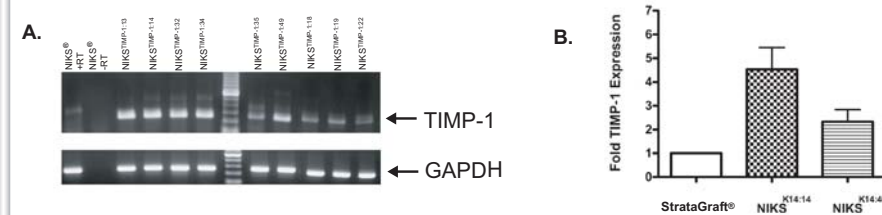
To produce a genetically engineered skin substitute tissue that expresses elevated levels of TIMP-1 and demonstrates enhanced protease inhibition both *in vitro* and *in vivo*.

## Experimental Design



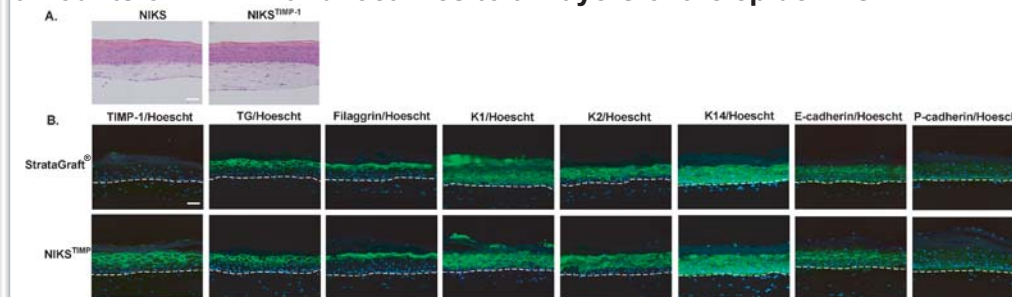
## Results

**Figure 1: Screening of NIKS<sup>TIMP-1</sup> tissue by PCR**



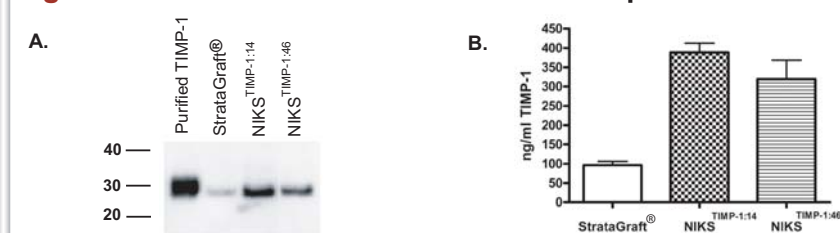
**A. RT-PCR screen for TIMP-1 transgene expression.** The forward primer anneals to the TIMP-1 coding region and the reverse primer anneals to the rabbit  $\beta$ -globin poly A fragment of the vector. Since one primer anneals to the vector, this primer set did not amplify endogenous TIMP-1 mRNA. **B. qPCR screen for total TIMP-1 expression.** NIKS<sup>TIMP-1</sup> tissue can produce over 4.5 fold more TIMP-1 RNA than NIKS<sup>®</sup> tissue (StrataGraft<sup>®</sup> (unmodified NIKS<sup>®</sup> tissue)). StrataGraft<sup>®</sup> was arbitrarily set to 1. Results reported as mean + SEM. n=4

**Figure 2: NIKS<sup>TIMP-1</sup> forms differentiated skin tissue that produces high amounts of TIMP-1 and localizes to all layers of the epidermis**



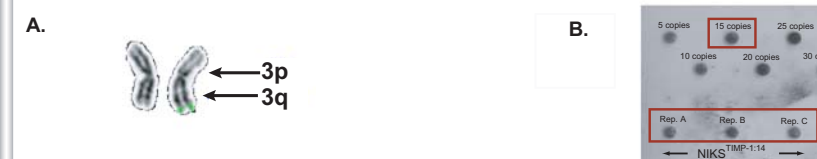
**A. NIKS<sup>TIMP-1</sup> forms normal skin tissue including basal, spinous, granular and cornified layers comparable to StrataGraft<sup>®</sup>.** Representative hematoxylin and eosin stained tissue sections. **B. A TIMP-1 clone produces high amounts of TIMP-1 protein and differentiates properly.** TIMP-1, Transglutaminase (TG), Filaggrin, K1, K2, K14, E-cadherin and P-cadherin (green) and Hoescht nuclei staining (blue). StrataGraft<sup>®</sup> tissue displays little TIMP-1 in the epidermis. NIKS<sup>TIMP-1</sup> produces high levels of TIMP-1 found in all layers of the epidermis. Markers indicate that NIKS<sup>TIMP-1</sup> tissue is formed properly and is comparable to StrataGraft<sup>®</sup>. Dotted white lines indicate epidermal/dermal junction. Bars equals 50 $\mu$ m

**Figure 3: NIKS<sup>TIMP-1</sup> tissue secretes TIMP-1 protein**



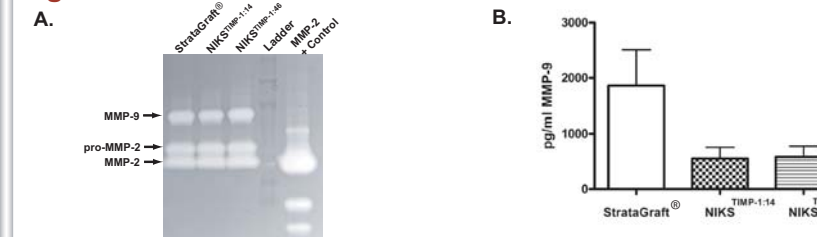
**A. Immunoblot of conditioned media indicates proper secretion of TIMP-1.** After 24 hrs of culture, media from beneath the tissue was collected and subjected to protein analysis. A band of the correct size (~29kD) is detected in the conditioned media. Note the large amount of TIMP-1 in NIKS<sup>TIMP-1</sup> clones compared to StrataGraft<sup>®</sup> tissue. **B. ELISA analysis of 24 hr conditioned media from tissue.** Approximately 4-fold more TIMP-1 is produced by NIKS<sup>TIMP-1</sup> than by StrataGraft<sup>®</sup>. Results reported as mean + SEM. n  $\geq$  4

**Figure 4: For clone NIKS<sup>TIMP-1:14</sup>, the transgene localizes to the long arm of chromosome 3 and 15-20 copies of the transgene are present**



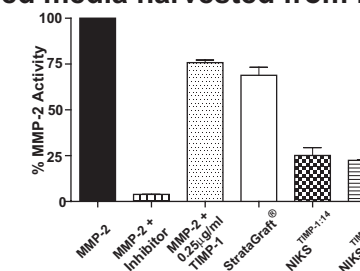
**A. Fluorescent in situ hybridization (FISH) was performed to determine the transgene insertion site.** FISH analysis was conducted by Cell Line Genetics (Madison, WI) and indicated that the transgene insertion site for NIKS<sup>TIMP-1:14</sup> is located on chromosome 3. For NIKS<sup>TIMP-1:46</sup> the transgene was not detected using FISH, presumably due to low copy number. **B. Dot blot analysis was performed to determine transgene copy number.** Analysis indicated that NIKS<sup>TIMP-1:14</sup> contains 15-20 copies of the TIMP-1 transgene (red boxes) and that NIKS<sup>TIMP-1:46</sup> possesses 1-2 copies, consistent with FISH analysis (data not shown). For dot blot, all samples and vector copies were diluted 5X.

**Figure 5: Conditioned media from NIKS<sup>TIMP-1</sup> tissue binds to MMP-9**



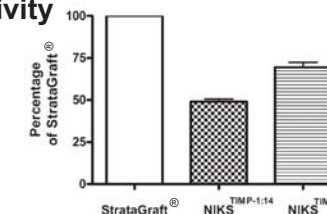
**A. Gelatin zymography of conditioned media from StrataGraft<sup>®</sup> and NIKS<sup>TIMP-1</sup> tissue was performed.** Clear bands indicate approximately equal amounts of enzymatic activity from MMP-2 and MMP-9 for all 72 hr conditioned media tested. **B. ELISA results indicate that secreted TIMP-1 interferes with the detectable MMP-9 in 24 hr conditioned media by over 70% compared to StrataGraft<sup>®</sup> conditioned media.** Manufacturer's instructions indicate interference can occur when over 1.56 ng/ml of TIMP-1 is present. Results reported as mean + SEM. n  $\geq$  3.

**Figure 6: Conditioned media harvested from NIKS<sup>TIMP-1</sup> tissue inhibits MMP-2 activity**



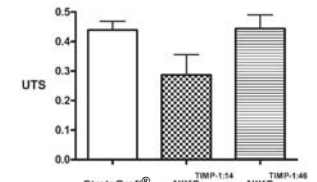
The EnzChek Gelatinase / Collagenase Assay (Molecular Probes, Eugene, OR) was used to assess protease inhibition activity. The DQ gelatin substrate provided with this kit fluoresces only when enzymatically cleaved. A standard concentration of MMP-2 (5 nM) was used to prepare the proteinase/substrate/24 hr conditioned medium suspension. A substantial inhibition of MMP activity was consistently obtained with the positive control (1, 10-phenanthroline), confirming our confidence in this assay. Inhibition of MMP-2 activity by as much as 48% was observed for the NIKS<sup>TIMP-1</sup> tissue compared to StrataGraft<sup>®</sup>. Results reported as mean + SD. n=2 for MMP-2, Inhibitor and TIMP-1. n=3 for conditioned media collected from separate tissue batches.

**Figure 7: NIKS<sup>TIMP-1</sup> tissue conditioned media inhibits chronic wound fluid proteolytic activity**



**Preliminary Data:** The EnzChek Gelatinase / Collagenase Assay was again used to assess protease inhibition activity. Chronic wound fluid was obtained from patient bandages. The bandage was soaked in 5 ml of PBS at 4°C for 1 hr. Samples were spun down to pellet any particulates and wound fluid was frozen at -80°C. Ten  $\mu$ l of chronic wound fluid was used to prepare the proteinase/substrate/24 hr conditioned medium suspension and incubated for 10 hrs at ambient temperature. For each experiment, proteolytic activity after incubation with StrataGraft<sup>®</sup> conditioned media was set at 100%. Conditioned media from NIKS<sup>TIMP-1:14</sup> tissue and NIKS<sup>TIMP-1:46</sup> tissue inhibited chronic wound fluid protease activity by more than 50% and 30% respectively, compared to conditioned media from StrataGraft<sup>®</sup>. Results reported as mean + SEM. n=4 (conditioned media batches)

**Figure 8: NIKS<sup>TIMP-1</sup> tissue displays similar tensile strength to StrataGraft<sup>®</sup>**



Briefly, standard dog-bone shaped tensile specimens were cut using an ASTM custom manufactured stainless steel die mounted on a manual toggle press. Specimens were pulled to failure at a strain rate of 100% per minute on an Insight 1 Bionix tensiometer (MTS Systems, Eden Prairie, MN) and ultimate tensile strength was determined from load and displacement data acquired using Testworks 4 software. \*One Way ANOVA analysis indicated that 5 batches of NIKS<sup>TIMP-1</sup> tissues were not significantly different from StrataGraft<sup>®</sup>. Results reported as mean + SEM

## Additional Results

Anchorage independent growth potential of both clones was negative (no tumorigenic potential *in vitro*). Assessed by WuXi Apptec (Philadelphia, PA).

Assessment to ensure that neither clone has any tumorigenic potential *in vivo* is currently in progress

## Summary

- \*A stably transfected clonal population of cells expressing TIMP-1 was generated using non-viral methods.
- \*NIKS<sup>TIMP-1</sup> cells undergo normal squamous differentiation to produce a skin substitute possessing the characteristics of human skin.
- \*The NIKS<sup>TIMP-1</sup> tissue secretes elevated TIMP-1 compared to StrataGraft<sup>®</sup> tissues.
- \*For clone NIKS<sup>TIMP-1:14</sup>, ~15-20 copies of the TIMP-1 transgene are located on chromosome 3
- \*TIMP-1 from NIKS<sup>TIMP-1</sup> tissue binds to MMP-9.
- \*Conditioned media from NIKS<sup>TIMP-1</sup> tissue inhibits MMP-2 activity.
- \*Conditioned media from NIKS<sup>TIMP-1</sup> tissue can potentially inhibit proteases from chronic wound fluid
- \*NIKS<sup>TIMP-1</sup> clones are non-tumorigenic *in vitro* and *in vivo* testing is ongoing.

## Future Directions

- \*Continued *in vitro* experiments using human chronic wound fluid to test inhibition by NIKS<sup>TIMP-1</sup> tissue.
- \**In vivo* testing of NIKS<sup>TIMP-1</sup> tissue in a rat chronic wound model will be assessed.

## Acknowledgements

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## References

- Allen-Hoffmann *et al.*, J of Inv Derm, 2000;114(3):444-455
- Schurr *et al.*, J of Trauma, 2009; 66(3): 866-874