Aus dem Zentrum für Biochemie
der Universität zu Köln
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The effect of hexosamine biosynthetic pathway (HBP) activation on wound healing

Inaugural-Dissertation zur Erlangung der Doktorwürde
der Medizinischen Fakultät
der Universität zu Köln

vorgelegt von
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promoviert am 26.10.2022

Gedruckt mit Genehmigung der Medizinischen Fakultät der Universität zu Köln
2023

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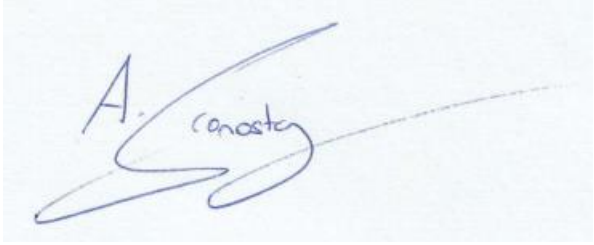
Unterschrift: 

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Danksagung

Ein herzlicher Dank geht an meine Doktorväter Herr Prof. M.-E. Paulsson sowie Herr Dr. M. Denzel für die Ermöglichung dieser Doktorarbeit und für die fachliche Betreuung. Bezüglich der finanziellen Förderung bedanke ich mich bei der SFB 829.
Ebenso bedanke ich mich herzlich bei meiner Betreuerin Frau Dr. K. Allmeroth für die Begleitung und Unterstützung auf diesem Weg sowie beim gesamten Labor der AG Denzel für die gemeinsame Zeit.
Weiterhin bedanke ich mich bei meiner Familie und meinen engsten Freunden für die stetige Unterstützung.

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Table of abbreviations

$ dollar
% percent
°C degree Celsius
4-MU 4-methylumbelliferone
AA antibiotic antimycotic solution
AMP adenosine monophosphate
AMPK AMP-activated protein kinase
ATP adenosine triphosphate
C. elegans Caenorhabditis elegans
cm centimeter
Da dalton
DMEM Dulbecco's modified eagle medium
DMSO dimethyl sulfoxide
DNA deoxyribonucleic acid
DPBS Dulbecco's phosphate-buffered saline
E. coli Escherichia coli
ECM extracellular matrix
EDTA ethylenediaminetetraacetic acid
EGF epidermal growth factor
ER endoplasmic reticulum
ERAD ER-associated degradation
EZM Extrazelluläre Matrix
FCS fetal calf serum
FGF fibroblast growth factor
Fru6P fructose-6-phosphate
g gram
GAG glycosaminoglycan
Gal galectin
G-CSF granulocyte colony-stimulating factor
GFAT-1 glutamine-fructose 6-phosphate amidotransferase
Glc D-glucose
Glc6P D-glucose-6-phosphate
GlcN D-glucosamine
GlcN6P D-glucosamine 6-phosphate
GlcNAc N-acetyl-D-glucosamine
GlcNAc1P N-acetyl-D-glucosamine-1-phosphate
GlcNAc6P N-acetyl-D-glucosamine-6-phosphate
GNA-1 glucosamine-6-phosphate N-acetyltransferase
GNPDA glucosamine-6-phosphate deaminase
GPI glucosamine-6-phosphate deaminase
h hour
HA hyaluronic acid
HAS hyaluronan synthase
HBP hexosamine biosynthetic pathway
HIF-1 hypoxia-inducible factor-1
HMW HA high-molecular weight hyaluronan
IL-1 interleukin-1
IL-6 interleukin-6
KGF keratinocyte growth factor
l liter
LC-MS liquid chromatography–mass spectrometry
LMW HA low-molecular weight hyaluronan
m meter
M molar

Man mannose
min minute
ml milliliter
mm millimeter
mM millimolar
MMP-1 matrix metalloproteinase-1
mRNA messenger ribonucleic acid
NaCl sodium chloride
NET neutrophil extracellular trap
ng nanogram
OGA O-GlcNAc hydrolase
OGT O-GlcNAc transferase
PDGF platelet derived growth factor
PEDF pigment epithelium-derived factor
PFA paraformaldehyde
PGM-3 phosphoglucomutase
RIPA Radio Immuno Precipitation Assay
rpm rounds per minute
RT room temperature, 15°C to 25°C
SPRY2 sprouty RTK signaling antagonist 2
TF tissue factor
TGF-beta tumor growth factor beta
TNFα tumor necrosis factor alpha
U unit
UAP-1 UDP-N-acetylglucosamine pyrophosphorylase
UDP-GalNAc uridine 5’-diphospho-N-acetyl-D-galactosamine
UDP-GlcNAc uridine 5’-diphospho-N-acetyl-D-glucosamine
UDP-GlcUA UDP-glucuronic acid
UDP-HexNAc uridine 5’-diphospho-N-acetyl-D-hexosamine
UGT UDP-glucuronosyltransferase
UPR unfolded protein response
UTP uridin triphosphate
UV ultraviolet
VEGF vascular endothelial cell growth factor
µg microgram
µl microliter

1. Summary

1.1 English Summary

Wound healing is a not completely unraveled complex interplay of cells, extracellular matrix (ECM) and mediators. As ineffective wound healing is an increasing global medical and economical problem, research is highly required to develop new therapeutic strategies. The ECM consists of glycosaminoglycans (GAG) which are produced in the intracellular, ubiquitous hexosamine biosynthetic pathway (HBP). Its end product, UDP-N-acetylglucosamine (UDP-GlcNAc), is required for posttranslational modification.

This project investigates the role of HBP activation in wound healing *in vitro*. Therefore, we tested the modulation of GlcNAc, UDP-HexNAc and hyaluronic acid (HA) by GlcNAc supplementation in both immortalized and primary murine fibroblasts and keratinocytes. Then, wound healing assays were performed in these cell types. The second part of the project focuses on the effect of HBP activation on cell proliferation potential on primary murine keratinocytes performing *colony formation assays* with either immortalized (NIH3T3 cell) or primary murine fibroblasts as feeder layer.

HBP activation by 50 mM GlcNAc supplementation caused only a mild increase of UDP-HexNAc and HA concentration in fibroblasts, yet a significant raise in keratinocytes. Interestingly, this led to a slowing effect in keratinocyte migration and consequently slowed wound closing (HaCaT cells: 80% of the wounds in the control group closed within 72 h vs. 45% of the tested group; primary keratinocytes: 80% vs. 50%). There was no significant dose-dependent effect for GlcNAc supplementation. As HA is known to accelerate wound healing, we evaluated the effect of substrates with a similar viscosity and showed this also for dextran, being therefore a possible therapeutical strategy for chronic wounds.
The second part of the project demonstrated no significant effect of 50 mM GlcNAc or 50 mM glucose on proliferative potential in keratinocytes. Surprisingly, 50 mM mannose supplementation caused a significant reduction.

In conclusion, HBP activation seems to affect wound healing *in vitro* negatively. However, my project underlines the significance of the HBP for effective cellular function including migration and proliferation, which are required for wound healing. In the future, more experiments should be performed to unravel its complete effect and to possibly identify targets for clinical application.

1.2 Deutsche Zusammenfassung

Das Endprodukt des intrazellulären, ubiquitären Hexosamin-Stoffwechselwegs (hexosamine biosynthetic pathway, HBP) ist UDP-N-acetylglucosamin (UDP-GlcNAc), welches für posttranslationale Modifizierungen notwendig ist und dessen Bedeutung immer weiter erforscht wird.[1] So wurde bereits beschrieben, dass dieser Stoffwechselweg einen positiven Einfluss auf die Proteinqualitätskontrolle und auf die Widerstandsfähigkeit gegenüber Stress in *C. elegans* hat.[2,3] Bei Aktivierung des HBP wird die Produktion von Glykosaminoglykanen stimuliert, darunter auch Hyaluronsäure (HA) mit positiv regulatorischer Funktion auf die Wundheilung.[4–8] Diese stellen wichtige Bestandteile der extrazellulären Matrix (EZM) der Haut dar. In letzter Zeit wurde vermehrt die vehemente Bedeutung des Zusammenspiels der EZM der Haut, regulierender Enzyme und der eigentlichen Hautzellen, den Fibroblasten und Keratinozyten, erforscht, welches für eine regelrechte und effiziente Wundheilung unabdingbar ist. Eine Erhöhung der Glykosaminoglykane und demnach eine Veränderung der EZM kann die Wundheilungsprozesse beeinflussen. Da nicht abheilende Wunden eine zunehmende globale medizinische und ökonomische Herausforderung darstellen,[9,10] sollten die noch nicht vollständig verstandenen Prozesse der Wundheilung tiefergehend erforscht werden.

In dieser Doktorarbeit wurde der Einfluss der Aktivierung des HBP auf die Wundheilung *in vitro* untersucht. Dafür prüfte ich im ersten Teil zunächst die mögliche Modulation von GlcNAc-, UDP-GlcNAc- und HA-Konzentrationen durch GlcNAc-Supplementation in den beiden Hauptzelltypen der Wundheilung per LC-MS und HA *quantification* ELISA Tests. Im nächsten Schritt wurde der Effekt dieser Aktivierung auf die Zellmigration in *wound healing assays* betrachtet.
Der zweite Teil der Arbeit überprüfte den Effekt des HBP auf die Zellproliferation, welche in der Wundheilung für den Verschluss der Wunde, für die Reepithelisation, notwendig ist. Auch hier spielt die EZM eine wichtige Rolle in der Regulierung von Zellen sowie von mechanischen und chemischen Signalen,[11] sodass ich die Hypothese aufstellte, eine Modulation dieser durch HBP-Aktivierung könnte das proliferative Potential von Keratinozyten steigern. Für die Testung verwendete ich *colony formation assays* mit sowohl immortalisierten (NIH3T3 Zellen) als auch primären murinen Fibroblasten als *feeder layer* und primären murinen Keratinozyten.

Interessanterweise zeigten meine Experimente, dass die HBP-Aktivität in Fibroblasten durch 50 mM GlcNAc-Zusatz nur mäßig, aber in Keratinozyten signifikant erhöht werden kann. Hier fand sich ein signifikanter Anstieg der UDP-HexNAc- und HA-Konzentrationen sowohl in HaCaT Zellen als auch in primären murinen Keratinozyten (UDP-HexNAc-Level in HaCaT: Kontrolle: 0.3 µg UDP-HexNAc/mg Protein vs. 10 mM GlcNAc: 1.4 µg UDP-HexNAc/mg Protein vs. 50 mM GlcNAc: 3.7 µg UDP-HexNAc/mg Protein, HA-Level in HaCaT (n=2):

Kontrolle: 160.2 bzw. 477.8 ng HA/mg/Protein/h, 10 mM GlcNAc: 1057.7 bzw. 851.1 ng HA/mg Protein/h, 50 mM GlcNAc: 7379.3 bzw. 4097.8 ng HA/mg Protein/h, UDP-HexNAc-Level in primären murinen Keratinozyten: Kontrolle: 0.105 µg GlcNAc/mg Protein; 10 mM GlcNAc: 0.441 µg GlcNAc/mg Protein, 50 mM GlcNAc: 1.0 µg GlcNAc/mg Protein; HA-Level in primären murinen Keratinozyten (n=2): Kontrolle: 72.3 bzw. 13.5 ng HA/mg/Protein/h, 10 mM GlcNAc: 225.8 bzw. 36.1 ng HA/mg/Protein/h, 50 mM GlcNAc: 326.9 bzw. 34.1 ng HA/mg/Protein/h). Erstaunlicherweise ergab diese Modulation eine verlangsamte Migration der Keratinozyten und einen damit einhergehenden verlangsamten Verschluss der Wunde: 80% der Wunden der Kontrollgruppe in HaCaT Zellen waren innerhalb 72 Stunden geschlossen, währenddessen es nur 45% der Wunden der Testgruppen waren. Um einen potenziellen dosisabhängigen Effekt der GlcNAc-Zugabe zu prüfen, testeten wir im nächsten Schritt zudem den Effekt von 10 mM, 20 mM, 30 mM und 40 mM GlcNAc auf den Wundverschluss innerhalb 72 Stunden (10 mM: 38%, 20 mM: 70%, 30 mM: 29%, 40 mM: 29%). Ebenso fand sich eine Verlangsamung des Wundschluss innerhalb 72 Stunden bei den primären Keratinozyten im Vergleich zur Kontrolle (50% vs. 80%).

Eine Zugabe von HA induzierte eine deutliche Beschleunigung des Wundschlusses. Bei 1% HA waren 61.5% der Wunden innerhalb 48 Stunden verschlossen. Dass HA einen positiven Einfluss auf die Wundheilung hat, ist bereits bekannt. Wir wollten jedoch zusätzlich herausfinden, ob Substanzen einer ähnlichen Viskosität gleichsam die Wundheilung beschleunigten, sodass wir zusätzlich die Gabe von 0.5% HA, 0.5% HMW Dextran, 1% HMW Dextran, 5% HMW Dextran, 10% HMW Dextran sowie 0.5% und 1% LMW Dextran in *wound healing assays* in HaCaT Zellen testeten. Ähnliche Ergebnisse fanden sich bei HA und HMW Dextran (0.5% HA: 44% Wundschluss innerhalb 48 Stunden, 0.5% HMW Dextran: 38.5%, 1% HMW Dextran 43%; 1% HA: 61.5%, 5% HMW Dextran: 67%). Eine weitere Steigerung der Viskosität mit 10% HMW Dextran-Zugabe war nicht möglich, keine Wunde verschloss sich in der vorgegebenen Zeit. LMW Dextran bewirkte kaum eine Beschleunigung der Wundheilung (0.5% LMW Dextran: 33%, 1% LMW Dextran: 33%). Zusammengefasst zeigte diese Untersuchung, dass HA effektiver in der Wundheilung ist, da es weniger hohe Dosen für die Ergebnisse brauchte. Jedoch beschleunigten auch Zugaben von Dextran den Wundschluss und zeigten auf, dass sie als therapeutische Möglichkeit genutzt werden können. Zudem betont dieser Test, dass nicht nur die biologischen Funktionen von HA, sondern auch die Viskosität des Mediums im Allgemeinen die Wundheilung beeinflussen kann.

Der zweite Teil dieser Arbeit beschäftigte sich mit der Auswirkung von HBP-Aktivierung auf die Proliferation von Keratinozyten, welches ich mittels *colony formation assays* mit NIH3T3 Zellen bzw. primären murinen Fibroblasten als *feeder layer* an primären murinen Keratinozyten untersuchte. Neben 50 mM GlcNAc verwendete ich zudem Glukose (Glc) und Mannose (Man),

da alle drei Hexosen in vorherigen Experimenten die Anzahl der Haarfollikelstammzellen erhöhen konnten.[12] Mit NIH3T3 Zellen fanden sich folgende Ergebnisse: Die GlcNAc-Zugabe zeigte kaum einen Unterschied im proliferativen Potential der Keratinozyten (GlcNAc vs. Kontrolle: 92.2% vs. 95%), wohingegen die Glc-Zugabe eine signifikante Reduktion um 32.9% (Glc vs. Kontrolle: 51% vs. 83.9%) und die Man-Zugabe eine signifikante Reduktion um 74.7% erwirkten (Man vs. Kontrolle: 1.2% vs. 75.9%, p-Wert < 0.0001). Dieser Trend wurde in den *colony formation assays* mit primären murinen Fibroblasten bestätigt: In der GlcNAc-Gruppe fand sich eine Reduktion um 31.4% (GlcNAc vs. Kontrolle: 58.9% vs. 90.3%). Glc reduzierte das proliferative Potential hier geringer um 13.2% (Glc vs. Kontrolle: 70.7% vs. 83.9%). Die Zugabe von Man erbrachte wiederum eine signifikante Reduktion um 76.3% (Man vs. Kontrolle: 9.4% vs. 85.6%). Dementsprechend fand sich kein signifikanter modulierender Effekt auf das proliferative Potential von Keratinozyten bei Aktivierung des HBP durch GlcNAc-Zugabe. Man hingegen reduzierte dieses signifikant.

Zusammenfassend offenbarte der erste Teil des Projekts unterschiedliche Ergebnisse in Fibroblasten und Keratinozyten. HBP-Aktivierung durch GlcNAc-Zugabe modulierte GlcNAc-, UPD-HexNAc- und HA-Konzentration deutlicher in Keratinozyten, welches durch die unterschiedlichen physiologischen Eigenschaften und Funktionen in dem Wundheilungsprozess bedingt sein kann. Interessanterweise verursachte eine Zugabe von 50 mM GlcNAc eine Verlangsamung im Wundschluss in Keratinozyten, in Fibroblasten zeigte sich keine Veränderung. *Lamers et al*. sahen einen verlangsamten Wundschluss in Fibroblasten in einem Medium von 25 mM Glukose.[13] Möglicherweise liegt in meinem Experiment in einem Medium von 25 mM Glukose plus Zugabe von 50 mM GlcNAc auch ein protektiver Einfluss auf die Migration der Fibroblasten vor, sodass keine Reduktion des Wundschlusses sichtbar ist. Hier wäre es interessant, beide Experimentbedingungen kombiniert zu testen.
Der Grund für eine verlangsamte Migration von Keratinozyten durch Aktivierung des HBP könnten Modifikationen von Zelladhäsionen oder in Zytokinkonzentrationen in der EZM sein. Bei HBP-Aktivierung kommt es zu einer vermehrten O-GlcNAcylation, welche wiederum Einfluss auf die EZM hat. Um dies genauer zu untersuchen, könnten wir verschiedene Konzentrationen vor und nach GlcNAc-Zugabe sowie Zell-Zell- und Zell-Matrix-Interaktionen messen. Ich konnte aufzeigen, dass HMW Dextran die Zellmigration in Keratinozyten wie HA beschleunigte. Da beide Substanzen ähnliche visköse Eigenschaften haben, sollte der Effekt der Viskosität auf die Wundheilung weiter erforscht werden. Durch Steigerung von UDP-HexNAc und HA durch GlcNAc-Zugabe könnte die Viskosität zu sehr erhöht werden, sodass dies einen negativen Einfluss auf die Wundheilung haben könnte. Daher bedarf es weiterer Forschung auf diesem Gebiet und wie medizinische Therapien hiervon profitieren könnten.

Schließlich fanden wir in den *colony formation assays* zur Untersuchung der Keratinozytenproliferation unterschiedliche Ergebnisse in den drei Testgruppen mit GlcNAc, Glc und Man. Erstens, HBP-Aktivierung durch GlcNAc-Zugabe hatte keinen Effekt auf das proliferative Potential. Im Vergleich zu den beiden anderen Zuckern könnte hier sogar ein protektiver Effekt zum Erhalt der normalen Proliferationsrate vorliegen. Inwieweit diese HBP-Aktivierung in die EZM eingreift, bleibt unklar. Zweitens, die Zugabe von Glc zeigte eine leichte Reduktion. Aus vorherigen Studien ist bekannt, dass eine erhöhte Glukosekonzentration zur verminderten Proliferation führt und in Diabetes generell einen negativen Einfluss auf Wundheilung hat. Letztendlich könnte man dies auch von der GlcNAc-Zugabe annehmen. Drittens, Man inhibiert Keratinozytenproliferation. Es scheint, als verringere Man die UDP-HexNAc- und HA-Spiegel und arbeite gegensätzlich zur Aktivierung des HBP. Dementsprechend unterstreicht es die Wichtigkeit einer hohen UDP-HexNAc-Konzentration für effektive Zellproliferation.

Schlussfolgernd stellte diese Arbeit dar, dass eine HBP-Aktivierung interessanterweise den Wundheilungsprozess negativ beeinflusst. Auch wenn Studien über das Altern und Proteinqualitätskontrolle in *C. elegans* vielversprechend schienen, dass der HBP auch Einfluss auf die Zellmigration und -proliferation habe, so fand sich hier keine therapeutisch mögliche Anwendung in der Wundheilung. Jedoch unterstreicht meine Arbeit die große Bedeutung des HBP für effektive zelluläre Funktionen wie Migration und Proliferation, welche für die Wundheilung notwendig sind. Umso wichtiger sind weitere Forschungen bezüglich des tieferen Verständnisses der Wundheilung, der Rolle der EZM und der Therapiemöglichkeiten von chronischen Wunden in der Zukunft.

2. Introduction

2.1 The skin

The skin is our largest organ, covering the entire surface of the human body with a surface area of 2 m^2. It constitutes about 15% of the total body weight of an adult. Being called integument which originates from the Latin word “integere”, and which means to cover, it illustrates the main function of the skin. It covers the bones, muscles, tendons, blood and lymph vessels, and it connects the exterior with the mucous system, respiratory system and urogenital system.[14]

With its various functions, the skin is a particularly important organ for humans. Figure 1 gives a detailed overview. The principal function is to provide a physical barrier to protect the organism. It prevents the loss of fluid, electrolytes, and proteins by means of the impermeable stratum corneum. Moreover, the skin serves as a mechanical and chemical barrier against infections, toxic chemicals, and ultraviolet radiation. As it covers the body, it blocks the penetration of pathogenic organisms already through its structure. Antimicrobial peptides that point against gram-positive and gram negative organisms, fungi and some viruses,[15] as well as cathelicidins and sebaceous lipids fight infections.[14,16] Consequently, it belongs to the first defense, to the innate immune system, by its mechanical and chemical features. In terms of ultraviolet (UV) protection, the first layer of the epidermis, the stratum corneum, reflects UV rays so that it reduces the exposure dose in lower layers. UV rays activate melanocytes, located in the epidermis, and increase the number of melanosomes and therefore the production of the deoxyribonucleic acid (DNA) protecting melanin.[15] Further, the skin is needed for vitamin D synthesis. Both epidermal keratinocytes and dermal fibroblasts carry 7-dehydrocholesterol in their plasma membranes which is converted to provitamin D and then released into the systemic circulation.[17] Next, hepatic and renal enzymes convert the provitamin into the active form 1,25OH_2 vitamin D.[18] Furthermore, we need the skin for sensory perception. Several specialized cells of the skin detect the single features of sense of pressure, stretch, vibration, and fine touch. The mechanoreceptors, called Merkel cells, in the stratum basale of the epidermis receive information about pressure. Ruffini bodies in the dermis sense stretch. Pacini corpuscles in the subcutis perceive vibration. Meissner corpuscles are only found in palmoplantar skin, enabling us to feel touch.[19]

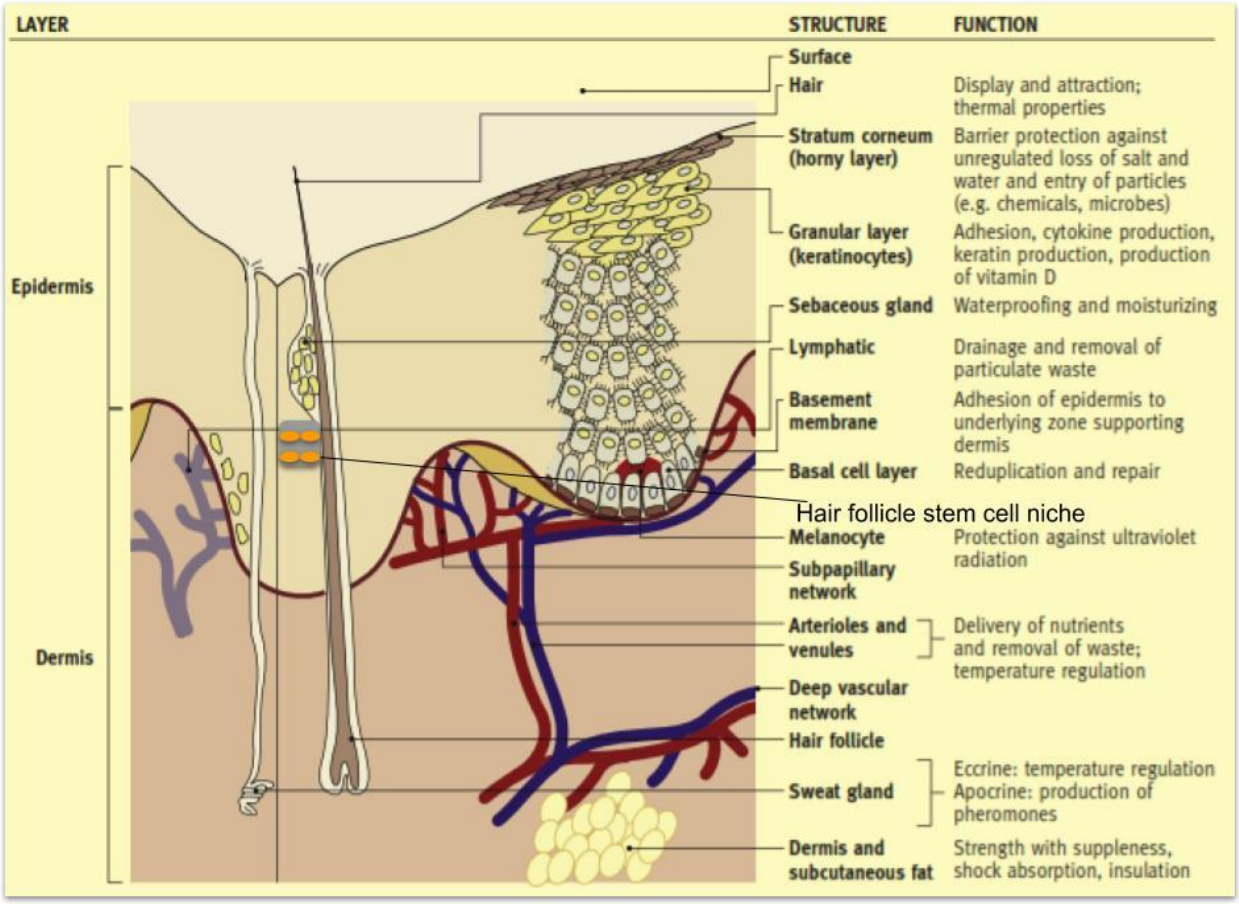


Figure 1: Schematic overview of skin structure and functions of its components

Modified from Venus et al. 2011.[15]

The skin is structured into three layers: epidermis, dermis and hypodermis (Figure 2). The most superficial layer is the epidermis. It consists mainly of keratinocytes (95%), melanocytes, antigen presenting Langerhans cells and Merkel cells.[19] Its thickness varies between 0.8 mm on the palms and 1.4 mm on the soles.[14,20] Figure 2 shows, the epidermis is subdivided into stratum corneum, stratum granulosum, stratum spinosum and stratum basale.[21] In palmoplantar skin, there is an additional layer between the stratum granulosum and stratum corneum, which is called stratum lucidum and which serves as an additional protection against mechanical loading.[19]

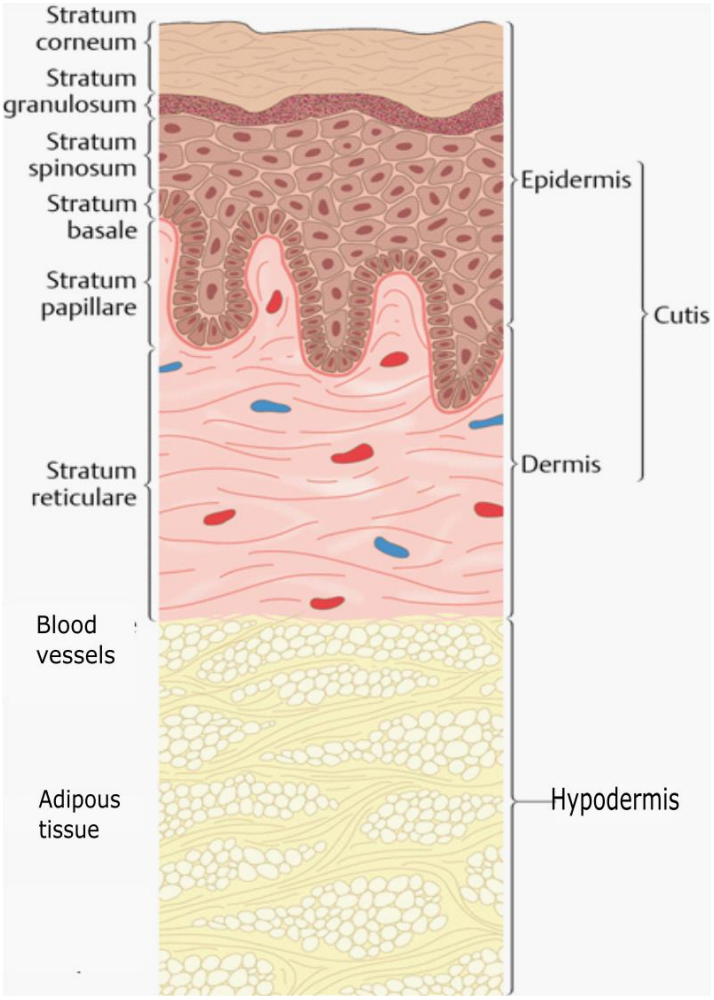


Figure 2: Histological drawing of the skin

Modified from Aumüller. 2014.[21]

Beginning with the deepest layer, the stratum basale is a single layer of stem cells which differentiate while moving towards the surface. Hemidesmosomes provide a connection to the dermis and create therefore more stability between the layers of the skin.

The stratum spinosum contains polyhedral cells migrating upwards and antigen presenting Langerhans cells. Keratinocytes are fully differentiated in the next layer, the stratum granulosum. It consists of one to five layers of flattened polygonal granulated cells.[15,19]

The stratum corneum is an amorphous matrix and has 15-20 layers of flattened dead cells, called corneocytes. Migrating upwards from the stratum spinosum to the stratum corneum, keratinocytes lose their nuclei and other cytoplasmic organelles. They exfoliate continuously. The cycle of cell proliferation just until exfoliation takes approximately 28 days but can also vary up to 40 days. This depends on age, infection and disease. Psoriasis, for example is a chronic inflammatory skin disease in which it takes around six days. [14,22] The disease presents itself in several forms, but mostly with the presence of plaques and thickened epidermis by uncontrolled proliferation and differentiation of keratinocytes.[23,24]

The dermis supports and nourishes the epidermis and connects it to the subcutis. Dermal papillae reach into the epidermis and the two layers are connected by the epidermal-dermal junction, thereby guaranteeing a high mechanical resistance of the skin.[19] Depending on the localization, the dermis is 0.55-5 mm thick.[25] It consists of two layers: the papillary and the reticular dermis.[19,26]

The papillary dermis binds to the epidermis in contact to the basement membrane zone. It also contains blood vessels for its nourishment and the immune response, and sensory nerve ends.[19] The reticular dermis, on the other side, supports the contact with the subcutis. In general, the dermis consists of interstitial components such as collagen fibers, predominantly types I and III, elastic tissue and ground substance, and of cellular components like fibroblasts, mast cells, plasma cells, dermal dendritic cells and histiocytes.[25,27] In terms of cellular components, it is the fibroblast which is the main cell type.[25] It secretes collagen, elastic fibers and ground substance.[19,25,28]

The deepest dermal layer is the hypodermis which consists of adipocytes that are arranged in fat lobules separated by fibrous septae. The bundles of fibers originate from the dermis and reach into the hypodermis to provide extra bonding to the skin layers. As the hypodermis is also connected to fascia and periosteum, it enables them to shift against each other and serves as a pressure pad.[19,25]

The skin also comprises sensory components (e.g. Pacinian corpuscles, Meissner corpuscles), blood and lymphatic vessels as well as the skin adnexa hair follicles, sweat glands, sebaceous glands and nails.[14,25]

Hair follicles originate from the epidermis and invaginate into the dermis. Each hair follicle consists of hair shaft, hair radix, hair bulb and hair papilla (Figure 3).[19,25]

Hair grows discontinuously in three phases: anagen, catagen and telogen. The first phase is characterized by active growth which regresses in the second phase. Whereas cellular activity continues within the germinal center of the hair follicle, there is no more growth nor cell activity in the final stage.[14,25]

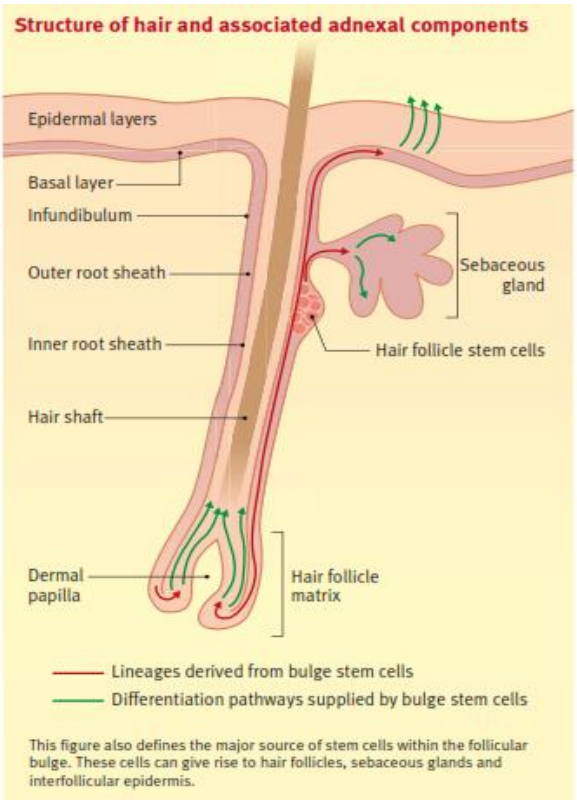


Figure 3: Hair follicle stem cells contribute to hair growth and wound healing

The stem cells of the hair follicle bulge migrate to the hair follicle to regenerate the matrix as well to the epidermal stratum basale to reepithelialize skin lesion. The red line depicts the migration of hair follicle stem cells.

Cited from Lai-Cheong and McGrath.[25]

Epithelial and melanocytic stem cell populations are found in the bulge region of the hair follicle (Figure 1 + Figure 3).[15,25] They play an important role in generating hair follicles and sebaceous glands but also in renewing the interfollicular epithelium during wound healing. The stem cell niche is located below the sebaceous gland and the arrector pili muscle.[25]

2.2 Wound healing

A wound is defined as the disruption of the integrity of the skin which leads consequently to structural and functional dysfunction not guaranteeing the barrier function anymore.[29] Therefore, this barrier defect needs to be quickly restored by reepithelization.[30,31] Reepithelization begins hours after lesion and is usually completed within 8-10 days.[32]

There are four known responses to skin injury: complete regeneration, normal repair leading to a scar, excessive repair leading to hypertrophic scars or keloids, or deficient repair, seen in

chronic wounds.[33] Only when the compartments of the skin and the extracellular matrix (ECM) work together in tight communication, wound healing can succeed. Several cytokines control this interaction which is described below.

If a wound occurs in the epidermis, the restoration is fast and efficient whereas the healing of a wound of the deeper dermal layers can be less effective and results in scar formation.[33] The definition of a scar is the replacement of physiological skin by fibrous tissue after any kind of injury. As the collagen fibers are not aligned in a reticular pattern, as they do in healthy tissue, but in just one direction, densely packed, the functional quality found in scars is inferior.[34,35] Myofibroblasts contribute to wound healing by contraction and interaction with the ECM. This pulls the tissue towards the center of lesion, which reduces the size of the wound area and finally the scar.[35,36]

The actual process of wound healing consists of three overlapping phases (Figure 4): hemostasis, proliferation and migration, and ECM remodeling.[33]

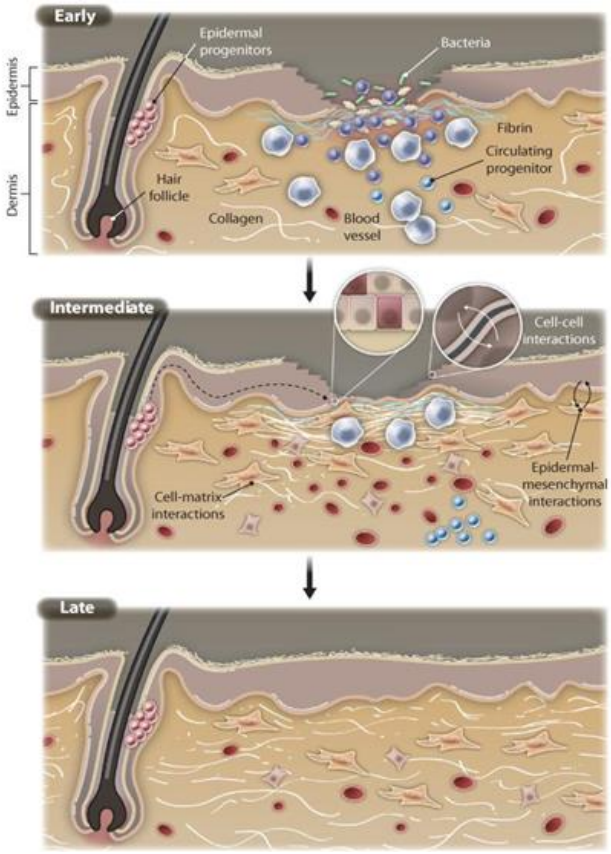


Figure 4: The wound healing process

1) Hemostasis and activation of keratinocytes and inflammatory cells

2) Proliferation and migration, matrix deposition and angiogenesis

3) ECM remodeling.

Cited from Eming et al. 2014.[33]

The first phase is the response to tissue injury with hemostasis to stop the bleeding. The activation of the hemostasis cascade is necessary to prevent excessive blood loss as well as loss of fluid and electrolytes. As maintaining the barrier between the organism and the exterior is an essential task of the skin, this step is important. When cells of vessel walls, including vascular smooth muscle cells, adventitial fibroblasts and pericytes get damaged, they induce the cascade of the hemostasis: vasoconstriction, primary and secondary hemostasis.[37,38] This happens by tissue factor (TF) that is present in subendothelial tissue. When TF is exposed to blood, it binds plasma factor VIIa and this complex sets off enzymatic reactions resulting in clot formation.[39,40] When platelets become exposed to collagen by a lesion, they release clotting factors, platelet derived growth factor (PDGF) and tumor growth factor beta (TGF-β).[41–43] PDGF initiates chemotaxis of smooth muscle cells, neutrophils and of macrophages as well as the mitogenesis of fibroblasts. TGF-β signals macrophages to migrate in and to secrete fibroblast growth factor (FGF), PDGF, tumor necrosis factor alpha (TNF-α) and interleukin-1 (IL-1).[41] Together, this further boosts chemotaxis of fibroblasts and smooth muscle cells, and likewise, the modulation of collagen and its expression.[41] TGF-β induces the transcription of collagen, proteoglycans, and fibronectin genes to build up the matrix. Also, it reduces the secretion of proteases like the metalloproteases which decompose the ECM components as well as the stimulation of the tissue inhibitor of metalloprotease.[44] Early in the first 24 h, there is an increased epidermal expression of IL-1 β and IL-6 and a dermal expression of TGF-β1.[45] Meanwhile, inflammatory cells, fibroblasts and keratinocytes get activated. Neutrophils phagocytose bacteria and damaged tissue. They also release toxic granules as part of the immune system and they build neutrophil extracellular traps (NETs). By spreading chromatin filaments with cytosolic proteins and proteases, these NETs can catch and destroy infectious pathogens like bacteria, viruses and fungi.[46]

In the second phase, cellular proliferation and migration, matrix deposition and angiogenesis dominate. IL-1, secreted by fibroblasts and neutrophils, stimulates keratinocyte growth and fibroblast proliferation (Table 1).[41] Late in the second 24 h after injury, there is elevated epidermal expression of TGF-α, epidermal and dermal expression of IL-6 and suprabasal expression of K16 (a marker of keratinocyte hyperproliferation) and dermal expression of keratinocyte growth factor (KGF).[45] IL-6 induces the mitogenesis and motility of keratinocytes (Table 1). Keratinocytes roll or slide over one another which initializes reepithelialization.[45]
Migration continues toward the wound site by three possible involved mechanisms: leapfrog-like epidermal tongue, lamellipodial crawling and shuffling.[32,35,47] The concept of the leapfrog-like epidermal tongue describes that the keratinocytes directly next to the wound site migrate towards the wound site first. To be able to migrate, they loosen the cell-cell and cell-matrix adhesion to the basal lamina. This epidermal tongue of the first row is used by other

keratinocytes to advance and to reepithelialize the wound site.[48] Then, lamellipodial crawling supports the wound closure by pushing the cells out of migration-hindering material of the fibrin clot. This happens by reorganizing cell shape and again loosening adhesions so that keratinocytes can pass the front (shuffling).[47] When the keratinocytes arrive in the middle of the wound, the migration stops by contact inhibition and their contacts towards each other and the basal lamina gets firmed.[35,49] When the wound site is finally reepithelialized, keratinocyte migration stops, whereas mitosis and differentiation continue for two more weeks.[45] Of note, stem cells from the hair follicle bulge can contribute to the regeneration of the interfollicular epidermis.[50–52] In case of dysfunctional cells or regulation of the cellular interplay, stem cells can replace missing cells and take over their function.[52]

Table 1: List of selected growth factor–secreting cell types and the impact of the factors on cell behavior

Modified from Safferling et al. 2013.[53]

Cytokine	Secreted by	Effect	Literature
IL-1α	Fibroblasts, Neutrophils	Induction of KGF, FGF	Werner and Grose, 2003; Werner et al., 2007
IL-3	Keratinocytes, Basophils	Basophil activation	McKay and Leigh, 1991; Schroeder et al., 2009
IL-6	Keratinocytes, Fibroblasts, Macrophages	Keratinocyte mitogen, motogen	Werner and Grose, 2003; Werner et al., 2007; Grossman et al., 1989
IL-8	Keratinocytes, Fibroblasts, Polymorphonuclear cells	Keratinocyte mitogen, Fibroblast motogen	Werner and Grose, 2003; Rennekampff et al., 2000; Gilitzer and Goebeler, 2001; Boxman et al., 1996
MCP-1	Keratinocytes, Fibroblasts, Macrophages	Monocyte attractant	Werner and Grose, 2003; Yadav et al., 2010
G-CSF	Keratinocytes, Fibroblasts	Keratinocyte and Fibroblast mitogen, motogen	Kaushansky et al., 1988; Kawada et al., 1997; Mansbridge et al., 1999; Mueller and Fusenig, 1999

The effective epithelialization relies on the groundwork of the ECM to loosen cell anchorage.[30,37,54] This is accomplished by metalloproteases. One important metalloprotease is matrix metalloproteinase-1 (MMP-1) which loosens contacts between collagen fibers so that keratinocytes can migrate through the fibrin clot and the granulation tissue.[55–57] Keratinocytes themselves express metalloproteases when they are activated to reepithelialize the wound area.[57]

Furthermore, mast cells release enzymes after injury, which lead to inflammatory response around the wound site (rubor, tumor, calor, dolor and functio laesa). The mediators (histamine, serotonin, TNF, tryptase, e.g.) loosen the vessel layers so that mononuclear cells can pass easier and arrive at the wound site.[41,58–61] These mediators are important for stimulation of keratinocyte reepithelialization, like histamine,[62] for stimulation of fibroblast proliferation and collagen synthesis, like tryptase and histamine,[63,64] as well for degradation of ECM for remodeling.[37,65,66]

Monocytes, that become macrophages at around 48 h after injury, release PDGF and TGF-β to support the ongoing phagocytosis by attracting fibroblasts.[41,67] Macrophages arrive from new capillaries and loose connective tissue to establish granulation tissue.[68] They also synthesize metalloproteases degrading the ECM and fibrin clot for an effective migration of fibroblasts and keratinocytes.[69]

Angiogenesis plays an important role in wound healing. By generating new vessels, nutrient and oxygen supply can be guaranteed which is important for cellular functions.[70] An injury leads to hypoxia which activates the endothelial cells lining the blood vessels to degrade ECM but also to build new capillaries.[71,72] Consequently, several pro-angiogenic factors are produced. These include vascular endothelial cell growth factor (VEGF) and hypoxia-inducible factor-1 (HIF-1). This transcriptional activator induces the expression of VEGF-A genes and thereby promotes angiogenesis.[73] Especially inflammatory monocytes/macrophages secrete VEGF-A in the early phase which is later on performed by keratinocytes.[74] Stimulated by these factors, vascular growth occurs in the wound periphery and continues in the center of lesion.[71,75]

Finally, the third phase, the remodeling phase occurs. While angiogenesis is characterized by disorganized and immature capillaries in the proliferative phase, anti-angiogenic factors like pigment epithelium-derived factor (PEDF) and sprouty RTK signaling antagonist 2 (SPRY2) cause apoptosis and degrade no longer needed capillaries in the third and last phase of the wound healing process. Maturation factors help to stabilize the new vessels so that the capillary bed provides a sufficient perfusion.[76]

In addition, the wound site gets cleared of cell debris and bacteria to gain space for fibroblasts to migrate in and to produce collagen for a provisional ECM. To complete the repair, the provisional ECM needs to be remodeled. This includes constant synthesis and degradation of the ECM components by metalloproteases aiming to establish a new equilibrium.[77,78] The granulation tissue consists mainly of collagen III and gets replaced by collagen I.[70] Briefly, the new collagen matrix is organized and cross-linked. The N- and C-terminal peptides of the procollagen are cleaved to form the mature collagen. The enzyme lysyl oxidase stabilizes the collagen fibrils by cross-linking. Over time, the developed intra- and intermolecular collagen cross links become densely packed and provide strength to the ECM.[35,79] Over the following two weeks, the hemostasis and cellular activation normalizes.[45]

What happens when the wound healing process is not effective? As mentioned, deficient repair may follow tissue injury. Chronic wounds are lasting barrier defects resulting from interrupted or deregulated wound healing.[33] They often present a prolonged inflammation with an imbalance between inflammatory and anti-inflammatory signals. It seems like they are trapped in this state.[80–82] Chronic wounds present also elevated concentrations of IL-1β, TNF-α, MMPs, fibronectin, collagenase and gelatinases A and B. On the other hand, EGF, FGF, TGF-β, PDGF and VEGF concentration decrease. IL-6 and G-CSF are assumed to be diminished.[83–85] IL-1β and TNF-α increase MMPs which can disassemble the ECM and consequently cause chronic wounds.[83] Other reasons are neutrophil infiltration or associated reactive oxygen species. Healing is then only possible when inflammation subsides.[41] Especially advanced age, diabetes, vascular diseases, comprised nutritional and immunological status and comorbidities disturb the efficient wound healing process described above.[33] Non-healing wounds are not only a cosmetic but also a medical and global economic problem. An analysis for 2014 showed a total medical cost for the treatment of acute as well as chronic wounds (subdivided into several categories) of $28.1 billion to $96.8 billion.[9] Also, a study of *Guest et al*. underlined that the annual prevalence of wounds in the UK demonstrated an increase by 71% between 2012/2013 and 2017/2018.[86]

2.3 The hexosamine biosynthetic pathway

The hexosamine biosynthetic pathway (HBP) is an intracellular, ubiquitous pathway whose product, uridine 5'-diphospho-N-acetyl-D-glucosamine (UDP-GlcNAc), is needed for posttranslational modifications. Figure 5 shows that fructose-6-phosphate (Fru6P) is converted by the enzyme glutamine-fructose 6-phosphate amidotransferase (GFAT-1) into glucosamine 6-phosphate (GlcN6P). Thereby, L-glutamine is used and L-glutamate gets released.[87] Adding Acetyl-CoA, GlcN6P reacts to form N-acetyl-D-glucosamine-6-phosphate (GlcNAc6P) via glucosamine-6-phosphate N-acetyltransferase (GNA-1).

Phosphoglucomutase (PGM-3) converts GlcNAc6P to N-acetyl-D-glucosamine-1-phosphate (GlcNAc1P). The next enzyme, UDP-N-acetylglucosamine pyrophosphorylase (UAP-1), uses UTP to activate GlcNAc1P by phosphorylation to UDP-GlcNAc.[1]

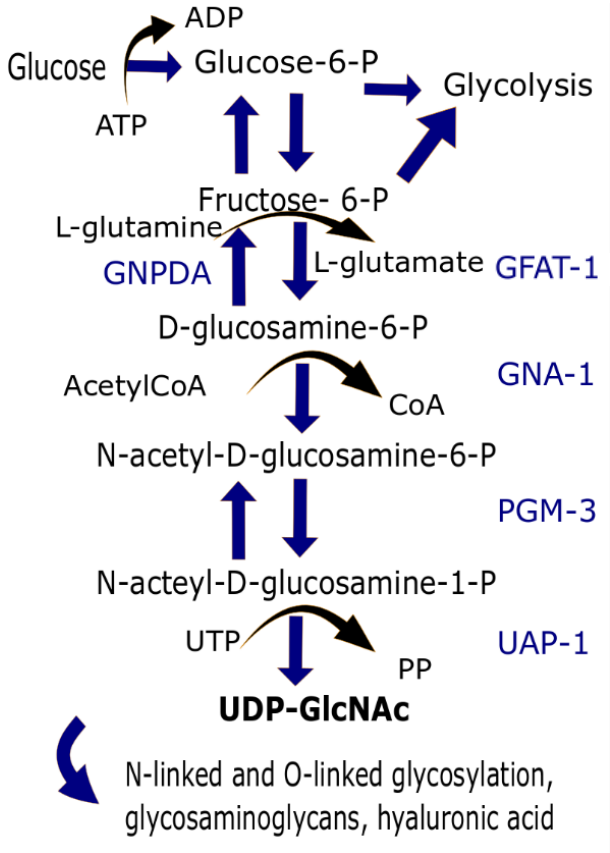


Figure 5: The hexosamine biosynthetic pathway

Glucose gets shunted into the HBP. D-glucose-6-phosphate (Glc6P) and fructose-6-phosphate (Fru6P) are converted by the enzyme glutamine-fructose 6-phosphate amidotransferase 1 (GFAT-1) into glucosamine 6-phosphate (GlcN6P). GNPDA can dose-dependently reverse this reaction backwards. The next step requires AcetylCoA to react to N-acetyl-D-glucosamine-6-phosphate (GlcNAc6P). Phosphoglucomutase (PGM-3) converts GlcNAc6P to N-acetyl-D-glucosamine-1-phosphate (GlcNAc1P). Adding UTP, UDP-N-acetylglucosamine (UDP-GlcNAc) origins, which is used for posttranslational modifications. Modified from Denzel et al. 2014.[2]

GFAT-1 is the key enzyme of the HBP which is under negative feedback regulation by UDP-GlcNAc.[88–90] It can itself be inhibited by phosphorylation by the energy-sensing AMP-activated protein kinase (AMPK).[91]

Uridine 5'-diphospho-N-acetyl-D-galactosamine (UDP-GalNAc) is also an acetylated amino sugar which is required for mucin type O-glycosylation and which is therefore important for posttranslational modifications including the regulation of protein processing and folding. The

epimerase GALE-1 converts UDP-GlcNAc to UDP-GalNAc.[92] Both, UDP-GalNAc and UDP-GlcNAc pools are taken together as uridine 5'-diphospho-N-acetyl-D-hexosamine (UDP-HexNAc). Figure 6 depicts the structure of the substrates GlcNAc and UDP-GlcNAc.[93,94]

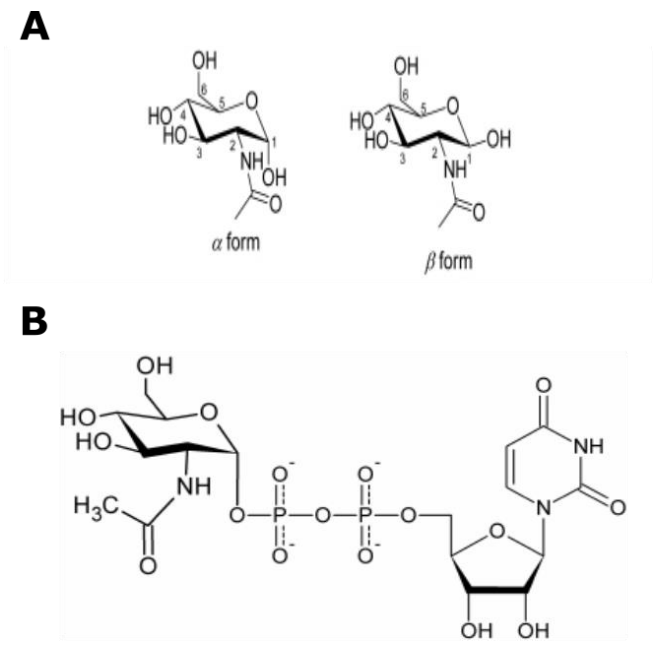


Figure 6: The structure of N-acetyl-D-glucosamine (GlcNAc) and UDP-N-acetyl-D-glucosamine (UDP-GlcNAc)

A *Structure of N-acetyl-D-glucosamine (GlcNAc). Modified from Liu et al. 2011.*[93]

B *Structure of UDP-GlcNAc. Cited from Held et al. 2007.*[94]

Figure 5 demonstrates the tight connection of the HBP with glycolysis. Research has shown that HBP flux reflects the cell's energy availability and this dependence might have pro-survival function. A study observed that ischemia and reperfusion stress induces O-GlcNAcylation in mouse heart and acts protectively regarding the survival of cardiomyocytes.[95]

The amino sugars are needed for glycosylation reactions such as N-glycosylation in the endoplasmic reticulum (ER) and O-GlcNAcylation in the cytoplasm and in the nucleus. Protein folding, maturation and posttranslational modifications require protein glycosylation. Thus, the HBP is indispensable to keep up a functional proteome.

The ER is the site of protein synthesis and maturation of membrane proteins. As a checkpoint, it controls the quality of protein folding and reacts to disruption in protein quality control.[96]

N-glycosylation in the ER provides structure and keeps up the function of cell surfaces. It also gives structure to secreted proteins and plays a part in the protein maturation and signaling for

protein folding in the ER.[97,98] Protein processing and folding in the Golgi apparatus depend on O-linked glycosylation.[92] O-GlcNAcylation has furthermore regulatory functions on proteins.[99] Overall, HBP activity influences protein folding and processing as well as protein function via different types of glycosylation reactions.

Interestingly, it was shown that HBP activation leads to lifespan extension and improved protein quality control in the nematode *Caenorhabditis elegans* (*C. elegans*).[2] Gain-of-function mutations in GFAT-1, which result in elevated UDP-GlcNAc production, activate ER-associated degradation (ERAD). ERAD, in turn, influences proteasome activity and autophagy. This improves protein quality control and ensures alleviation of proteotoxicity of e.g. polyglutamine peptides or amyloid beta.[2] Unfolded protein stress triggers a signaling cascade of the ER, the unfolded protein response (UPR), which restores physiological cell function by inhibiting translation, by degrading misfolded proteins, and by inducing cell apoptosis when repair seems inefficient.[100]

To sum up, the HBP is a longevity and stress response pathway, which presents itself as a favorable target for research regarding aging and age-related diseases. As UDP-HexNAc is indispensable for regulating different processes, an elevation could lead to changes in glycosylation, substrate modulation and in ECM structure and function, which is described in detail below.

2.4 The extracellular matrix

The extracellular matrix (ECM) is found in almost all tissues, but is particularly abundant in bone, cartilage, and loose connective tissue. As there are differences in structure and function of these tissues, the composition of the ECM is adapted, especially regarding the collagen and elastin concentration.[101] The ECM consists mainly of interstitial matrix and structural proteins like collagen and elastin, adhesive glycoproteins including fibronectin and laminin, as well as glycosaminoglycans (GAGs) (Figure 7).[102–104]

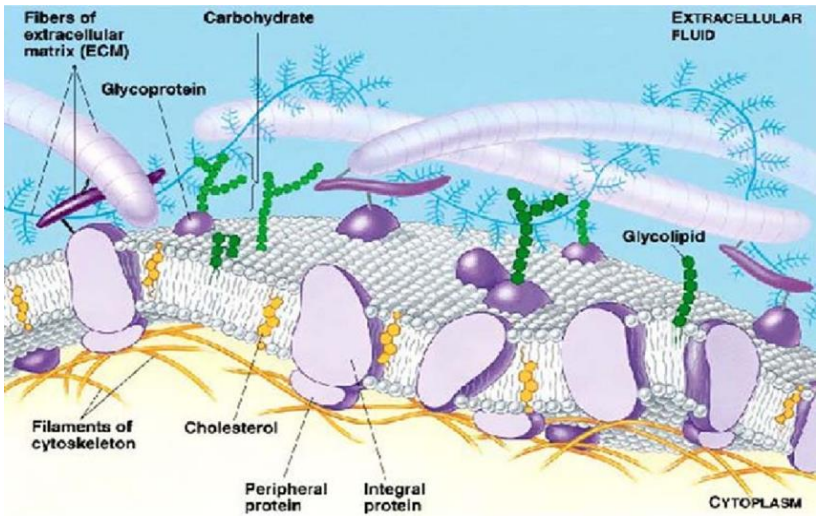


Figure 7: The extracellular matrix of the skin

It consists mainly of structural proteins (collagen, elastin), adhesive glycoproteins (fibronectin, laminin) and proteoglycans (heparan sulfate, chondroitin sulfate).
Cited from Mishra. 2015.[104]

GAGs are long linear disaccharide chains that contain acetylated amino sugars (Figure 8) which are generated in the HBP (as described in 2. 3.).[105] All GAGs are bound to proteins, expect for hyaluronic acid (HA).

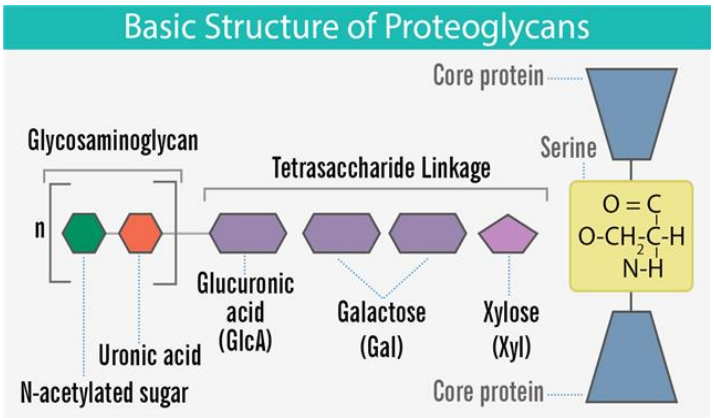


Figure 8: The structure of proteoglycans

Proteoglycans consists of a core protein, tetrasaccharide linkage and glycosaminoglycans. Cited from https://biologywise.com/structure-function-of-proteoglycans (accessed Feb 20, 2022).[105]

HA is a non-sulfated, linear GAG of high molecular weight (379.32 g/mol; 2,28 x 10 [26] Da) (Figure 9).[106] It consists of repeating units of (b,1-4) glucuronic acid and (b, 1-3)-N-acetyl glucosamine (Figure 10).[107]

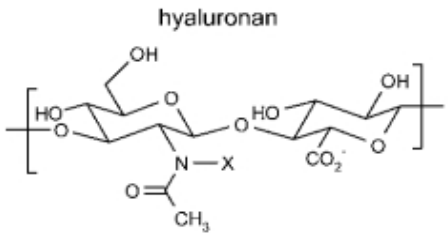


Figure 9: The structure of hyaluronic acid

X=H, native glycosaminoglycan.

Cited from Rees et al. 2004.[106]

HA is a component of most forms of ECM, not just in the skin, but also in the brain and in the central nervous system.[108] It is also found on the surface of many cell types. About half of the total HA, though, is located in the skin.[109–112] As a structural component of the ECM, it plays an important role in wound healing, inflammation, and embryonic development. HA gives structure as it is fundamental for the viscoelasticity of joint synovial fluid and the arrangement of cartilage.[108,113,114]

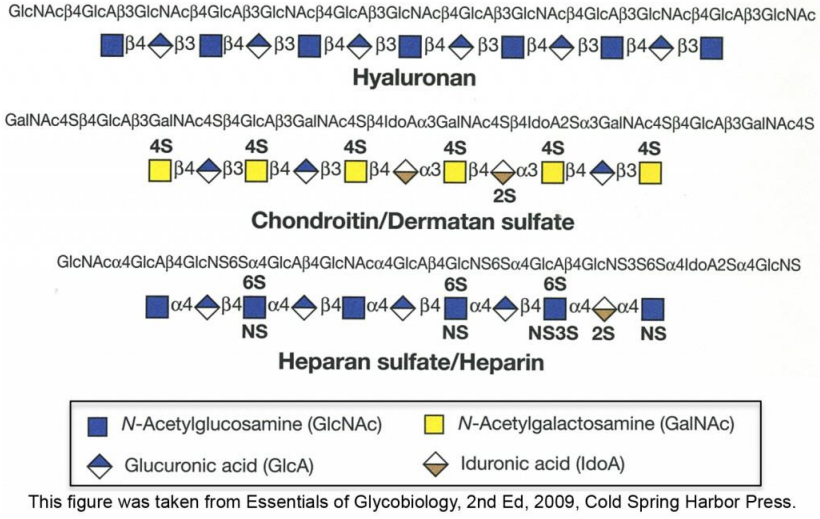


Figure 10: The hyaluronic acid in connection with UDP-HexNAc

The figure shows that GlcNAc is an essential component of HA.

Cited from Varki et al. 2009.[107]

HA is used clinically as a lubricating agent in osteoarthritis treatment, ocular surgery, tissue augmentation and as a template for tissue engineering, and to prevent adhesions in abdominal surgery.[115,116]

Moreover, topically applied, an acceleration of wound healing in mice and rat studies were observed.[5] HA's hydrophilic and highly osmotic features are keystones in the wound healing process as they help loosen cell attachment to the ECM, making it easier for cells to migrate.[117] HA influences cell migration and proliferation by interaction with receptors and activating signaling cascades.[118,119] HA levels are higher in normal healing wounds compared to pressure ulcers.[120] This all highlights its importance in the complex process of wound healing.

It was shown that high and low-molecular weight HA molecules have different functions in the wound healing process. Whereas the high-molecular weight hyaluronan (HMW HA, 1400 kDa, used in their experiment) is indispensable for modulating the ECM, the low-molecular weight hyaluronan (LMW HA, 35-280 kDa) induces inflammatory processes.[4] Right after the injury, a sharp increase in HMW HA is seen. HMW HA weakens cell attachment to the ECM so that leukocytes can easier access the wound to remove the damaged tissue, debris and bacteria. At the same time, nutritional supplies can be transported to the wound site. An increase of LMW HA is a starting signal for early inflammatory response.[4,121] It induces cytokine release as TNF-α, IL-1b and IL-8 stimulating cell migration and proliferation and angiogenesis.[5,121,122]

To sum up, the skin ECM plays an important role in many cellular functions: it provides the mechanical properties and tissue integrity. In terms of mechanical properties, it gives stability, elasticity, resistance to compression and cell anchorage.[11,102] In addition, it controls cell behavior regarding maintenance, growth, and differentiation through mechanical and chemical signals.[11,33] The ECM was ignored as irrelevant for a long time, but research has begun to discover its role and the interactions within the ECM. Nowadays, we find ECM-derived products, mostly collagen- and fibrin-based products in clinical and scientific use regarding tissue repair, for example in skin, heart valves, trachea, muscle and tendon.[123,124] These studies expose the complexity of the three-dimensional ECM and its various functions that influence wound healing but also the importance of the ECM in skin homeostasis.

2.5 Research questions and aims

Fibrous proteins (such as collagen, elastin, fibronectin, laminin) and proteoglycans are proteins which are conjugated to GAG chains and which are major components of the ECM that interact with each other and that regulate the ECM.[102,125,126] Among GAGs, there are high levels of HA.[8] Right after injury, HA shows an increase and is present in all phases of wound healing in which it modulates several pro-healing functions.[4–7] Effective wound healing can only occur if the complex system of cells, ECM and mediators work neatly in tight communication.

HBP activation has a positive effect on protein quality control and therefore also on stress resistance as shown in *C. elegans.*[2] Interestingly, it was also observed that the production of GAGs is stimulated by HBP activation.[127–129] As the essential role of the skin ECM in wound healing is more and more unraveled, it is promising to test the effect of HBP activation on ECM function. I hypothesize that an activated HBP that influences the ECM could also modify wound healing. Research on wound healing is of tremendous interest regarding the increasing global problem of chronic wounds.[10] New and effective therapeutic strategies are required to cope non-healing wounds. Therefore, my aim is to investigate the role of HBP activation in wound healing by performing wound healing assays *in vitro*, using both immortalized (NIH3T3, HaCaT cells) as well as primary murine fibroblasts and keratinocytes.

The second part of the project focuses on the effect of the HBP on cell proliferation. Cell proliferation is needed for wound healing and mainly takes part in the proliferative phase.[33] Being the major cell type in the epidermis, keratinocytes require effective migration and proliferation for reepithelialization. If functional deficiency or dysregulation occurs, it can lead to chronic wounds.[130] Again, the ECM is directly related to the regulation of cell proliferation through mechanical and chemical signals affecting fibroblasts, keratinocytes, and other cells involved in wound repair.[11] Since the ECM plays a pivotal role in cell proliferation and this feature of keratinocytes is strongly needed in wound healing, I hypothesized that HBP activation results in increased proliferative potential of primary murine keratinocytes.

3. Materials and Methods

3.1 Material

3.1.1. Cell culture

substance/material	information	Provider
Dulbecco's modified eagle medium (DMEM)	high glucose (4.5 g/l) + FCS (10% or 1%) + penicillin (1%) + streptomycin (1%)	Gibco
fetal calf serum (FCS)	10% or 1%, 10270-106	Gibco
penicillin, streptomycin	P4333, 100 ml, lot 067M4819V	Sigma-Aldrich
Dulbecco's phosphate-buffered saline (DPBS)	without calcium or magnesium	Gibco
5% trypsin-EDTA (10x)	1x → 0.5%, 10x: trypsin 20%, PBS 80% (40 ml), ref. 15400-054, 40 ml	Gibco
2.5% trypsin (10x)	1x → 0.25%, ref. 15090-046, 100 ml	Gibco
cell culture dish	PS, 100x20 mm, with vents, sterile	Greiner Bio-one
tissue culture dish	with 20 mm grid, 150x25 mm	Falcon, Corning incorporated
falcon tubes	a) 50 ml, 114x28 mm, PP b) 15 ml, 120x17mm, PP	Sarstedt
incubator	HERAcell 240, CO_2 incubator	Thermo Fisher Scientific
bead bath		Labarmor
vortex	Vortexgenie2	Scientific Industries
counting chamber	0.1 mm depth, 0.0025 mm^2	Neubauer
freezing medium	1 ml DMSO (10%) (lot. SZBF2310V) + 4 ml FCS (40%) + 5 ml DMEM (50%)	Sigma-Aldrich
CryoTube vials		Thermo Fisher Scientific
DMEM/Ham's F12	3.5:1.1, low calcium (0.05 mM Ca2+) 460 ml DMEM (2/3) HAM's F 12 (1/3) 50 ml FCS gold, chelated 10 ml supplement Z (penicillin + streptomycin, glutamine ascorbic acid) 50 µl EGF 550 µl insulin 750 µl adenine 5 µl cholera toxin	custom made by Biochrom, Berlin final concentration 1x 1x 5 ng/ml 10 ng/ml 5 µg/ml $0.27*10^{-4}$ M 10 ng/ml

	50 µl hydrocortisone	0.5 µg/ml
FCS Gold	50 ml FCS + 2 g Chelex® (100 Resin, 500 g, 100-200 mesh, sodium form)	Bio-Rad
adenine	BioReagent, suitable for cell culture	Sigma-Aldrich
insulin	recombinant, expressed in yeast, y-irradiated	Sigma-Aldrich
cholera toxin	from Vibrio cholerae	Sigma-Aldrich
epidermal growth factor (EGF)	recombinant, expressed in E. coli	Sigma-Aldrich
hydrocortisone	50 µM, sterile-filtered	Sigma-Aldrich
collagen G	approx. 4 mg/ml, from bovine calf skin, Cat.-No. L7213 100 ml 100 ml PBS 750 µl Collagen G	Biochrom
TS Nalgen Filter	TS Nalgen Filtration Products Filter	TS Nalgen

3.1.2. Cell viability assay (XTT)

substance/material in addition	information	provider
cell proliferation Kit II (XTT)	REF11465015001	Roche

3.1.3. Wound healing assay

substance/material in addition	Information	provider
hyaluronic acid	Lot 433363; 100 mg; > 950 kDa. This lot has a mass of 1.60×10^6 Da.	RD systems, USA
25 Culture-Inserts 2 Well for self-insertion	80209	Ibidi
mitomycin C	needed 4 µg/ml, for a volume of 70 µl: 400 µg/ml → 1:100 4 µg/ml so 0.7 µl	Sigma-Aldrich
N-acetyl-D-glucosamine (1 M in DMEM HG 10% or 1% FCS or in FAD)	A8625, 25 g	Sigma-Aldrich
dextran (35.000-45.000 mol wt), dextran (1.500.000-2.800.000 mol wt)	D1662-10G, D from Leuconostoc mesenteroides D5376-100G	Sigma-Aldrich

3.1.4. Liquid chromatography–mass spectrometry (LC-MS)

substance/material	information	provider

in addition		
nuclease free H_2O	AM9937	Ambion
Pierce BCA protein assay kit		Thermo Fisher Scientific
chloroform: methanol (1:2) mix	chloroform MKBX7489V methanol STBG0971V	Sigma-Aldrich
glass vial	vial short thread, 1.5 ml cl. glass + label	VWR
GeneVac EZ-2 Series Personal Evaporator		GeneVac

3.1.5. HA quantification ELISA

substance/material in addition	information	provider
Radio Immuno Precipitation Assay (RIPA) +/+ buffer	cOmplete™, EDTA-free Protease Inhibitor Cocktail Tablets, 1183617001 stock solution - 1 tablet - 10 ml PBS	Roche
Quantikine® ELISA hyaluronan, DHYALO		R&D systems

3.1.6. Isolation of primary skin cells of murine skin

substance/material in addition	information	provider
betaisodona	povidon iodine, solution	Mundipharma GmbH
octenidine dihydrochloride		Ambeed
antibiotic antimycotic solution (AA)	with 10. 000 units penicillin, 10 mg streptomycin and 25 µg amphotericin B per mL, sterile filtered	Sigma-Aldrich
Dispase® II protease	Dispase® II, D4693-1G	Sigma-Aldrich
Gibco TrypLE Express (trypsin)	0.25% trypsin without EDTA with Dispase at a 1:1 ratio, Cat. No. 12604-013, 100 ml	Thermo Fisher Scientific
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid, N-(2-Hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid)	Sigma-Aldrich
NaCl	powder	Sigma-Aldrich
collagenase	concentration 0.25%, 0.125 g of collagenase type I to 50 ml of FAD−Ca basal medium C0130-500MG	Sigma-Aldrich
70 µm cell strainer	Ref. 352 350	Falcon

3.1.7. Colony formation assay

substance/material in addition	information	provider
D-mannose	(1 M in FAD), 25 g, M8574	Sigma-Aldrich
D-glucose	(1 M in FAD), 1 g, X997.2	Carl Roth
N-acetyl-D-glucosamine	(1 M in FAD), 25 g, A8625,	Sigma- Aldrich
crystal violet solution	1% 99% PBS	Sigma-Aldrich
6 well cell culture plates	Cellstar, Cat. No. 657 160	Falcon
paraformaldehyde (PFA)	4% 96% PBS	Sigma-Aldrich

3.2 Methods

3.2.1. Cell culture

NIH3T3 cells, immortalized cell line from mouse embryonic fibroblasts,[131] and primary fibroblasts were cultured in DMEM at 37°C and 5% CO_2 on non-coated cell culture dishes of 10 cm (Greiner Bio-One) and of 15 cm diameter (Falcon). For passaging, media was removed, and cells were washed once with PBS before adding 2 ml trypsin (0.25%) for 10 cm or 4 ml trypsin (0.25%) for 15 cm plates. After incubating the cells at 37°C for 1-2 minutes, the cells were collected with 7 ml DMEM, transferred into a Falcon tube and centrifuged at 900 rpm for 5 min. Then the supernatant was taken off, resuspended in 10 ml of fresh medium and seeded on fresh plates, depending on the needs.

HaCaT cells, an immortal cell line of human adult keratinocytes, were cultured the same.[132,133] Yet, 2 ml trypsin 0.5% were used for trypsinization, and incubating took 10-15 min before the reaction was stopped.

Primary keratinocytes were cultured in FAD at 32°C, 5% CO_2, on collagen G coated 15 cm cell culture dishes (Falcon). For passaging, media was removed, and cells were washed once with PBS before adding 2 ml trypsin 0.5%. After incubating the cells at 32°C for 10-15 minutes, the cells were collected with 4 ml FAD, then the cell suspension was centrifuged in a Falcon tube for 4 min at 900 rpm, the supernatant was discarded, resuspended in 10 ml FAD and seeded on fresh plates, depending on the needs.

Freezing requires the same steps. After trypsinizing and resuspending in fresh DMEM, cells were centrifuged for 4 min at 900 rpm. Then, the supernatant was discarded, and the pellet had to be resuspended in 1 ml freezing medium and had to be transferred into a Cryotube Vial (Thermo Fisher Scientific), which should be transported fast in a freezing container because of the DMSO in the freezing medium. The cells need to be stored at -80°C. Vials are transferred to liquid nitrogen if long-term storage is envisaged.

The cell thawing also must be performed quickly. The cell suspension is transferred into a falcon which contains warm medium. Then, the cells are seeded onto a plate with fresh medium.

3.2.2. Cell viability assay (XTT)

The XTT assay (Roche) was performed to assess the efficiency of mitomycin C as a proliferation inhibitor by measuring relative cell viability. NIH3T3 cells were seeded out at a density of

20.000 cells/ml each on six 96-well plates and were incubated with mitomycin C either for 4 h or for 24 h. Cell viability was measured at 24 h, 48 h and 72 h by following the protocol of the cell proliferation Kit II (Roche) using a plate reader (SparkControl). Each treatment was tested once with control and treatment group (n=1).

3.2.3. Wound healing assay

The experiment was performed as illustrated in Figure 11. Cells were seeded into Culture-Inserts 2 Well for self-insertion (ibidi) on 24 well plate (falcon) at certain densities: a) NIH3T3 50.000 cells/insert half, b) primary fibroblasts 35.000 cells/insert half, c) HaCaT 30.000 cells/insert half, d) primary keratinocytes 100.000 cells/well as the wells had to be treated with collagen G as a substrate for adherent cells at least 30 min before. 1 ml of the cell specified medium was added around the inserts or in the wells. After 4 h incubation at 37°C or 32°C for the primary keratinocytes, cells were treated with 4 µg/ml mitomycin C. The next day, inserts were removed with a disinfected tweezer, detached cells and debris were washed away with PBS and 1 ml DMEM 10% FCS (for NIH3T3 and primary keratinocytes) or 1% FCS (for HaCaT) was added. Cells were treated with 50 mM GlcNAc while GlcNAc was omitted in control wells. When using 1% HA, the controls got 0.5 ml medium, and the HA wells 0.5 ml of 1% HA in DMEM 1% FCS aliquots.

For the dose response assays, 10 mM, 20 mM, 30 mM, 40 mM or 50 mM GlcNAc (stock of 1 M in DMEM 1% FCS) were added at this step.

For 1 M GlcNAc, 2 g of N-acetyl-D-glucosamine (molecular weight 221.21 mol/g) were solved in 9 ml DMEM 10% or 1% FCS. 1 ml aliquots were stored at -20°C.

For 1% HA, 100 mg hyaluronic acid were solved in 10 ml DMEM 1% FCS. Aliquots were stored at -20°C and thawed at 65°C in shaker for around 60 min.

For the assays with dextran, I used dextran 35.000-45.000 mol wt., dextran 1.500.000-2.800.000 mol wt. (Sigma). For both, aliquots of 1% were prepared in DMEM 1% FCS and stored at -20°C. For the higher mol wt. dextran, also 5% and 10% were prepared.

Images were taken every hour by EVOS FL Auto 2 (Thermo Fisher Scientific), an inverted microscope, with a 4x objective (4x PLAN/0.13NA long working distance objective) by the EVOS software's 'Scan' function over a period of 72 h to observe and evaluate wound closure. This inverted optical microscope is equipped with a monochrome camera (high-sensitivity 1,3MP CMOS, 1.328x1.048 pixels) and a stage incubator (CO_2, temperature and air control) which supplies the required environmental conditions for the examined cell cultures.

The location to be imaged was selected by the criteria of accurate wound edges and cell free wound gaps. For analyzing purpose we focused on the wound gaps so that we set the scan "Center Region Only", which saved 4x3 tiled images of the center of each well, like described in *Klimaj et al.*[134] Triplicates were performed for each wound healing assay.

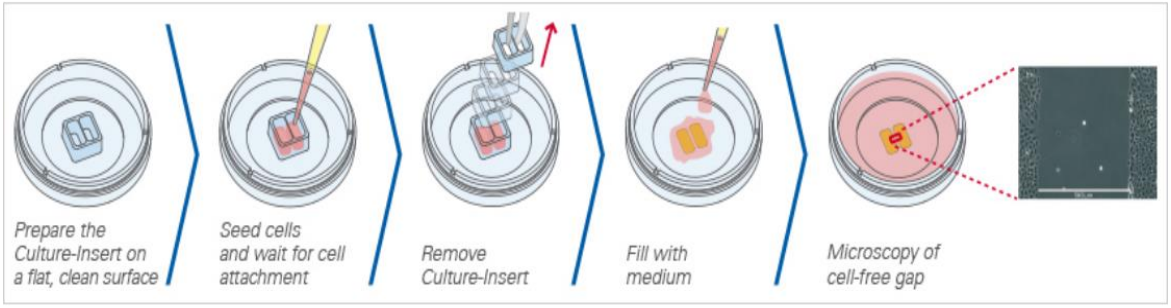


Figure 11: Schematic representation of wound healing assays using culture-Inserts

A *The cells were seeded into the pockets of the insert and treated with mitomycin C. On the next day, the inserts were removed and thereby the wound gap was created. The medium was renewed. 50 mM GlcNAc was additionally given to the test group. The cell migration was analyzed using an inverted microscope. (Cited from https://ibidi.com/removable-chambers/25-25-culture-inserts-2-well-for-self-insertion.html.(accessed Aug, 23, 2020).*[135]

B *Images of the wound closure were taken every hour over a period of 72 h by EVOS FL Auto 2 at 4x magnification. This inverted microscope contains a high sensitivity monochrome camera. The plate lays within an incubator which provides a physiological environment to the cells regarding the temperature, the CO_2 concentration and the humidity.*

Photograph taken by A. Gronostay

3.2.4. Liquid chromatography–mass spectrometry (LC-MS)

For measuring the metabolic levels of GlcNAc and UDP-HexNAc, which stands for both UDP-GlcNAc and UDP-GalNAc pools, as a control of HBP activation, liquid chromatography-mass spectrometry (LC-MS) was used. For preparation of samples a) 500.000 NIH3T3 cells, b) 750.000 primary fibroblasts, c) 500.000 HaCaT cells, d) 750.000 primary keratinocytes were each plated into a 10 cm cell culture dish (Greiner Bio-One). 24 h after seeding, cells were either left untreated or treated with 10 mM or 50 mM GlcNAc and collected 24 h later. 2 ml 1x trypsin were added to cells for trypsinization. After 2 min (or 20 min for keratinocytes) incubation at 37°C (or 32°C), 4 ml DMEM (or FAD) was added to stop the reaction. Cells were centrifuged, washed once with 5 ml PBS and again centrifuged before the supernatant was discarded. Until further processing, cells were stored at -20°C.

After thawing the cell pellets and adding 250 µl nuclease free water (Ambion) per tube, they were vortexed and frozen in liquid N_2 and thawed in a water bath at 37°C. These three steps had to be repeated three times with thorough vortexing in between. The Pierce BCA Protein Assay Kit (Thermo Fisher) was used to measure protein concentrations.

All samples were adjusted to 100 µg/ml (diluted in nuclease free H_2O to a final volume of 200 µl). 1 ml chloroform: methanol (1:2) mix was added to each sample. They were incubated for at least 1 h at room temperature (RT) on a nutator mixer. After centrifugation at full speed for 5 min, the supernatant was transferred into a glass vial. GeneVac EZ-2 Series Personal Evaporator performed the vaporization of the liquid for 4 h (1003 mbar/30°C). Absolute UDP-HexNAc levels were measured using an Acquity UPLC (Waters) connected to a Xevo TQ Mass Spectrometer (Waters). The Metabolomics Core Facility of the Max Planck Institute for Biology of Ageing (Cologne, Germany) performed the spectrometric measurements and the final analysis of GlcNAc and UDP-HexNAc concentration, as previously described by *Denzel et al.*[2] Triplicates were performed for each condition (n=3).

3.2.5. HA quantification ELISA

In order to measure the HA levels in the samples, cells were collected as for LC-MS and treated on day 2 as mentioned above. On day 3, 20 h after treatment, medium was removed, the plate was washed once with PBS and 10 ml pure DMEM 4.5 g/l glucose (or pure FAD for primary keratinocytes) were added. Four hours later, five times 1 ml of the medium was collected in Eppendorf tubes. The rest of the medium on the plates was removed, the plates washed once with 10 ml PBS and 1 ml Radio Immuno Precipitation Assay (RIPA) buffer was added. RIPA buffer is a reagent for cell lysis, so that intracellular protein can be measured. The plates were then placed on ice for 10 min. Then, 1 ml of the protein extract was collected in one Eppendorf tube for each plate. The samples were stored at -80°C until further processing.

First, the protein concentrations of the samples were measured from the Eppendorf tube containing the sample in RIPA buffer via Pierce BCA Protein Assay Kit (Thermo Fisher).
The ELISA measurement was performed as recommended from Quantikine® ELISA hyaluronan (R&D systems). The dilution for the samples was performed as following: NIH3T3 control 1:5, 10 mM GlcNAc 1:5, 50 mM GlcNAc 1:10, primary fibroblasts control 1:50, 10 mM GlcNAc 1:50, 50 mM GlcNAc 1:100. Three biological replicates were performed for each condition (n=3). HaCaT control 1:10, 10 mM GlcNAc 1:20, 50 mM GlcNAc 1:50, primary keratinocytes control 1:2, 10 mM GlcNAc 1:5, 50 mM GlcNAc 1:10. Two biological replicates were used for analysis (n=2).

3.2.6. Isolation of primary skin cells of murine skin

Primary skin cells were isolated from newborn wild type mice according to *Jensen et al.*[136] Animals were housed and maintained according to FELASA guidelines in the animal facility of the Max Planck Institute for Biology of Ageing, Cologne, Germany. All experiments were approved by local authorities.
The mice were sacrificed and put them in a disinfection line: 10 ml betaisodona +10 ml PBS (betadine 10%), PBS, PBS, octenidine dihydrochloride, PBS, 70% EtOH, PBS, PBS, PBS+ antibiotic antimycotic solution (AA) 1:50. The skin was separated from the body with two tweezers and transferred to an Eppendorf tube containing 2 ml dispase solution (5 mg/ml). Dispase is a protease that separates the epidermis from the dermis. After incubating at 4°C for around 15 h, the epidermis was separated from the dermis and the two skin layers were treated separately. The epidermis was transferred with the basal side down on a 0.25% TrypLE drop on a dish culture plate and left incubating for 20-30 min at RT. The cells were washed off with medium and collected in a 15 ml Falcon. The next steps were centrifugation for 4 min at 900 rpm, discarding supernatant and resuspending in 10 ml medium. Then the cell mix was transferred in medium on collagen G coated plates (at least 30 min) and incubated at 32°C and 5% CO_2.
The dermis was cut with two scalpels into small pieces and transferred in a 15 ml Falcon with 4 ml collagenase (400 U/ml) which was put in a bead bath (37°C) for 1.5 h by shaking it occasionally. Afterwards, the cell suspension was pipetted through a 70 µm cell strainer in a 50 ml Falcon and the strainer was washed with 8 ml DMEM. After centrifugation for 10 min at 1.000 rpm at RT, the supernatant had to be discarded and the pellet was resuspended in 10 ml DMEM before transferring it on a cell culture dish, which was incubated at 37°C and 5% CO_2 until further processing.

3.2.7. Colony formation assay

These assays were used as a read-out for proliferative potential of primary keratinocytes. The protocol by *Chacón-Martínez et al.* was adapted.[137]

Either immortalized or primary murine fibroblasts provided the feeder layer for primary murine keratinocytes. Figure 12 illustrates the steps of the experiment. First, 2 ml of the fibroblasts DMEM cell mix (either 150.000 cells/well NIH3T3 or 100.000 cells/well primary fibroblasts) were seeded in 6-well plates (Greiner). When the cell confluency reached 100%, the cells were treated with 4 µg/ml mitomycin C. The next day, primary keratinocytes were added on top at a density of 4.000 cells/well. As the primary keratinocytes need a different medium, DMEM had to be removed, the wells had to be washed twice with PBS and then 2 ml of the cell-FAD mix was added per well. One plate was taken for one of the three sugar treatments, having three technical replicates per plate. The first row was always the untreated control and the second either 50 mM GlcNAc, 50 mM glucose or 50 mM mannose. Every other day, the medium was changed and the supplemented treatment repeated until colonies emerged. This varied from 10 to 14 days. Next, the fibroblasts were washed off the plates by removing the medium, washing once with PBS, adding 0.2 ml trypsin and incubating for 2-3 min. The reaction was stopped by 1 ml medium and washing twice with PBS to remove the fibroblasts. In the safety cabinet, 1 ml paraformaldehyde (PFA, 4% in PBS) was added and incubated for 15 min. Afterwards, PFA was carefully removed, washing twice with 2 ml PBS. For the staining, 1 ml 1% crystal violet/PBS was added and the plates were put on a shaker for 1 h. The color was washed away with tap water. After letting the plates dry, images were taken by EVOS using the 4x objective and colonies were counted by hand (Figure 11B). The actual colony number was set relative to the highest colony number in the control (for each test group per plate n=3) and the averages from each condition per plate. Six biological replicates were performed for NIH3T3 cells as feeder layer and three for primary fibroblasts.

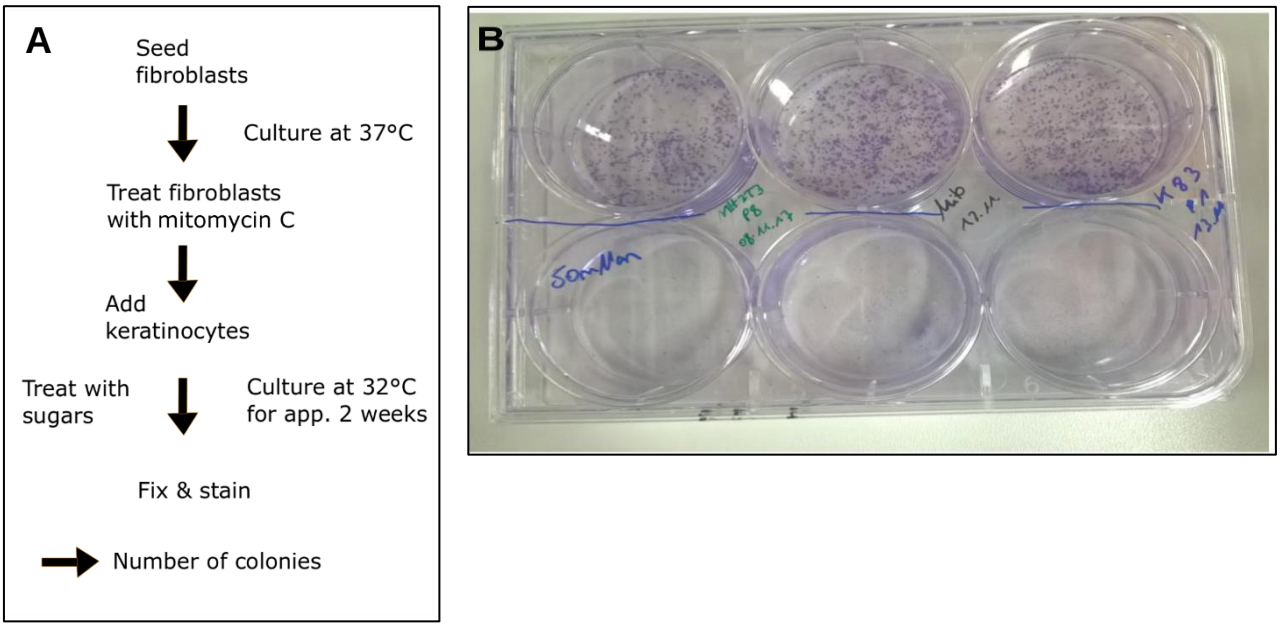


Figure 12: Steps for colony formation assay

A *Schematic workflow of colony formation assay.*

B *One representative picture of control group (upper row) and mannose treatment (lower row) of NIH3T3 feeder layer. Photograph taken by A. Gronostay*

3.2.8. Statistics

The statistical analyses were performed using GraphPad Prism Software (GraphPad, version 5.0f). Statistical significance was determined by repeated measures ANOVA with Dunnett's post-test as indicated in the corresponding figure legends.

4. Results

4.1 The role of HBP activation in wound healing

The first part of the project focused on the question whether HBP activation by GlcNAc supplementation can influence wound healing *in vitro*. Therefore, wound healing assays in fibroblasts and keratinocytes were performed.

4.1.1. Cells treated with mitomycin C with 24 h pre-incubation and cultured in 10% FCS showed the best conditions for further experiments

First, the best conditions had to be established for the cell culture. In order to exclude cell proliferation to investigate migration only, mitomycin C treatment, which inhibits cell growth, was used. Mitomycin C is an antibiotic produced by *Streptomyces caespitosus* that causes cross-linking of DNA and inhibition of DNA synthesis. Cell viability assays (XTT) tested the efficiency, which are colorimetric assays for the nonradioactive quantification of cellular proliferation, viability, and cytotoxicity.

The control and the treated group (4 h pre-incubation treatment) showed an absorbance of around 0.5 after 24 h (Figure 13A). The absorbance increased at 48 h for both groups (1.1 vs. 0.7), which indicates that cell proliferation was still ongoing. At 72 h, the absorbance for the treated cell population did not change (0.7), whereas the absorbance of the control group raised to 1.4 (linear slope). In conclusion, the inhibitor decelerated cell proliferation in the treated cell population by around 50% after 72 h. Yet, the cells were still capable to proliferate diligently for one day.

Therefore, as the focus of the wound healing assays should stay on cell migration, the XTT viability assay was repeated with a 24 h pre-incubation treatment of mitomycin C (Figure 13B). The control group showed a slightly lower absorbance (0.4) compared to Figure 13A. The absorbance of the treated cells (24 h pre-incubation treatment) was lower than in the 4 h XTT assay (0.1) and also the difference to the control cells increased. The difference in the absorbance of the control cells reflects biological variability. Despite the same number of cells seeded, a cell damage during the steps of the experiment cannot be excluded and the control group of 24 h XTT assay therefore started with a lower proliferation level. Nevertheless, the control group showed a linear growth: the absorbance doubled from 24 h to 48 h (0.4 to 0.7) and raised to 1.4 after 72 h (Figure 13A). Treated cells showed a small increase from 0.07 to 0.09 from 24 h to 48 h. The absorbance is close to 0 (0.04) at 72 h, showing that 24 h pre-treatment with mitomycin C is more efficient in inhibiting proliferation (Figure 13B).

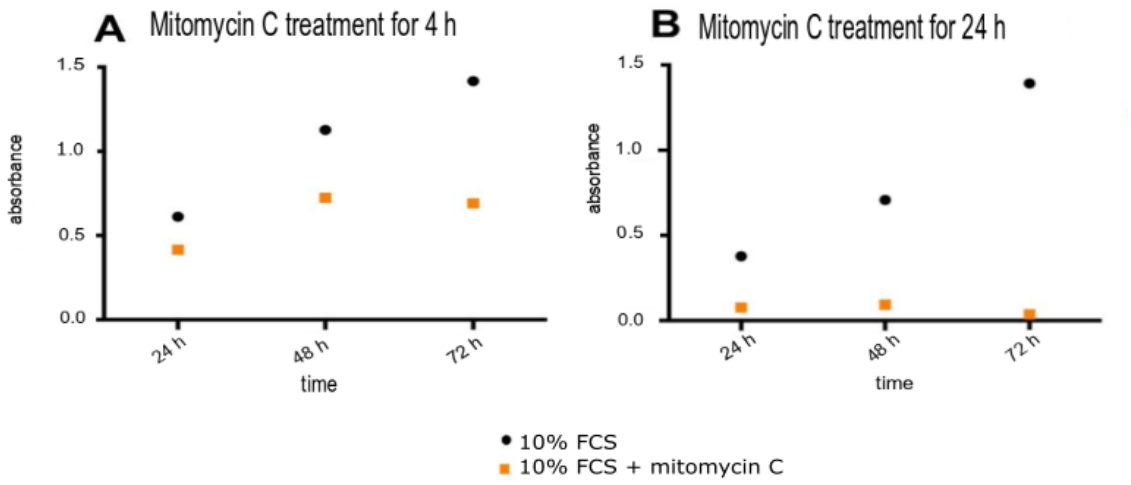


Figure 13: XTT cell viability assay of NIH3T3 fibroblasts

Cell proliferation assays at three time points (24 h, 48 h, 72 h) for control group (10% FCS medium =black dot) and for test group (additionally treated with mitomycin C 1:100 = orange square). Data are shown from two independent experiments.

***A** Mitomycin C treatment for 4 h shows that cell proliferation was slightly inhibited compared to control.*

***B** In comparison to A, the inhibition of cell proliferation by mitomycin C treatment for 24 h is stronger.*

Summing up, we ensured the inhibitory effect of mitomycin C on cell proliferation with a 24 h pre-incubation treatment. Therefore, pre-treating of cells was performed with mitomycin C for 24 h in further experiments.

Next, after having found the right condition for mitomycin C treatment, I tested different amounts of FCS in the medium to define the optimal environment for healthy and alive cells for the following experiments to guarantee viability. Cell survival was used as a read-out. I analyzed cell populations in four different conditions over a period of 48 h: FCS 10%, FCS 1%, each with or without mitomycin C (4 µg/ml). The experiment was performed once (n=1).

When comparing between FCS 10% and FCS 10% with mitomycin C treatment, the treated group showed lower viability (Figure 14A + B). Hence, mitomycin C, as a proliferation inhibitor, prevented an increase of the cell amount which explains the difference (Figure 13).
With regard to the choice of which medium to take for the wound healing assays, the comparison between FCS 10% and FCS 1% medium is relevant. As FCS already contains HA, we aimed for a HA concentration as low as possible for our medium, in which the experiments

could be performed, to check on HA effect by exterior supplementation. Fewer cells were alive after 48 h in the medium containing 1% FCS (Figure 14C). Moreover, they appeared smaller, rounder and more spread, which are signs of dying cells. Therefore, medium containing 1% FCS is less convenient to cultivate fibroblasts. This was even more striking for the combination of 1% FCS and mitomycin C (Figure 14D). The poor quality in contrast of the pictures made it impossible to quantify the exact cell amount in each group. For the assays, I selected the medium containing 10% FCS as it guarantees healthy cells over the period of experiments.

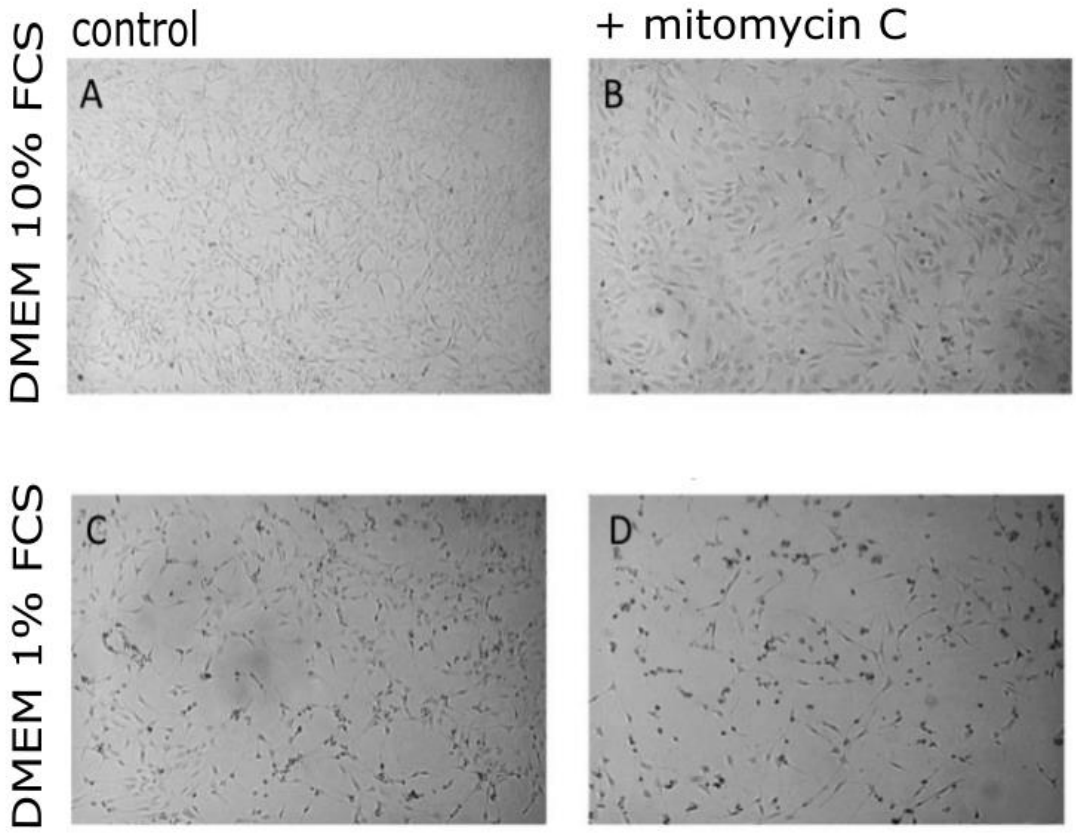


Figure 14: **NIH3T3 fibroblasts were best cultivated in 10% FCS medium**

Cell survival was tested in cell culture by different conditions. Images were taken after 48 h using EVOS FL Auto 2 at 4x magnification (n=1).

***A** DMEM 10% FCS, **B** DMEM 10% FCS with mitomycin C, **C** DMEM 1% FCS, **D** DMEM 1% FCS with mitomycin C. The cell morphology clearly showed that the NIH3T3 cells tolerated the addition of mitomycin C in DMEM containing 10% FCS better than in 1% FCS medium.*

4.1.2. Although GlcNAc supplementation increased GlcNAc and UDP-HexNAc levels significantly in NIH3T3 cells, there was no effect on cell migration

The next question was if GlcNAc supplementation could influence the cellular concentrations of GlcNAc and UDP-HexNAc. Therefore, liquid chromatography-mass spectrometry (LC-MS) measurements were performed. Since previous research had demonstrated that HA influences wound healing positively, I wondered if GlcNAc supplementation could also modulate the HA secretion, which could affect the foreseen cell migration assays. For that reason, HA ELISA was also realized.

Figure 15A shows the following results: 10 mM GlcNAc supplementation raised the GlcNAc level 1.7-fold (0.046 µg GlcNAc/mg protein vs. 0.078 µg/mg protein). A significant increase by more than 4-fold was found by 50 mM GlcNAc supplementation (0.2 µg/mg protein). These results confirm that a modulation of GlcNAc levels in NIH3T3 fibroblasts is possible and therefore that they take up GlcNAc from the medium.
Likewise, the GlcNAc supplementation raised the UDP-HexNAc level confirming that GlcNAc feeds into the HBP (Figure 15B). In comparison to the control, there is a 3.5-fold increase for 10 mM GlcNAc treatment (1.4 µg UDP-HexNAc/mg protein vs. 4.9 µg UDP-HexNAc/mg protein), and of 9-fold for 50 mM significantly (12.9 µg UDP-HexNAc/mg protein).
Interestingly, the HA measurements did not correlate with the LC-MS measurements of UDP-GlcNAc (Figure 15C). The 10 mM GlcNAc treatment showed about the same amount of HA as the control (214.9 ng HA/mg protein/h vs. 212.6 ng HA/mg protein/h). There was a mild but not significant increase of 1.6-fold for the 50 mM GlcNAc treatment (355.4 ng HA/mg protein/h). In consequence, it is unclear if such a supplementation really modulates HA secretion. Given that the HA level of the control (214.9 ng HA/mg protein/h) was already quite high, GlcNAc treatment might not be sufficient to raise HA secretion even more.
Taken together, the LC-MS measurement verified the significant modulation of GlcNAc and UDP-HexNAc level by GlcNAc supplementation. A significant increase of HA concentration was not found, although 50 mM GlcNAc raised HA concentration. Despite this finding, we decided to continue the experiments with 50 mM GlcNAc supplementation in fibroblasts as a significant increase in GlcNAc and UDP-HexNAc level was seen which indicated HBP activation. This might conceivably cause changes in ECM and affect the wound healing process.

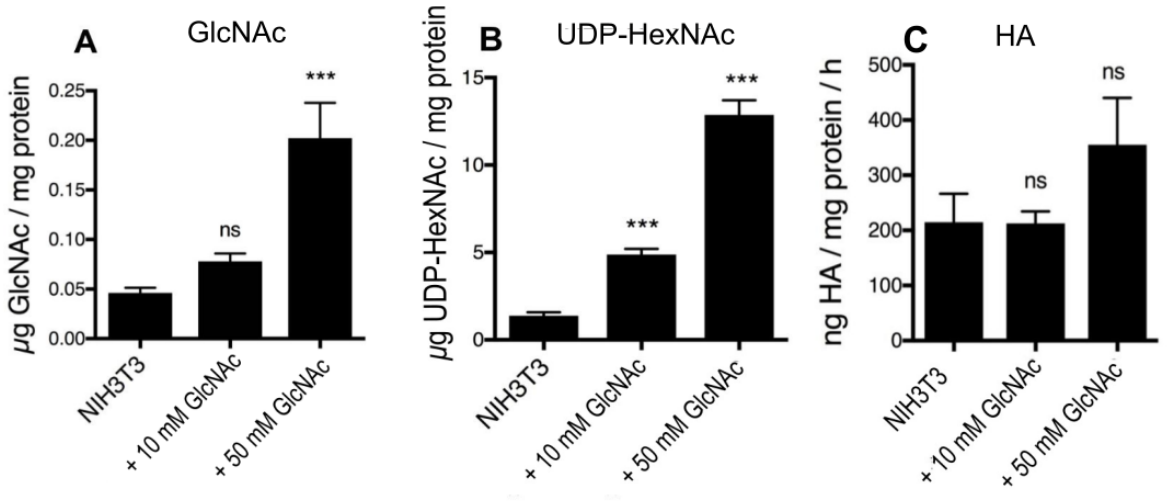


Figure 15: GlcNAc supplementation significantly increases GlcNAc and UDP-HexNAc levels in NIH3T3 cells

The GlcNAc and UDP-HexNAc levels were measured by LC-MS and the HA level by ELISA.

***A** GlcNAc level was measured by LC-MS for control, 10 mM GlcNAc and 50 mM GlcNAc treated cells. The levels were raised by 1.7-fold for 10 mM GlcNAc supplementation and more than 4-fold for 50 mM GlcNAc.*

***B** UDP-HexNAc levels were significantly increased by 3.5- and 9-fold. Data are shown as means ± SD (n=5)*

***C** Measurement of HA level by ELISA for control, 10 mM and 50 mM GlcNAc treatment. No significant modulation was detected. Data are shown as means ± SD (n=3)*

*ns = not significant, * p< 0.05; ** p< 0.001; *** p< 0.0001*

Next, I investigated the effect of HBP activation by GlcNAc supplementation on immortalized NIH3T3 fibroblasts in wound healing assays. Like introduced in Figure 11, wound healing assays ran over 72 h. Wound closure occurred both in control and GlcNAc treated fibroblasts (Figure 16). Although the wound gap of the control cells was already more closed at 12 h, the two groups showed similar behavior and pace in closing the gap later on. Both groups closed the wound gap at 32 h. Hence, we cannot see any modulating effect of HBP activation on wound closure in NIH3T3 fibroblasts.

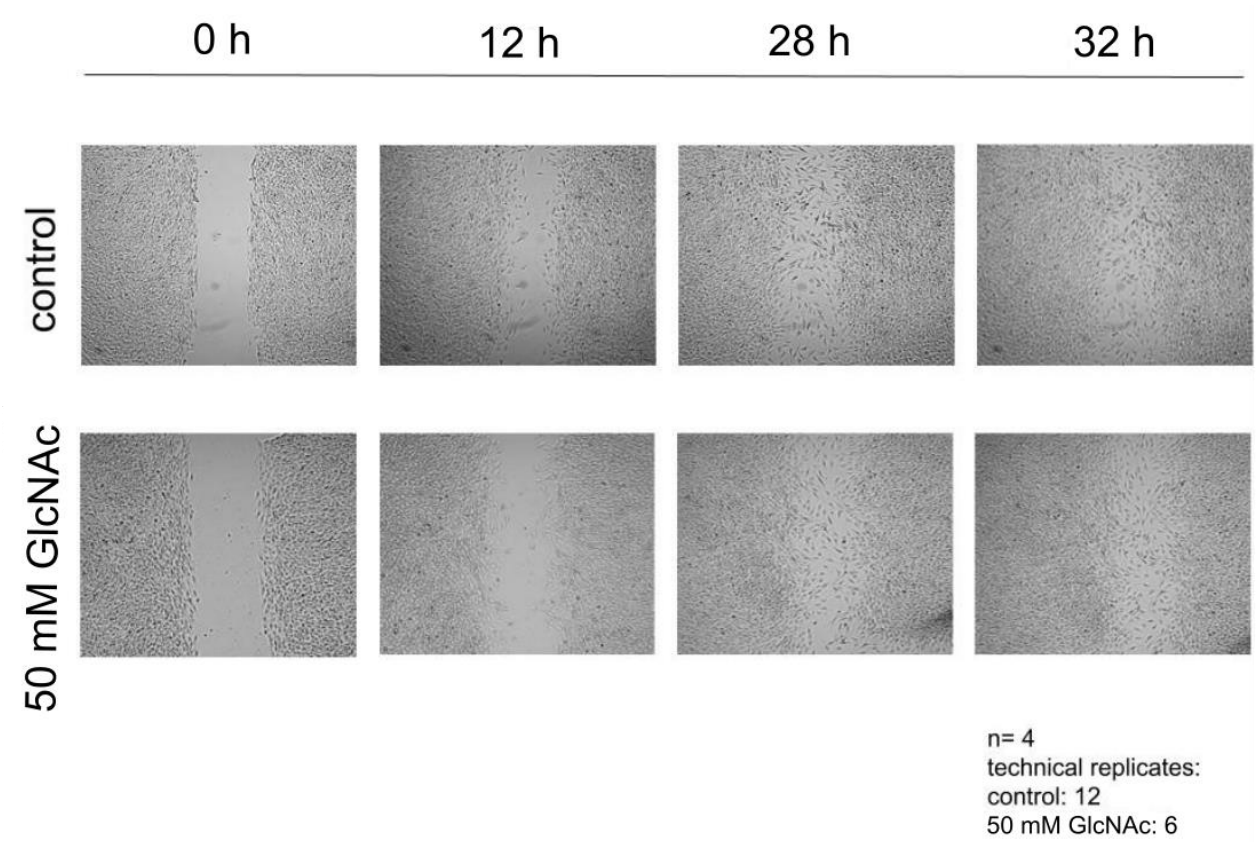


Figure 16: No effect of GlcNAc supplementation was seen in the wound healing assay of NIH3T3 cells

Images of wound healing assays of control and 50mM GlcNAc cells. The 50 mM GlcNAc treatment was compared to the control group. While the wounds of both groups closed, an influence on cell migration caused by GlcNAc supplementation was not given.

Representative pictures are shown of four independent experiments.

4.1.3. Primary murine fibroblasts showed no different behavior in wound healing assay by GlcNAc supplementation

Since NIH3T3 fibroblasts showed no effect upon GlcNAc treatment which might be due to their immortal nature, I tested primary murine fibroblasts next in the same setting to get closer to biological conditions. As for NIH3T3 cells, it was necessary to measure the modulation of the GlcNAc, UDP-HexNAc and HA levels by LC-MS and HA ELISA in primary fibroblasts by GlcNAc supplementation.

Figure 17A shows the elevation of GlcNAc level by 2.3-fold by 10 mM GlcNAc supplementation (0.075 µg GlcNAc/mg protein vs. 0.175 µg GlcNAc/mg protein).

In fact, the increase of GlcNAc levels is higher for the 10 mM treatment than for the 50 mM treatment which is only 1.7-fold (0.125 µg GlcNAc/mg protein).

In comparison to the measurements of NIH3T3 cells (Figure 15A), the baseline level of GlcNAc was about 2-fold higher in primary fibroblasts (0.046 and 0.078 µg GlcNAc/mg protein). This could indicate why a 2-fold higher raise by 50 mM GlcNAc was possible in NIH3T3 cells (0.2 vs 0.1 µg GlcNAc/mg protein).

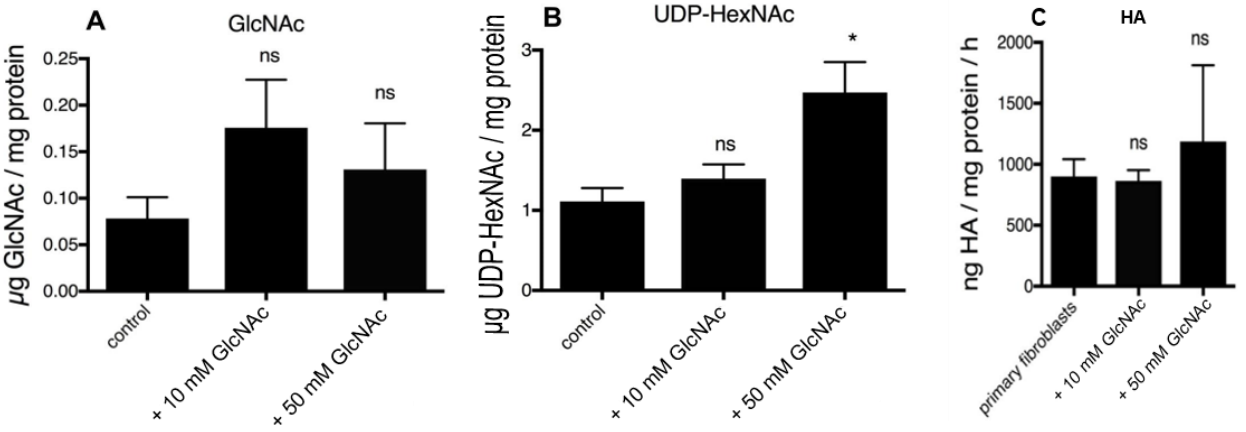


Figure 17*:* No significant increase of GlcNAc, UDP-HexNAc or HA concentrations in primary murine fibroblasts was observed by GlcNAc supplementation

GlcNAc and UDP-HexNAc levels were measured by LC-MS and the HA level by ELISA.

***A** The 10 mM GlcNAc supplementation can increase the GlcNAc level 2.3-fold, yet not significantly. In the 50 mM GlcNAc group was only a raise of the GlcNAc level by 1,7-fold.*

***B** For the 10 mM GlcNAc treatment, there was no significant rising effect (1.3-fold) and there is only a mild increase for the 50 mM treatment (2.2-fold). Data are shown as means ± SD (n=5)*

***C** Neither an increase of HA levels could be modulated by 10 mM GlcNAc treatment, nor a strong effect on 50 mM GlcNAc supplementation. Data are shown as means ± SD (n=3)*

*ns = not significant, * p< 0.05; ** p< 0.001; *** p< 0.0001*

A similar effect was seen for the UDP-HexNAc levels in primary fibroblasts (Figure 17B).
While 10 mM GlcNAc treatment had almost no effect (1.3-fold) on UDP-HexNAc level, there is only a mild increase for the 50 mM treatment (2.2-fold, p-value < 0.05).
Compared to the immortalized fibroblasts (Figure 15B), the controls of NIH3T3 cells and primary murine fibroblasts show almost the same level (1.4 vs 1.1 µg UDP-HexNAc/mg protein). Still, the increase of UDP-HexNAc level is for both supplementations, 10 mM and 50 mM GlcNAc, higher in the NIH3T3 cells than in the primary murine fibroblasts. It can be concluded that a significant modulation is possible in immortalized NIH3T3 cells, whereas only a mild increase in primary fibroblasts seems feasible.

Remarkably, the basic value of HA level for primary fibroblasts (900.2 ng HA/mg protein/h) is 4.2-fold higher than in the immortalized cells (Figure 17C and Figure 15C). There is a slight decrease for the 10 mM GlcNAc supplementation to 864.1 ng HA/mg protein/h and a 1.3-fold raise for 50 mM GlcNAc supplementation to 1188.0 ng HA/mg protein/h. Therefore, no significant modulation is possible. The reason could be that the HA level in primary fibroblasts is already quite high in untreated cells.

In conclusion, there is only a slight trend for increasing the GlcNAc level whereas a significant raise of UDP-HexNAc is given by 50 mM GlcNAc supplementation. The HA concentration was not affected in primary fibroblasts.

Next, primary murine fibroblasts were tested in the cell migration assay in the same way as for NIH3T3 cells to approach the usual skin physiology. Over the whole testing time, the control as well as the 50 mM GlcNAc group both showed the same behavior and speed in wound closure (Figure 18).

Because of the fine shapes of the fibroblasts, it was difficult to define the exact closing time. It was approximately between 34 h and 40 h. Again, we see no modulating effect upon GlcNAc supplementation on the wound closing behavior of the treated cells which corresponds to the findings of unchanged GlcNAc, UDP-GlcNAc and HA levels.

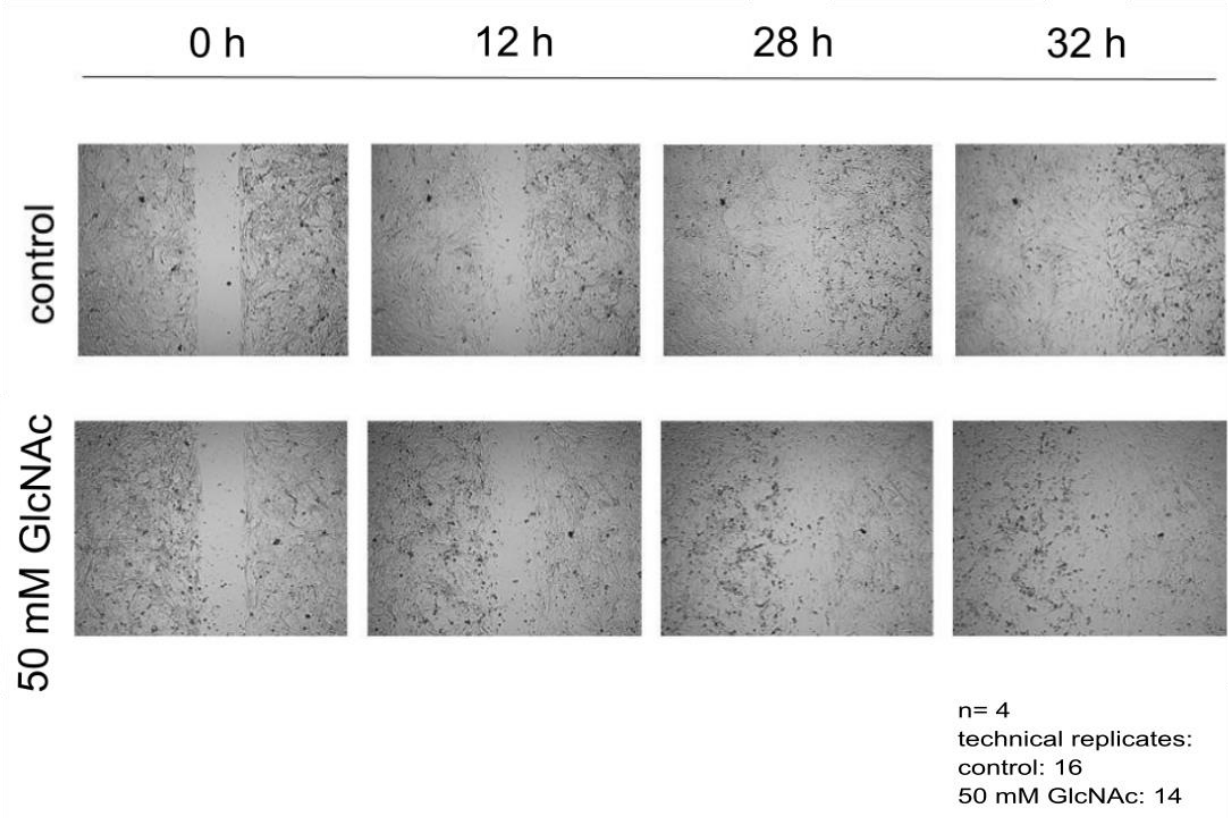


Figure 18: No effect of GlcNAc supplementation was seen in the wound healing assay of primary murine fibroblasts

The control wounds in DMEM 10% FCS and the additionally with 50 mM GlcNAc treated wounds were imaged over 72 h. Both groups show a similar behavior in cell migration towards the gap. No modulating effect by activation of the HBP was found. Representative pictures are shown of four independent experiments.

4.1.4. GlcNAc supplementation slowed cell migration in HaCaT cells

After completing the experiments in fibroblasts, the same experiments were performed with keratinocytes to answer the question if HBP activation influences wound healing. As keratinocytes play a major role in reepithelialization after wound injury, we hypothesized that a modulation of the ECM by activating the HBP would show a more significant change than we saw in the assays with fibroblasts.

Again, we started by testing the modulation of the levels of GlcNAc, UDP-HexNAc and HA in HaCaT cells by LC-MS and HA ELISA (Figure 19). Although both treatments of 10 and 50 mM GlcNAc (Figure 19A) showed a slight increase of 1.6-fold, no significant modulation of GlcNAc levels was detectable (control: 0.1 µg GlcNAc/mg protein; 10 mM: 0.3 µg GlcNAc/mg protein; 50 mM: 0.3µg GlcNAc/mg protein). The UDP-HexNAc level of HaCaT cells (Figure 19B) presented a similar linear increase as seen for fibroblasts (Figure 15B and Figure 17B).

Supplementing with 10 mM GlcNAc caused a 5.5-fold increase (control: 0.3 µg UDP-HexNAc/mg protein vs. 10 mM: 1.4 µg UDP-HexNAc/mg protein) and supplementing with 50 mM GlcNAc led to a 14.7-fold raise to 3.7 µg UDP-HexNAc/mg protein. Consequently, there is no significant modulation for GlcNAc level, but for UDP-HexNAc level when supplementing with GlcNAc. HA ELISA measured HA levels (Figure 19C + D)*.* As one test group showed exceedingly high concentrations compared to the other two test groups, this one had to be excluded to avoid wrong conclusions. The concentrations of the two experiments could not be summed up in one single graph so that the results are presented in one graph for each test. Figure 19C shows a baseline HA level of 160.2 ng HA/mg/protein/h which was increased by 6.6-fold to 1057.7 ng HA/mg protein/h by the supplementation of 10 mM GlcNAc. A raise of 7-fold to 7379.3 ng HA/mg protein/h was seen by 50 mM GlcNAc supplementation. Figure 19D also presents an elevation of HA concentration level by GlcNAc supplementation. The baseline level of 477.8 ng HA/mg/protein/h increased by 1.8-fold to 851.1 ng HA/mg/protein/h by 10 mM GlcNAc and by 4.8-fold to 4097.8 ng HA/mg/protein/h. In conclusion, GlcNAc supplementation modulated HA levels in HaCaT cells.

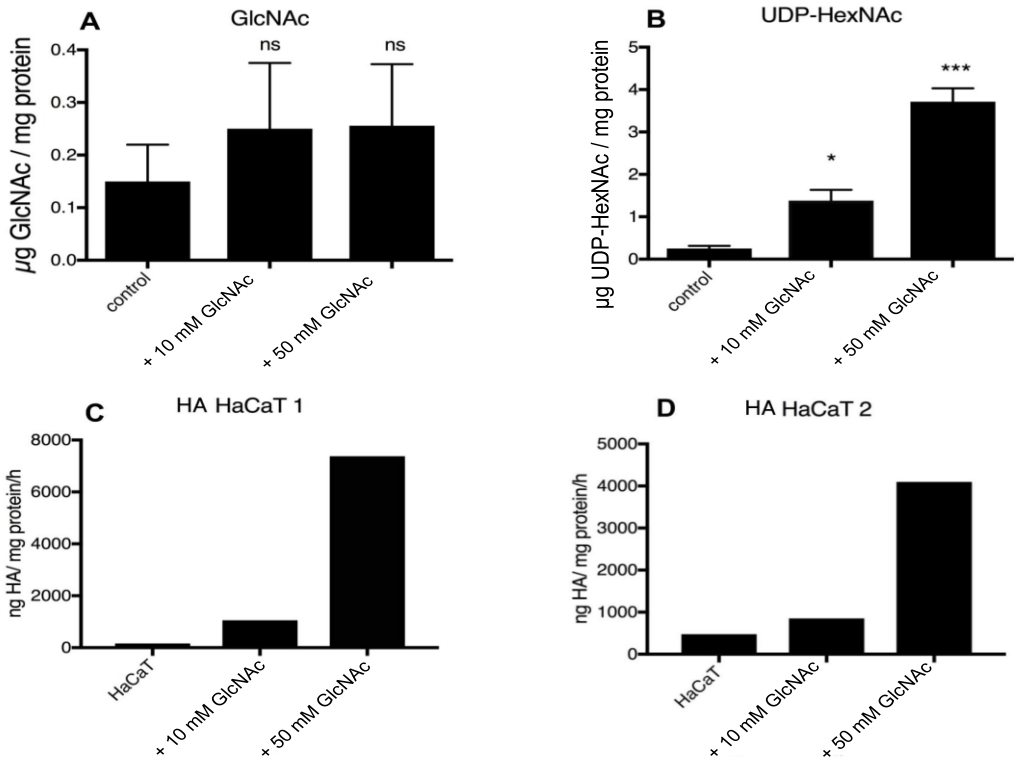


Figure 19: GlcNAc supplementation increases UDP-HexNAc level significantly in HaCaT cells

The GlcNAc and UDP-HexNAc levels were measured by LC-MS and the HA level by ELISA.

***A** No significant increase of GlcNAc level when supplementing with 10 or 50 mM GlcNAc (1.6-fold) was shown.*

***B** A raise of 5.5-fold with 10 mM GlcNAc and of 14.7-fold with 50 mM GlcNAc treatment was seen. Data are shown as means ± SD (n=5)*

***C + D** The HA level of two tests were each measured by HA ELISA. The tendency of the increasing effect of GlcNAc supplementation was found. Data are shown as means ± SD (n=2).*

*ns = not significant, * p< 0.05; ** p< 0.001; *** p< 0.0001*

Testing keratinocytes, the essential cell type that reepithelializes the skin lesion, the wound healing assays were performed with the immortalized HaCaT cell line (Figure 20). Additionally, we treated one group with 1% HA as a positive control for a better classification of our findings. The team of *D'Agostino et al.* verified scratch closure at around 20 h in HaCaT cells cultivated on collagen type 1 coats by HA supplementation (0.1% HMW HA, LMW HA and HMW HA/LMW HA in DMEM 1% FCS) compared to the control without any supplementation.[4]

As already seen in the pictures (Figure 20A), the wound treated with 1% HA was closed the fastest at 16 h. At the same time point, the control wound was almost closed whereas the GlcNAc treated wound was still open. At 28 h, the control wound closed. The GlcNAc treated

cells needed 70 h to close the wound. Based on these data, we concluded that 50 mM GlcNAc supplementation slows the cell migration in HaCaT cells.

Figure 20B + D depict the time when wound closure occurred as a relative proportion at the time point 24 h or 72 h. At 24 h, 10% of the control wells closed the gap. The HA treated cells closed all within 24 h (at 14 h, 16 h, and 21 h, n=3). It was described previously that HA accelerates wound healing and is already used clinically.[138] In my case, it functions as a positive control which indicates that an acceleration of wound closure in wound healing assays is possible and that consequently the effect seen by GlcNAc supplementation is not caused by cellular harm or errors in the method. Surprisingly, wounds treated with GlcNAc supplementation presented a slower closing behavior although HA level were shown to be increased by the treatment (Figure 20C + D). None of the wounds treated with 50 mM GlcNAc closed within 24 h. The earliest wound closure occurred at 37 h. Therefore, to find the wound closure time for GlcNAc treatment the imaging of the process was extended to 72 h (Figure 20C). The relative proportion of wound closure was calculated to the end point, here 72 h. 80% of the control wounds closed within 72 h. Upon treatment with 50 mM GlcNAc only 45% of the wounds were closed within 72 h, clearly showing a delay in cell migration. Yet, one wound did not close at all. This might stem from an error in cell numbers. In the 50 mM GlcNAc treatment, it was just 45% of the wounds that closed within 72 h. With a difference of 35% to the control, it shows that the GlcNAc treatment slows cell migration and it indicates that it has a negative influence on wound healing. Remarkably, these results indicate that 50 mM GlcNAc supplementation has a negative influence on wound healing although HA levels were increased.

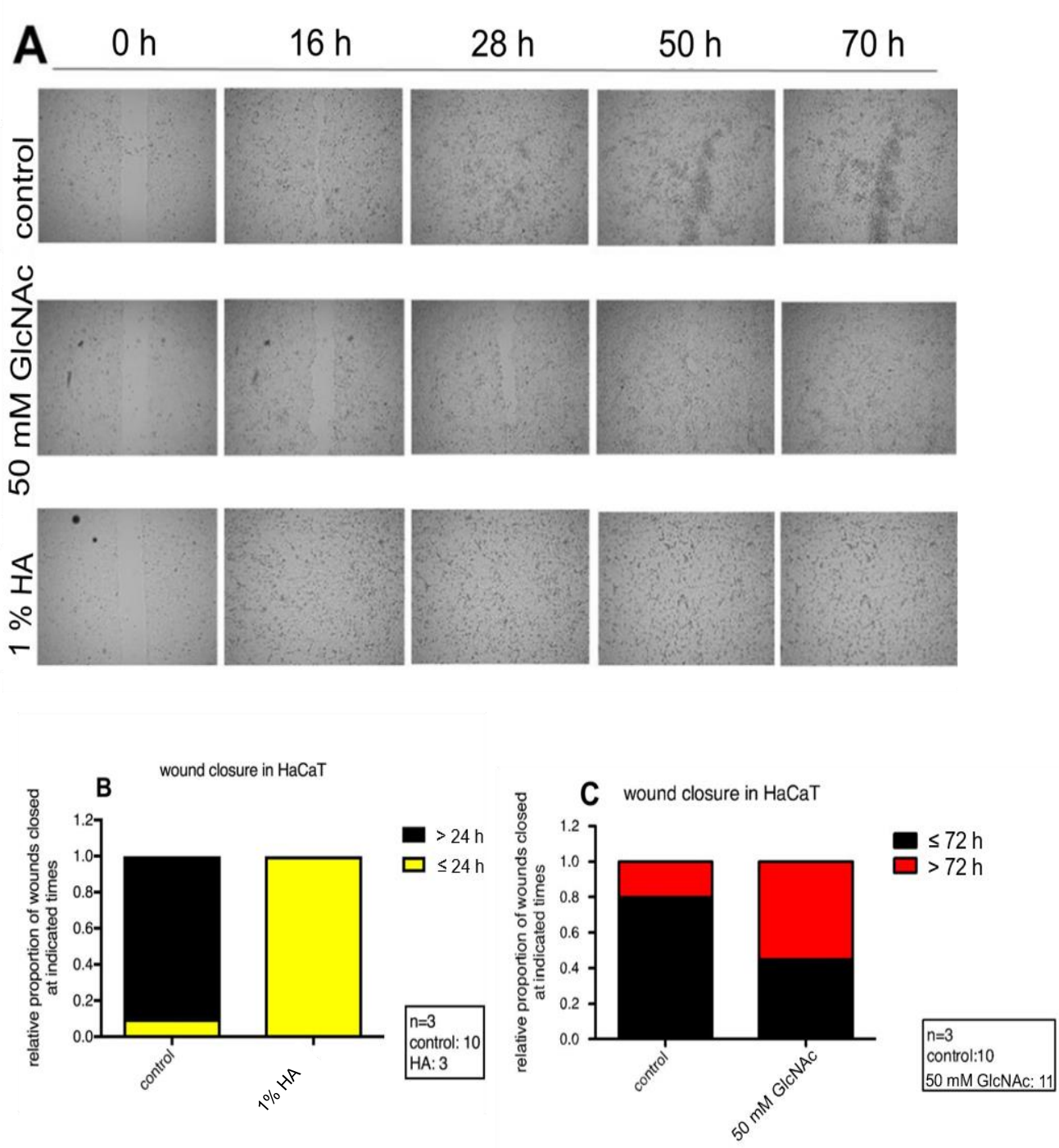


Figure 20: GlcNAc treatment slows the wound closure in HaCaT cells

A *Representative pictures of the wound healing assay of HaCaT cells for the control, the 50 mM GlcNAc and 1% HA groups are shown at given time points. The wound in the control group closed at 28 h, whereas the 50 mM GlcNAc wound closed at 70 h and the 1% HA wound closed at 16 h. n= 3, control 10 wells, 50 mM GlcNAc 11 wells, HA 3 wells. Images taken by EVOS at 4x magnification.*

B *The results of the cell migration assay of HaCaT cells with control and 1% HA group were taken as relative proportion of wound closure at indicated time. 100% of the HA treated wells showed a wound closure within 24 h indicating the fastening effect on wound healing. Yellow = wound closure occurred within 24 h; black= wound was still open at > 24 h. n= 3, control 10, HA 3*

C *The results of the wound healing assay of HaCaT cells of control and of 50 mM GlcNAc group were also taken as relative proportion of wound closure at 72 h. 80% of the control group closed the gap within 72 h whereas it was only 45% of the 50 mM GlcNAc treatment. This indicates its slowing effect. Black = wound closure occurred within 72 h; red = wound was still open at > 72 h. Representative pictures of three biological replicates are shown. n= 3, control 10, 50 mM GlcNAc 11*

As 50 mM GlcNAc is already a high concentration,[107] we wanted to analyze the possibility of a toxic effect and how lower doses could affect the closing velocity. Therefore, dose-dependency regarding wound closure was tested next. Three independent experiments were performed with the doses 10 mM, 20 mM, 30 mM, 40 mM and 50 mM GlcNAc (Figure 21). Treated with 10 mM GlcNAc, 38% of the wounds were closed within 72 h. Increasing the supplementation to 20 mM GlcNAc, the proportion of wound closure within the time raised to 70%. No further dose-dependent effect for neither 30, 40 or 50 mM GlcNAc (29%, 29%, 0%) occurred. Interestingly, 70% of the 20 mM GlcNAc treated wounds closed within 72 h whereas 0% of the 50 mM GlcNAc wounds closed. In addition, it was a better outcome than seen in Figure 20. Oddly, the control group had also a lower closing outcome with 43% within 72 h, whereas it was 80% in Figure 20. This might be explained by a higher passage number of the cells so that the cell population could have suffered damage.
In this case, it seems that 20 mM GlcNAc treatment influences the cell migration positively compared to the control. Therefore, it could be interesting to perform more assays with higher numbers of samples to verify if 20 mM GlcNAc supplementation could accelerate the wound healing process. However, comparing to the relative proportion of wound closure of the control (Figure 21), there is no significant acceleration of 20 mM GlcNAc treatment that could be extended.

Concluding, a linear dose-dependent effect was not seen by raising the supplementation from 10 to 50 mM GlcNAc. We could demonstrate an improvement of accelerated wound closure by 20 mM compared to the control. This effect just could not get expanded. In fact, elevating the dose of GlcNAc treatment further led to a slower wound closure.

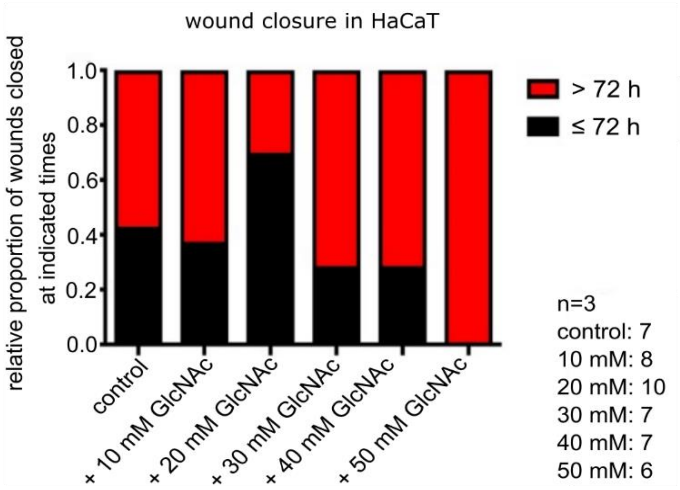


Figure 21: Dose-titration of GlcNAc treatment

The results of the wound healing assays with indicated doses of GlcNAc supplementation are shown in relative proportion of wound closure at indicated time. Black = wound closure occurred within 72 h; red = wound was still open at > 72 h. Results are representative of three biological replicates. The number of technological replicates is shown.

4.1.5. A similar slowing effect occurred in primary keratinocytes

The last step of this part of the project was to perform these experiments of measuring the GlcNAc, UDP-HexNAc and HA levels upon GlcNAc supplementation in primary keratinocytes to get more biological conditions.

Similar to the experiments in HaCaT cells, LC-MS and HA measurements were performed for the primary murine keratinocytes to investigate whether GlcNAc supplementation activated the HBP. An increase of the GlcNAc level of 1.3-fold for 10 mM and 1.7-fold for 50 mM GlcNAc supplementation was found, though it is not significant (control: 0.083 µg GlcNAc/mg protein; 10 mM: 0.111 µg GlcNAc/mg protein; 50 mM: 0.139 µg GlcNAc/mg protein) (Figure 22A). The UDP-HexNAc level (Figure 22B) was increased by 4.2-fold for the 10 mM GlcNAc treatment (control: 0.105 µg GlcNAc/mg protein; 10 mM: 0.441 µg GlcNAc/mg protein). A significant increase by 9.7-fold is shown by 50 mM GlcNAc supplementation to 1.0 µg GlcNAc/mg protein. Although the changes in GlcNAc and UDP-HexNAc were not always significant, a modulation of the concentrations seemed to be possible.

In comparison to HaCaT cells (Figure 19), GlcNAc levels were not significantly elevated in both cases, whereas a dose-dependent increase is observed for the primary cells. Yet, both analyses show big error bars which cannot eliminate that the actual increase might be covered. The LC-MS results for UDP-HexNAc levels showed again the dose-dependent increase in both cell populations. HaCaT cells have higher UDP-HexNAc levels than primary keratinocytes in

general which a comparison of their base line levels indicates (0.3 µg UDP-HexNAc/mg protein vs. 0.1 µg UDP-HexNAc/mg protein) (Figure 22B).
The HA concentrations were measured by HA ELISA. As one of the three replicates showed negative concentration levels, this test was excluded to avoid wrong conclusion. Therefore, the two experiments are depicted each in one graph.
Figure 22C presents the positive modulation effect of GlcNAc supplementation. 10 mM GlcNAc treatment increased the baseline level of 72.3 ng HA/mg/protein/h by 3-fold to 225.8 ng HA/mg/protein/h. 50 mM GlcNAc caused a raise of 1.4-fold to 326.9 ng HA/mg/protein/h.
The increase of 10 mM GlcNAc supplementation can also be seen: 13.5 ng HA/mg/protein/h to 36.1 ng HA/mg/protein/h (2.7-fold) (Figure 22D). Yet, the concentration decreased to 34.1 ng HA/mg/protein/h for 50 mM GlcNAc.

In conclusion, although there might be a modulating effect of GlcNAc supplementation on HA concentration levels, further verification is needed. Again, the physiological HA level of HaCaT cells (Figure 19C) are already higher than in primary keratinocytes.
Summing up, no significant modulating effect of GlcNAc supplementation was found in primary keratinocytes. 50 mM GlcNAc supplementation raised UDP-HexNAc levels significantly, and HA levels seemed to be increased. Overall, GlcNAc supplementation activated the HBP in primary keratinocytes.

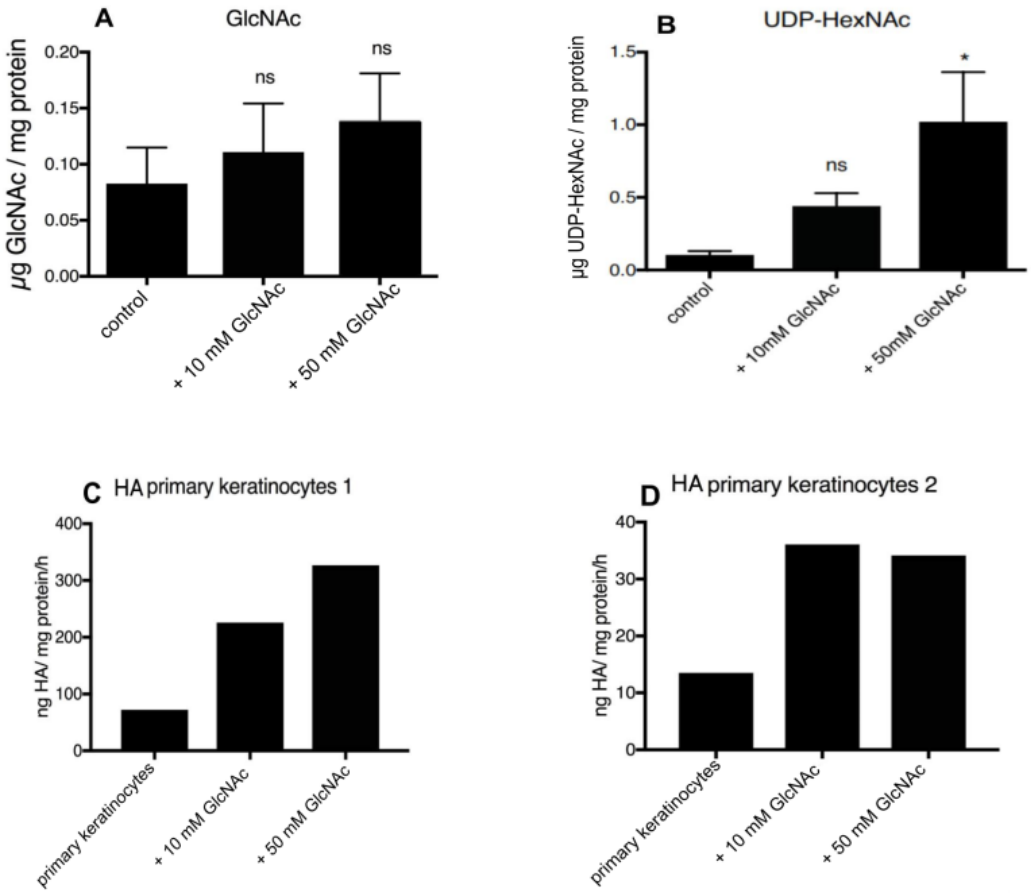


Figure 22: 50 mM GlcNAc supplementation increases UDP-HexNAc level significantly in primary murine keratinocytes

The GlcNAc and UDP-HexNAc levels were measured by LC-MS and the HA level by ELISA.

***A** No significant increase of GlcNAc level for neither treatment.*

***B** A significant 9.7-fold increase of UDP-HexNAc level is shown by 50 mM GlcNAc.*

Data shown as means ± SD (n=5)

***C + D** The HA levels were measured for two experiments by HA ELISA. 10 mM and 50 mM GlcNAc treatment seemed to increase the HA levels in C and in D, but no further raise is seen for 50 mM GlcNAc in D. Data shown as means ± SD (n=2)*

*ns = not significant, * p< 0.05; ** p< 0.001; *** p< 0.0001*

Finally, wound healing assays tested the effect of HBP activation on wound healing in primary keratinocytes. Since barely any cell migration was observed for the control group or the test group in three independent experiments, we feared that the cells were too susceptible to additional stress caused by mitomycin C. In conclusion, we omitted the 24 h mitomycin C pre-treatment in the following experiments so that the effect of cell proliferation might influence the results.

80% of the wounds in the control group closed within 70 h but only 50% for 50 mM GlcNAc (Figure 23).

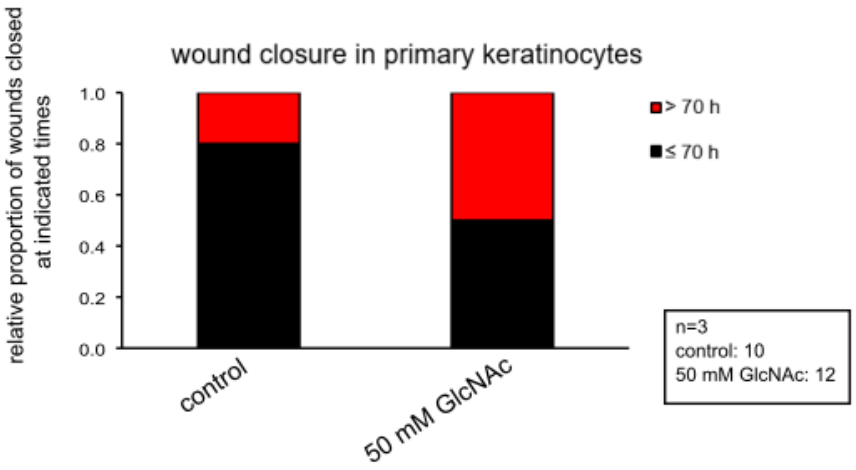


Figure 23: 50 mM GlcNAc treatment in primary keratinocytes seems to slow the wound closure

The time of wound closure of the wound healing assay of primary murine keratinocytes with control and 50 mM GlcNAc group is depicted as relative proportion of wound closure at 70 h. 80% of the control group closed the gap within 70 h whereas it was only 50% of the 50 mM GlcNAc treatment. This indicates its slowing effect.

Black = wound closure occurred within 70 h; red = wound was still open at 70 h.

Results are representative of three biological replicates.

It can be concluded that 50 mM GlcNAc treatment slows the migration also in primary keratinocytes, as seen in HaCaT cells (Figure 20). For a more precise conclusion, it would be necessary to perform further migration assays with mitomycin C treatment to make it equal to the conditions in the wound healing assays using HaCaT cells. When comparing to the outcome of the wound healing assay in HaCaT cells, in which 80% of the control wounds and 55% of 50 mM GlcNAc treated wounds closed within 72 h (Figure 20C), we observed the same trend in murine primary keratinocytes. This allows the conclusion of a similar slowing effect of HBP activation by 50 mM GlcNAc supplementation in these cells.

4.1.6. HA treatment was more effective than HMW dextran in accelerating wound closure

Our initial hypothesis was that GlcNAc supplementation would elevate HA in the ECM and would therefore accelerate wound healing. Although the sugar treatment can raise the HA level, the cell behavior is not similar in comparison to HA treatment. In contrast, 50 mM GlcNAc supplementation slowed wound closure in both HaCaT and primary murine keratinocytes. Thus, we wondered why HA supplementation itself had a positive effect in terms of accelerating the migration.

If the viscosity effect of HA treatment is the reason for speeding up the wound closure, it should be reproducible by treatments of similar viscosity. To test if viscosity might affect cell migration independently from the chemical nature of the supplement, we used dextran. Different concentrations of HA, low-molecular weight dextran (dextran 35.000-45.000 mol wt., LMW dextran) and high-molecular weight dextran (dextran 1.500.000-2.800.000 mol wt., HMW dextran) were used as treatments in wound healing assays in HaCaT cells. Figure 24 illustrates the relative proportion of wound closure within 48 h for three independent experiments.
The HMW dextran groups showed comparable closing rates to HA treatment for both concentrations. 44% of the wounds in the 0.5% HA group, 38.5% of the wounds in the 0.5% HMW dextran group and 43% of the wounds in the 1% HMW dextran group closed within 48 h. It was 61.5% in the 1% HA group and 67% in the 5% HMW dextran group were wound closure occurred within 48 h. Surprisingly, a further increase in viscosity had no favorable effect as none of the 10% HMW dextran treated wounds closed within 48 h. LMW dextran treated wounds closed by 33% for 0.5% and 1% LMW dextran treatment each within the given time, which is still higher than for the control group.
Although similar wound closure rates can be seen for HA and HMW dextran treatment, HA treatment seems to be more effective, as a lower concentration can accelerate the wound closure. Nevertheless, dextran supplementation itself has a positive influence on wound healing and might therefore be useful as a therapeutic strategy. Of note, these results suggest that not only the biological function of HA but also the viscosity of the medium influence wound closure in this setting.

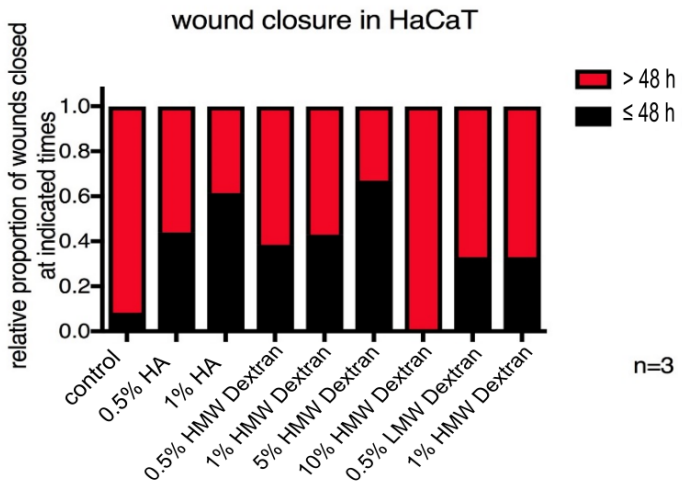


Figure 24: HA and HMW Dextran treatment show similar wound closure rates

Wound healing assays were performed in HaCaT cells to test different supplementations, as depicted. The results were taken as relative proportion of wound closure at indicated time 48 h. black = wound closure occurred within 48 h; red = wound was still open at > 48 h. Results are representative of three biological replicates.

4.2 The effect of sugar supplementation on proliferative potential of keratinocytes

The second part of the project tested the effect on proliferative potential of primary murine keratinocytes by treatment with three different sugars: 50 mM GlcNAc, 50 mM glucose, 50 mM mannose. Colony formation assays with either NIH3T3 cells or primary murine fibroblasts as feeder layers were performed.

4.2.1. The proliferative potential of keratinocytes is reduced by glucose and mannose

In the second part of the project, I was determined to find out if HBP activation would influence the proliferative potential of primary keratinocytes since both proliferation and migration are essential for effective wound healing.

A good method to evaluate proliferative potential is to perform colony formation assays, as described in Figure 12. Due to the low cell number in such an assay, colonies are formed from a single proliferating cell. Therefore, the number of colonies can be used as read-out for proliferation. Both immortalized NIH3T3 fibroblasts and primary murine fibroblasts functioned

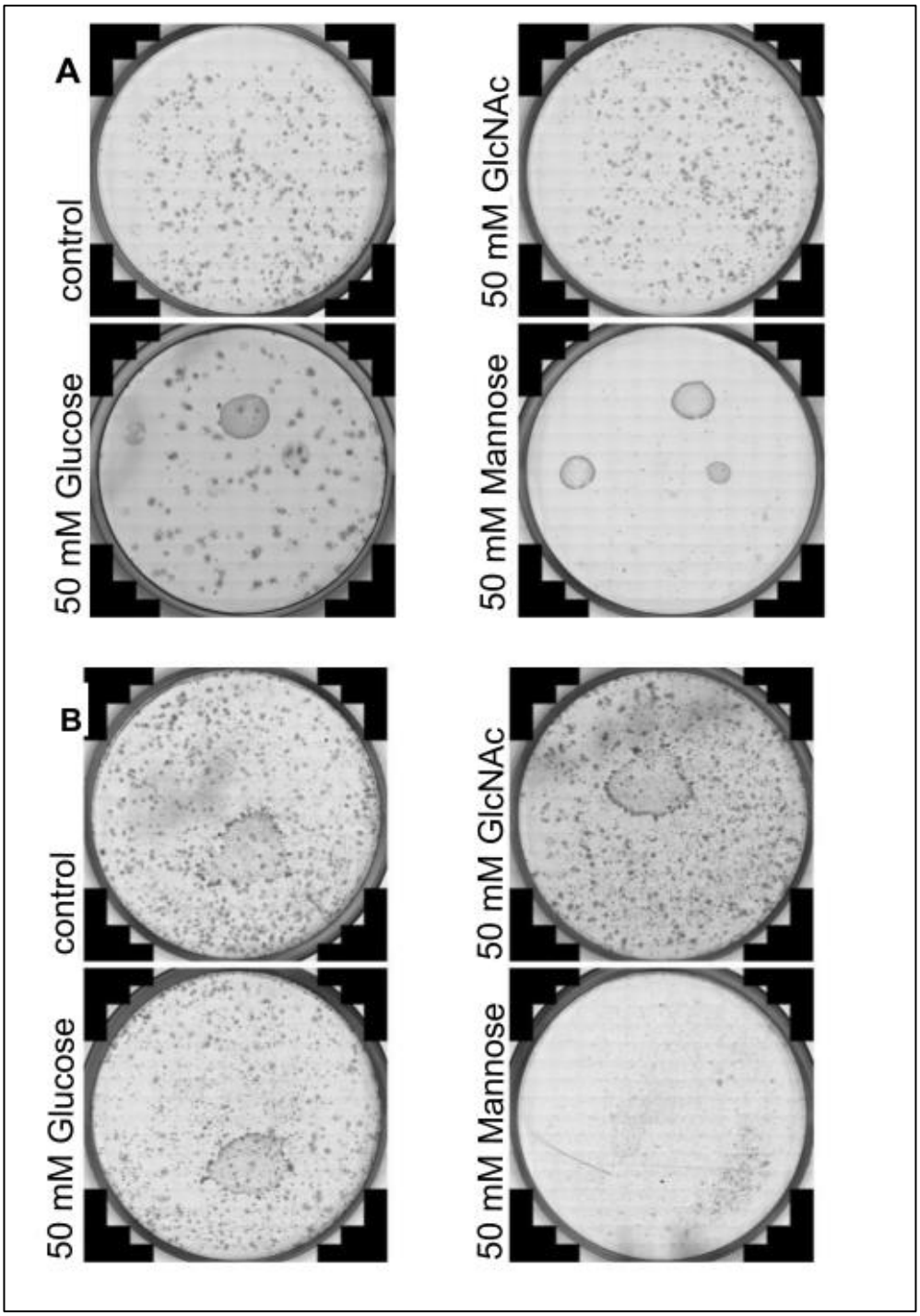
as a feeder layer to investigate the effect on primary keratinocytes in a more stable and in one more biological relevant environment.

For the sugar treatments, GlcNAc, D-glucose (Glc) and D-mannose (Man) were used as hexoses, as previous studies in the laboratory showed that these treatments can increase the number of hair follicle stem cells in an *in vitro* culture system (Figure 25).[12]

Figure 25C shows that the colony number of GlcNAc treated cells was comparable to the untreated keratinocytes in the experiment with NIH3T3 cells as feeder layer (92.2% vs. control: 95%). The supplementation of Glc seemed to decrease the proliferative potential significantly regarding the 32.9% difference to the relative amount of the control group (51% vs. control: 83.9%). Yet, mannose treatment caused an even stronger decrease of 74.7% relative to its control group (1.2% vs. control: 75.9%, p-value < 0.0001).

The colony formation assays performed with murine primary fibroblasts as feeder cells verified a similar effect of each sugar supplementation (Figure 25D). GlcNAc treatment led to a decrease of 31.4% (58.9% vs. control: 90.3%). The effect of Glc treatment is less than with NIH3T3 feeder layer by 13.2% (70.7% vs. control: 83.9%). Again, Man reduced the number of colonies significantly by 76.3% (9.4% vs. control: 85.6%, p-value < 0.0001).

In conclusion, GlcNAc treatment and therefore HBP activation has no significant modulating effect on the proliferative potential of primary murine keratinocytes. Glc treatment seems to decrease but Man treatment reduces the proliferative potential significantly.


A
control
50 mM Glucose
50 mM GlcNAc
50 mM Mannose
B
control
50 mM Glucose
50 mM GlcNAc
50 mM Mannose

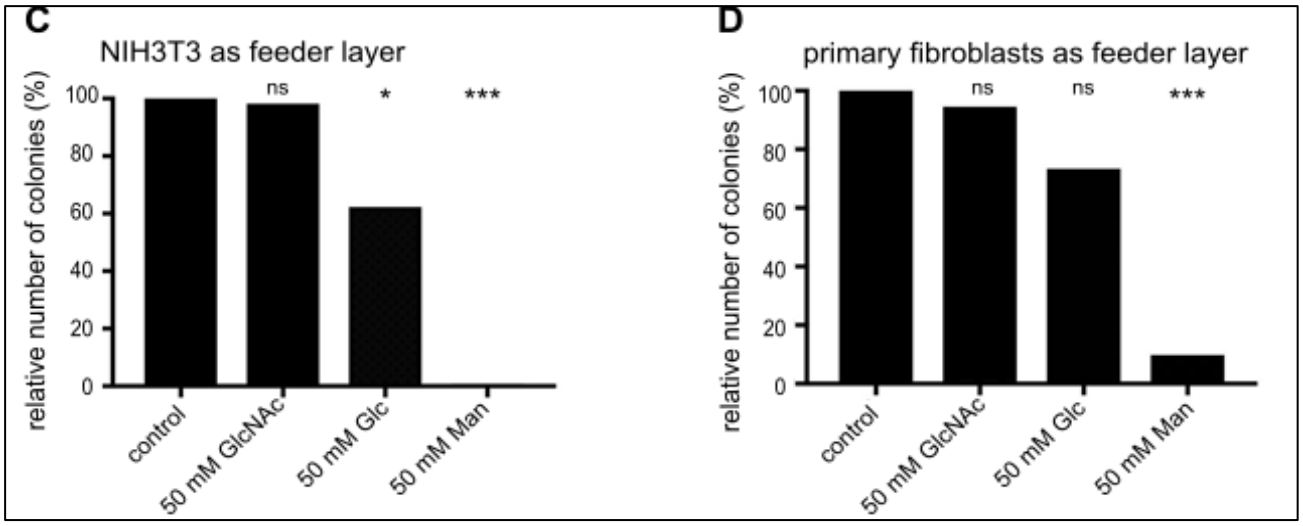


Figure 25: Mannose treatment significantly reduced the proliferative potential of primary keratinocytes

Colony formation assays were performed with NIH3T3 (A, C) or primary murine fibroblasts (B, D) as feeder layer for testing the effect of sugar supplementation on proliferative potential in primary murine keratinocytes. The number of colonies for each well of one group was counted, their average was set relative to the mean number of colonies in their corresponding control group in percentage. Sugar supplementation was either 50 mM GlcNAc, 50 mM Glc or 50 mM Man.

Data are shown for six (A, C) and three (B, D) independent experiments.

***A and B** Representative pictures of the experiments for NIH3T3 cells and primary murine fibroblasts as feeder layer. Images taken by EVOS Auto FL 2 at 4x magnification.*

***C** No effect of the GlcNAc treatment whereas Glc treatment showed a slight decrease and Man treatment a decrease of 74% relative to its control group.*

***D** A mild decrease was observed for GlcNAc and Glc treatment. The number of colonies was decreased by 76% relative to its control group for Man treatment.*

5. Discussion

5.1 The role of HBP activation in wound healing

The HBP, an intracellular, ubiquitous pathway, plays an important role in posttranslational modification through its end product UDP-GlcNAc (Figure 5). Glycolytic intermediates get shunted into the HBP, so that 2-5% of glucose, depending on the cell type, take this way of metabolism.[139] GFAT-1 is the key enzyme of the HBP which slows synthesis by negative feedback regulation.[88,89] It can itself be inhibited by phosphorylation by the energy-sensing AMPK.[91] As the HBP is sensitive to energy metabolites like nutrients and insulin, it is considered as a pro-survival pathway that regulates signaling and transcriptional modifications.[140,141]

As HBP activation showed a positive effect on protein quality control and on stress resistance in *C. elegans*,[2,3] in this thesis I addressed the hypothesis that HBP activation might affect wound healing. As non-healing wounds present an increasing medical and economic challenge worldwide,[9,10] it is of tremendous interest to unravel more of the wound healing process to develop effective therapeutic strategies to cope non-healing wounds. Activating HBP stimulates the production of GAGs which themselves are important components of the ECM. A modulation of the ECM can interact in the wound healing process. Therefore, I activated the HBP by GlcNAc supplementation in fibroblasts and keratinocytes, which are the main cell types involved in the wound healing process, and investigated the effect on cell migration and proliferation in this project. While HBP activity could not be increased in fibroblasts, I confirmed that GlcNAc supplementation increased UDP-HexNAc and HA levels in keratinocytes. Surprisingly, HBP activation slows migration of keratinocytes despite elevated HA levels, while their proliferation was not affected. Nevertheless, as described previously, addition of HA increased cell migration in the same system. Of note, I could demonstrate that this positive effect on wound healing was mediated by the viscosity of HA since also addition of HMW dextran accelerated cell migration.

5.1.1. GlcNAc supplementation activates the HBP in keratinocytes but not in fibroblasts

The first question was if GlcNAc supplementation could influence the cellular concentrations of GlcNAc, UDP-HexNAc and HA in the two cell lines being involved in wound healing: fibroblasts and keratinocytes. LC-MS and ELISA were therefore done.

First, I would like to discuss the results seen in fibroblasts: 50 mM GlcNAc supplementation of NIH3T3 cells increased GlcNAc and UDP-HexNAc concentrations significantly (Figure 15). It

also increased HA concentrations mildly but non-significantly. In contrast, the supplementation increased UDP-HexNAc levels significantly in primary murine fibroblasts (Figure 17). Yet, there are no alterations in GlcNAc or HA concentrations.

Why does the HBP activation increase HA concentrations stronger in immortalized cells than in primary cells? Hyaluronan synthases (HAS) use UDP-GlcNAc to produce HA by which they maintain a relative constant pool of cytoplasmic UDP-GlcNAc concentration.[142] With increased supply of UDP-GlcNAc, HAS can consequently elevate HA synthesis. Yet, it might be that a maximum is reached at some point and the enzyme is replete. The increase of UDP-HexNAc levels in the experiments supports this idea of such a limit. The HA level in primary fibroblasts is already quite high without treatment (mean 900 ng HA/mg protein/h) compared to NIH3T3 cells (mean 200 ng HA/mg protein/h) (Figure 15 and Figure 17). It seems that HA production cannot be raised further while UDP-GlcNAc level increased, as the substrate pool loses its balance. Moreover, O-GlcNAc transferase (OGT) takes UDP-GlcNAc for O-GlcNAcylation. It was observed that hyperglycemia or increased UDP-GlcNAc levels lead to O-GlcNAcylation of transcription factors that regulate HAS2 gene expression.[142] In consequence, its expression is lowered and HAS2 synthesizes less HA. Indeed, taking the experiment duration into account, this idea does not seem to be the most probable. Yet, we can consider the possibility of a maximum of HA synthesis.

Next, the experiments with keratinocytes (HaCaT cells and primary murine keratinocytes) modulate GlcNAc, UDP-HexNAc and HA concentration similarly (Figure 19 and Figure 22). 50 mM GlcNAc supplementation caused a significant increase of UDP-HexNAc and HA levels. Also, the treatment of primary keratinocytes showed a significant elevation of UDP-HexNAc concentration but only a mild modulation of the HA concentration compared to HaCaT cells. As there are only two results because one experiment had to be excluded, a significant change of HA level could not be demonstrated. Yet, I observed an increase of HA level by GlcNAc supplementation in both. Here, the physiological HA level of HaCaT cells is already higher than in primary keratinocytes (319 ng HA/mg protein/h vs. 43 ng HA/mg protein/h). Despite the lower HA basal level primary keratinocytes increase HA level only mildly compared to HaCaT cells.

When comparing immortalized and primary keratinocytes one must consider that immortalized cells (HaCaT) grow on non-coated plates due to their adaption to cell culture conditions. Primary cells, though, are cultured on coated plates as they depend on artificial ECM. Consequently, this coating already offers the required environment and primary cells are not forced to secrete as much HA for survival.

How can we explain the difference in modulation in fibroblasts and in keratinocytes? Regarding the physiological conditions, fibroblasts are the main cell type in dermis and keratinocytes in epidermis, whereas HA levels are higher in dermis compared to epidermis. The ratio of HA of glycosaminoglycan species (HA, heparan sulphate, dermatan sulphate, chondroitin sulphate and heparin-like glycosaminoglycan) in dermis/epidermis is 61%/44%. Also, the comparison of the concentration in uronic acid equivalents per mg of wet dermis and epidermis together confirm the higher proportion of HA in dermis to epidermis: 243/0.48.[8] A quantification of HA performed by LC-MS revealed 739 +/- 32 µg/g in dermis and 15 +/- 1.5 µg/g in epidermis.[143] This supports our findings that HA levels are higher in fibroblasts in general and that additional GlcNAc supplementation might not increase the HA concentration further. However, it can still raise the HA production in keratinocytes.

5.1.2. GlcNAc supplementation did not affect cell migration in NIH3T3 cells or in primary murine fibroblasts

My project tested the modulation in fibroblast migration by 50 mM GlcNAc supplementation. Neither treated immortalized nor primary fibroblasts showed a different behavior in migration assays compared to control (Figure 16 and Figure 17). As my previous experiments showed only a slight change in the concentration levels of GlcNAc, UDP-HexNAc and of HA this might explain this outcome.

When skin lesions occur, the availability of empty space stimulates the cells at the area of the wound gap to proliferate and to migrate towards the center of the lesion until closure. Also, the influx of cell matrix of damaged cells is discussed to trigger their proliferation.[144,145] Migration takes place in a cyclic process by cell polarization, extension and stabilization of the cell processes by adhesion to the ECM, followed by cell contraction and detachment leading to a forward movement.[146] In addition to chemokines, cytokines and growth factors, also changes in cell-cell contacts regulate migration.[147]

Interestingly, a previous study demonstrated, that NIH3T3 fibroblasts and primary skin fibroblasts from rats cultured in high glucose (25 mM D-glucose) showed a reduction by ~40% in migration speed. This effect is due to an elevation of reactive oxygen species production, harmed cell polarization, diminution of protrusion stability and adhesion maturation.[13] Also, fibroblasts in diabetic conditions demonstrate reduced migration and proliferation.[148] Although my cell culture experiments were done in high glucose medium (25 mM D-glucose), no difference in migration speed occurred in 50 mM GlcNAc treated fibroblasts compared to control. Thus, UDP-GlcNAc might in fact have a protective function by raising the GAG level and modulating the ECM. That is why migration is reduced by 25 mM D-glucose environment but not upon additional 50 mM GlcNAc supplementation. This outcome could be due to

alterations in cell-matrix adhesions. In normal state, fibroblasts rest in low-tension. After an injury, wound healing in terms of cell migration, proliferation and collagen synthesis require a high cell-matrix tension.[149] As HBP activation increases O-GlcNAcylation and it leads to substrate modification such a higher tension in adhesion might occur. This could be tested by adhesion assays.[150,151] Furthermore, it would be interesting to repeat the wound healing assays in low glucose medium (5 mM D-glucose) to check if GlcNAc supplementation has a beneficial effect on migration when the negative side effects of hyperglycemia are excluded.

To sum up, fibroblast migration is essential for wound healing and is mainly regulated by ECM. Increasing GlcNAc, UDP-HexNAc and HA concentrations does not seem to slow or accelerate migration. When considering a hyperglycemic state and the findings of the other research teams, it would be interesting to test a possible protective effect of GlcNAc supplementation on fibroblast migration in further experiments using low glucose medium.

5.1.3. GlcNAc treatment slows the wound closure in HaCaT cells and in primary murine keratinocytes

Keratinocytes are major players in reepithelialization in wound healing. Therefore, we expected an effect of cell migration in the wound healing assay because of the increase of UDP-HexNAc and HA level in HaCaT cells and in primary murine keratinocytes by GlcNAc supplementation (Figure 19 and Figure 22). Interestingly, we saw the following in the HaCaT experiments (Figure 20): 80% of the control wounds closed within 72 h. Only 45% of the wounds closed within 72 h upon treatment with 50 mM GlcNAc, clearly showing a delay in cell migration. We noticed the same trend in primary keratinocytes where 80% of the control and 50% of the 50 mM GlcNAc treated wounds closed within 70 h (Figure 23). This allows the conclusion of a similar attenuation of wound closure by HBP activation by GlcNAc supplementation in this cell type. GlcNAc supplementation modulates the UDP-HexNAc and HA levels in HaCaT cells best. It is therefore not surprising that we got a clearer and more reliable result in wound healing assays using HaCaT cells in which the modulation is highest.

When comparing the outcome of the wound closure ratio within 72 h in the control groups of the experiments in keratinocytes in comparison to the dose dependency experiment, one questions why the proportion of closed wounds is 80% compared to 43% (Figure 20 and Figure 21). The higher passage of the cells in contrast to the firstly performed wound healing assays might explain the difference. The cell population might have suffered damage over time. It should be repeated with lower passages. Figure 20 shows that around 80% of the control wounds of HaCaT cells closed within 72 h. Figure 24, though, presents only a closing rate of 8.3% within 48 h. This might indicate that wound closure occurs *in vitro* usually between 48 h and 72 h, as described in *Myers et al.*[45] This could explain why many control wounds were still

open at the end of the experiment period at 48 h. Reepithelialization begins hours after lesion and is usually completed within 8-10 days in human skin.[32] One could also think that the cell population might have suffered cell damage over the time of passaging and cultivating either due to biological or technical effects. Whereas around 80% closed (Figure 20), 43% control wounds closed (Figure 21) in one experiment later. As the first experiments used lower passages, the higher passage of the cells in contrast may explain this difference. In the future, we should repeat the experiments to exclude cell damage and an interference in the results.

Before discussing why HBP activation might slow migration process, I would like to take a step back and to regard impaired wound healing. The exact underlying mechanisms beyond poor wound healing are not yet unraveled. Despite intensive research, the wound repair remains complex as many cell types, chemical signals and the ECM are involved and entangled with each other. Comorbidities and patient individuality as well as not precise correlation of animal models with human conditions complicate therapeutic progress. Fact is that impaired or even non-healing wounds arise if at least one of the phases of wound healing (hemostasis, proliferation and migration, and ECM remodeling) gets interrupted or deregulated.[33]
Consequently, which risk factors lead to impaired wound healing? Infection, smoking, aging, malnutrition, immobilization, diabetes, vascular diseases (chronic venous insufficiency, peripheral artery disease), immunosuppressive therapy, and others. In my experiment, we can exclude infection, immunosuppression, smoking, aging (as control and treated group were from the same passage), immobilization (meaning an increased pressure on the wound) and difficulties of angiogenesis. This leaves diabetes like a hyperglycemic state as created in my experiments and changes in cytokines due to ECM alterations as possible reasons for a deceleration in wound closure. Thus, glucose and cytokine levels should be investigated and controlled in future experiments.

5.1.4. GlcNAc supplementation might mimic a diabetic-like state

Diabetes is one of the most important reasons for impaired healing. Deficient wound healing can take the form in foot ulcers, which present at 2 to 3%/year and up to 15%/life time prevalence among diabetic patients.[33] Hyperglycemia perpetuates the inflammatory response by inducing glycosylation.[152–154] Histopathological analysis of granulation tissue biopsies confirmed the maintenance in the inflammatory phase of diabetic wounds.[155]
Furthermore, hyperglycemia causes impaired growth factor production, angiogenesis and remodeling of the ECM.[156–160] Also, hyperglycemia affects macrophage function, migration and proliferation of fibroblasts and keratinocytes.[161,162] Keratinocytes demonstrate hyperproliferation but an absence of migration at the non-healing area of diabetic foot ulcers.[163,164]

Several papers link the HBP with diabetes.[99,165–168] In hyperglycemia, the HBP takes up glycolytic intermediates. Its product UDP-GlcNAc affects posttranslational modifications by OGT and an elevated flux increases O-GlcNAcylation.[169–171] Elevated O-GlcNAc and OGT levels appear to cause cell damage and oxidative stress.[171] For example, it leads to damage in the cornea leading to loss of vision in animal models.[172,173]

Keratinocytes having been cultivated in hyperglycemic condition demonstrate the relation between impaired wound healing in diabetes and increased O-GlcNAcylation by HBP activation also. They show an increase in O-GlcNAcylation and a slowed wound healing. An OGT knockdown via RNAi was able to reverse this phenomenon indicating the importance of a constant equilibrium of OGT and O-GlcNAc hydrolase (OGA).[174] As GlcNAc supplementation activates HBP and consequently raises UDP-GlcNAc concentrations, more substrate exists for OGT's disposal to take for substrate modifications. This results in delayed wound healing. My experiments in keratinocytes confirm a slowing effect in wound healing by HBP activation due to GlcNAc supplementation which raise GlcNAc and HA level. It seems promising to check on modulation of O-GlcNAcylation in non-healing wounds.

Considering a toxic effect of high glucose conditions, *Lamers et al*., showed a reduction by ~40% in migration speed. They cultured NIH3T3 fibroblasts and primary skin fibroblasts from rats in high glucose condition (25 mM D-glucose). Apparently, the decreased cell migration is due to an elevation of reactive oxygen species production, harmed cell polarization, diminution of protrusion stability and adhesion maturation.[13] Yet, my findings cannot exclude cell damage caused by HBP activation. Therefore, it would be interesting to test oxidative stress also for GlcNAc supplementation as well as apoptotic rate during wound healing assays.

To sum up, it appears likely that not only high glucose, but also high GlcNAc can trigger a diabetic-like state. My experiments were performed in high glucose medium (25 mM D-glucose) and the cells were treated with additional 50 mM GlcNAc. Consequently, the general high concentration of glucose might influence the slowing effect in the wound healing assays and the additional GlcNAc supplementation might boost this effect. Performing the experiments in low glucose medium (5 mM D-glucose) could help to dissect the GlcNAc-mediated effects from the ones of high glucose.

In this context, it would also be interesting to take biopsies of diabetic patients of non-damaged skin and wound areas as well of control tissue of non-diabetic patients to compare GlcNAc and HA levels. If GlcNAc levels are elevated in patients' skin, it would confirm its involvement in

diabetic wound healing processes. Especially, if an elevated GlcNAc pool is found in biopsies of wound areas in diabetic patients.
In addition, it is appealing to investigate if GlcNAc supplementation causes the same downstream pathology on a molecular level as hyperglycemia.

5.1.5. GlcNAc supplementation might strengthen cell adhesion

Effective reepithelialization necessitates accurate cell proliferation and migration, especially of keratinocytes. The migration may be even more important.[175] Appropriate migration relies on a precise interplay between strengthening and loosening of adhesion.[54] Keratinocytes at the wound margin downregulate adhesion to be able to migrate forward. Important modulators of cell-matrix interactions being involved in wound healing are galectins (Gal). A lack of Gal-3 or Gal-7 disturbs reepithelialization.[176,177] Both diabetic mice and keratinocytes in hyperglycemic condition (25 mM glucose) present reduced Gal-7 expression on mRNA and protein level caused by increased O-GlcNAcylation. Reduced Gal-7 expression, in turn, slowed wound closure by 50% to control after 17 h while Gal-7 overexpression fastened the process.[178] Thus, increased HBP activity might reduce Gal-7 expression via increased O-GlcNAcylation, which might contribute to the slowing effect of GlcNAc supplementation on keratinocyte migration in my experiments. Overall, the study of *Huang et al.* highlights the importance of the interplay between cell-cell and cell-matrix for cellular processes like wound healing.[178] Furthermore, it suggests that higher GlcNAc concentration cannot be set equal to favorable conditions in general. To demonstrate that increased O-GlcNAc occur in my experiments and a ECM alteration might take place, we could quantify the amount using binding probes, lectin (wheat germ agglutinin) or glycomic/mass spectrometric approaches.[99,179]

As mentioned, cell-matrix adhesion seems to be essential for wound healing. After injury, keratinocytes from the wound margin become activated to migrate into the center of the lesion. In order to move forward, there are alterations in cell-matrix adhesions required. These can be clipped or loosened by proteases, especially several MMPs which change the interplay between integrins and collagen in the advancing epidermis.[180]
After five days of hyperglycemic treatment, the ability to migrate, to proliferate, the activity and gene expressions of MMP-2 and MMP-9 decreased in human keratinocytes.[181] Both MMPs are essential but especially MMP-2 is associated with cell detachment from the matrix.[182] These reductions possibly contribute to the inadequate reepithelialization, providing a link between elevated O-GlcNAcylation and hyperglycemic deficient wound repair. Therefore, expression of MMP-2 and MMP-9 should be investigated upon GlcNAc supplementation since their downregulation might contribute to the reduced migration seen in keratinocytes.

Another example of the effect of hyperglycemia on cell adhesion was the experiment of *Lan et al.* which described a decrease of focal adhesion kinase ($p125^{FAK}$) by glucose treatment (12 and 25 mM L−glucose).[181] $p125^{FAK}$ is a protein-tyrosine kinase regulating cell migration.[183] As elevated phosphorylated $p125^{FAK}$ is indispensable for cytoskeletal protein modifications affecting cell migration,[184] it would be worth to test its concentration and expression in my experiments to depict an alteration eventually caused by elevated UDP-GlcNAc concentration. These examples point out how a change in adhesion can influence cell migration, especially due to hyperglycemia. Therefore, it is important to take such an effect also due to GlcNAc supplementation into account.

Furthermore, as GlcNAc supplementation increases O-GlcNAcylation and in consequence affects the balance of O-GlcNAc concentration maintained by OGT and OGA, we should emphasize the importance of dose dependency. For O-GlcNAcylation, there are two enzymes: O-GlcNAc transferase (OGT) adds and O-GlcNAc hydrolase (OGA) removes a single β-N-acetylglucosamine moiety.[185,186] O-GlcNAc level regulates their activity so that OGT and OGA guarantee a homeostatic concentration of O-GlcNAc. Any long-term disruption due to an imbalance between O-GlcNAcylation and phosphorylation is assumed to be associated with the prevalence of diabetes, cancer and Alzheimer's disease.[99,185,187,188] This indicates the importance to keep the O-GlcNAc level in a defined range. So far, about 1.500 O-GlcNAc sites from all organisms are known which show the same or competitive proximal binding site for O-GlcNAc and phosphate, demonstrating the competition of the two modulation ways.[99]
Denzel et al. have shown that the extension of life span in *C. elegans* was dose dependent.[2] It occurred for a dose between 2 mM and 10 mM GlcNAc but not at 25 mM GlcNAc. Higher levels may even diminish protein quality control mechanisms and result in pathologies. HBP activation is suspected to create itself cellular stress that might cause cell damage. It is assumed that it leads to energy imbalance by consuming metabolites as glucosamine supplementation increased ROS response.[189]

In my project, three independent experiments tested a dose-dependency of GlcNAc supplementation regarding wound closure with the doses 10 mM, 20 mM, 30 mM, 40 mM and 50 mM GlcNAc (Figure 21). While 10 mM GlcNAc treatment led to a wound closure of 38% within 72 h, 30 mM and 40 mM GlcNAc led to 29% each and no wound treated with 50 mM GlcNAc showed closure within this time. Leaving out 20 mM GlcNAc, a higher GlcNAc supplementation seems to increase the negative effect on cell migration. Yet, a treatment with 20 mM GlcNAc presented wound closure of 70% within 72 h, providing an acceleration compared to control (43%). The results support the hypothesis that effective cell function relies on a narrow range of UDP-GlcNAc concentration.[2,185] One could investigate the wound closure

rate using 10, 15, 20 and 25 mM GlcNAc supplementation to confirm a positive effect and to narrow down the best range of UDP-GlcNAc level.

In conclusion, we should consider changes in keratinocyte migration processes due to GlcNAc supplementation aside from the glucose-mediated direct toxicity on keratinocytes. It is known that wound healing is negatively affected by diabetes. As my results show also a delay in wound closure by GlcNAc supplementation, it seems that GlcNAc supplementation might mimic a diabetic-like state. Modulation of adhesion may explain the reduced cell migration causing a deficient wound reepithelialization and should therefore tested in further research.

5.1.6. Viscosity affects the wound healing process

Next, we tested the influence of viscosity as one possible reason for the accelerating effect on wound healing assays by HA supplementation. Viscosity is defined as *"the resistance to flow or alteration of shape by any substance as a result of molecular cohesion; most frequently applied to liquids as the resistance of a fluid to flow because of a shearing force"*.[190]

My experiments were intended to raise HA levels by GlcNAc treatment and consequently to affect wound healing. Whereas HA supplementation itself increased cell migration, GlcNAc supplementation slowed wound closure in keratinocytes.

That HA has a positive influence in wound healing has been shown in several publications.[138,191,192] *Dechert et al.* showed that the HA level in dermal biopsies taken from wounds of healthy adults were higher than in biopsies from pressure ulcers,[120] stating its correlation with proper wound repair. It is also known that HA provides viscoelasticity and hygroscopic properties.[108,113,114,117,193] Even a concentration of 0.1% HA leads already to a high viscosity.[194] To test whether HA's viscosity was underlying the increased wound healing speed, different concentrations of HA and of low-molecular weight (LMW) and high-molecular weight (HMW) dextran were added to wound healing assays in HaCaT cells. Dextran was chosen as a substrate as it is known for its viscous effect, but it does not influence the HPB. To test different levels of viscosity both HMW and LMW dextran were used. As the HMW dextran treated groups showed similar closing rates as the HA treated groups (Figure 24), we can consider a comparable viscosity and positive effect on cell migration. Accordingly, the viscosity likely contributed to the positive effect of HA treatment seen in the wound healing assays. LMW dextran treated wounds, though, showed slower closing rates compared to HMW dextran, which indicates that this treatment cannot reach the same viscosity. My data suggest that not only HA but also HMW dextran is a promising treatment for chronic wounds. Indeed, dextran

is already used as a substrate in wound dressings.[195] Due to its hydroxyl groups, dextran is capable of binding water which is favorable in creating and maintaining a moist environment.[196]

The question remains why raising HA concentration by adding GlcNAc does not lead to a positive effect on cell migration in keratinocytes. Does increasing the HA concentration by GlcNAc supplementation raise the viscosity too much so that no positive effect occurs any longer? It seems possible as no wounds closed in the group of 10% HMW dextran. That viscosity can actually be too high is illustrated by two examples: gall bladder stones due to increasing viscous bile sediment and infertility due to hyperviscous semen.[197,198] Raising UDP-HexNAc and HA concentration could also increase viscosity too much which negatively affects cell migration so that wound closure *in vitro* gets ineffective as we saw for 10% HMW dextran supplementation. It is also reported that dextran hindered cell attachment but not proliferation, which might explain a slower migration at high concentrations.[196] The same effect might occur with high concentrations of HA.

In conclusion, I could demonstrate that the viscous feature of the medium plays a major role in the wound healing process *in vitro*. In future experiments, the viscosity of the medium should be measured before and after GlcNAc supplementation to test whether this treatment indeed increases viscosity too much and therefore slows cell migration. Also, further research is needed to identify the downstream effects of more viscous medium and to investigate why increasing the viscosity too much has detrimental effects. Most importantly, HMW dextran should be tested as a treatment for chronic wounds in diabetic patients.

5.1.7. While HBP activation is detrimental, high viscosity accelerates wound healing

Summing up the first part of the project, HBP activation by GlcNAc supplementation altered GlcNAc, UDP-HexNAc and HA levels better in keratinocytes than in fibroblasts. This could rely on their different physiological property and also their function in wound healing.

Consequently, 50 mM GlcNAc supplementation caused a reduced migration speed in keratinocytes while fibroblast migration showed no difference to control. Regarding fibroblasts, the treatment with 50 mM GlcNAc might affect ECM positively in regard of abolishing the inhibitory influence of 25 mM glucose supplementation seen by *Lamers et al.*[13] Keratinocytes treated with 50 mM GlcNAc presented slower migration and slower wound closure. There is probably a change in ECM due to HBP activation. Which modification HBP activation induces and if it alters cell adhesions needs to be further investigated. If alterations in cytokine

concentration, in inflammatory signals or in cell adhesion or toxic effects by increased HBP flux cause the deficient wound healing remains to be discovered. To address this, we could measure cytokine and regulatory signal levels, search for alteration in cell-cell and cell-matrix interactions and for changes due to raised O-GlcNAcylation.

Finally, we saw no effect on wound healing in fibroblasts which might be due to an insufficient activation of the HBP. In keratinocytes, HBP activation influences the ECM and wound healing process negatively. Even though studies on longevity and protein quality control in *C. elegans* were promising that HBP activation might stimulate cell migration. It is important to unravel the present alterations, first for a deeper understanding of wound healing and secondly, for possible therapeutic strategies on non-healing wounds. Importantly, I could show that HMW dextran accelerated cell migration as also HA treatment did. As they both have a similar viscous feature, consequently, we should test the effect of viscosity on wound healing further. By raising UDP-HexNAc and HA concentration by GlcNAc supplementation it might increase viscosity too much affecting cell migration in the wound healing process *in vitro* negatively. Therefore, future research should focus on the viscosity effect in wound healing and how medical treatment could take advantage of it.

5.2 The effect of sugar supplementation on proliferative potential of keratinocytes

Wound healing depends on efficient cell migration and proliferation. The skin ECM plays an important role in many cellular functions providing mechanical properties as well as regulating cell behavior, including migration and proliferation.[11,33,102] It consists mainly of interstitial matrix and structural proteins, adhesive glycoproteins and proteoglycans like GAGs and HA.[102,103]
In the second part of the project, colony formation assays tested the effect of different sugars on proliferative potential in primary murine keratinocytes since an effective proliferation potential is essential for adequate reepithelialization after injury.

Either primary or immortalized fibroblasts were used as a feeder layer in previous experiments. *Kaviani et al*. proved that human fibroblasts treated with mitomycin C keep optimal conditions for such a test on proliferation.[199] *Jensen et al.* used murine fibroblasts effectively.[136] For the sugar treatments, we chose GlcNAc, Glc and Man because previous studies in the laboratory showed that these treatments increased the number of hair follicle stem cells in an *in vitro* culture system.[12] As proliferation is a key feature of stem cells which can be raised by these treatments, it was interesting to test their effect on keratinocytes whose proliferation is highly required in wound healing.

5.2.1. GlcNAc treatment does not change the proliferative potential of keratinocytes

Figure 19 depicts that GlcNAc supplementation increases UDP-HexNAc levels in keratinocytes in my experiments. Consequently, I hypothesized that an elevated UDP-GlcNAc level would lead to an increased cell proliferative potential. Surprisingly, this was not the case in the tissue culture monolayer experiment. 50 mM GlcNAc treatment showed no significant effect on proliferative potential of keratinocytes having been cultivated on NIH3T3 feeder layer (98% vs. control 100%) (Figure 25A + C). A diminution of 7% was seen for primary fibroblasts feeder layer (93% vs. 100%) (Figure 25B + D). As this experimental set up causes limitations, there need to be *in vivo* experiments for definitive conclusion about wound healing.

Previous studies suggested that O-GlcNAcylation is important for the regulation of cell growth: without the capability of O-GlcNAcylation mice embryo were not viable.[200,201] O-GlcNAc cycling is assumed to be crucial as a global regulator in the cell cycle.[202] Accordingly, increased O-GlcNAcylation is described in cancers.[203–208] At the same time, UDP-GlcNAc concentration was twelve times higher in breast cancer biopsies than in normal breast tissue.[209] An imbalance in terms of increased UDP-GlcNAc pool causes reduced ability of differentiation and cell growth arrest in human colon cancer HT-29 cells.[210] Moreover, decreased UDP-GlcNAc level seemed to lead to defects in proliferation of embryonic cells.[211]
It might be that a defined level of UDP-GlcNAc is important for the maintenance of cellular function as proliferation but even a raise does not increase the proliferative potential. Studies in cancer cells have showed that an increased O-GlcNAcylation can lead to an increased proliferation, although the mechanism is still not fully understood. Further research is needed to understand the underlying mechanism. To get a deeper understanding of our experiment, we could measure O-GlcNAcylation before and after treatment.

In sum, proliferation might not be affected by GlcNAc supplementation. Given the narrow range of the UDP-GlcNAc level that is needed for efficient cell function, it would be interesting to test different concentrations of GlcNAc in the future.

5.2.2. Glucose might reduce proliferation of keratinocytes

Supplementation of 50 mM Glc decreased the proliferative potential significantly regarding the 40% decrease to control group (60% vs. control 100%) with NIH3T3 feeder layer (Figure 25A + C) and 30% (70% vs. 100%) with primary fibroblasts (Figure 25B + D). One would expect the opposite effect of Glc treatment when taking into consideration that high glucose uptake is a key feature of tumor cells which present rapidly proliferating behavior. These cells and also

stem cells use aerobic glycolysis.[212,213] By using rather glycolysis, oxidative phosphorylation becomes reduced despite oxygen availability so that ATP synthesis compensates with an increased uptake of energy metabolites (the so-called Warburg effect).[214] Glucose belongs to the metabolites with an increased uptake as well as glutamine and fatty acids in cancer cells.[215,216] Yet, we did not use tumor cells for the experiment. This means that the Warburg effect does not consequently occur when cells are treated with glucose raising the available glucose concentration. The oxidative phosphorylation still seems to be the predominant metabolism pathway. This would not directly conclude a raise in proliferation due to an increased glucose level.

Glucose can be used to activate the HBP and therefore to test its effect as glucose, glutamine and UTP are used in the HBP to provide UDP-GlcNAc.[165] *Wice et al*. tested the effect of 25 mM fructose or glucose or +/- nucleosides on human colon cancer cells. Interestingly, there was an increase of UDP-HexNAc level in these cells.[210] It is therefore possible to activate the HBP by glucose supplementation. However, that only a raise of UDP-HexNAc is the reason for decreased proliferation does not seem to be possible, since GlcNAc treatment, which should be more efficient in raising UDP-HexNAc levels, did not affect the proliferative potential.

It seems reasonable that a diabetic-like state explains the finding. As we discussed the impact of high-glucose environment on migration in my first part of the project (compare 5.1.4.), a raised glucose concentration leads also to reduced proliferation of keratinocytes. Animal studies confirmed a decrease on proliferation in diabetic conditions.[217] *Lan et al*. showed an inhibition of proliferation in keratinocytes on day 5 after 25 mM and on day 7 also for 12 mM glucose treatment.[181] Cells treated with high glucose concentration (20 mM) compared to low glucose concentration (2 mM) presented differences in morphology. They expanded, flattened and orientation toward each other seemed to be reduced.[218] This might explain the decreased proliferation.

Other studies have revealed an ambivalence of glucose effect on proliferation. Whereas Glc treatment increased the proliferation rate in, for example, smooth muscle cells, it decreased it in endothelial cells or in dermal fibroblasts.[219,220] This all emphasizes the dependence on cell type. Moreover, studies illustrated even a dependency on incubation time and different serum concentration which affected the outcome of Glc treatment.[221–223]

In conclusion, Glc treatment seems to require an absolute fine tuning to guarantee an increased proliferation rate. One could try to establish the best condition for this in further steps. Yet, if this treatment is so susceptible to smallest changes in environment, one must doubt if this treatment could be effective in clinical use. Moreover, wound closure is already reduced in diabetic patients. Adding glucose would therefore probably amplify the negative effect.

For further investigation on decreased proliferation, we could compare Ki67 as proliferation marker in control group and keratinocytes treated with 50 mM Glc. If this treatment inhibits proliferation, we would expect a reduction of Ki67 level.

5.2.3. Mannose treatment decreases the proliferative potential of keratinocytes

My experiments performed with 50 mM Man showed a significant inhibition of proliferation to 0% in NIH3T3 (Figure 25A + C) and a decrease to 10% compared to control in primary fibroblasts feeder layer (Figure 25B + D).

Jokela et al. tested mannose's effect on HA synthesis and proliferation in rat epidermal keratinocytes.[127] They found no change in HA synthesis for 20 mM glucose, galactose, and fructose treatments and no change in proliferation for 20 mM galactose. But they described a reduction in HA synthesis of around 50% by a similar decrease of UDP-HexNAc level when they treated the cells with 20 mM Man. Concerning proliferation, 5 mM Man treatment caused a reduction of cell number of 33% over a period of five days. 10 and 20 mM treatment even led to a decrease by 58%.[127] As I used a higher mannose concentration for treatment and it resulted in a higher decrease, it supports the dose-dependent effect seen.

Mannose treatment in other cell lines (fibroblasts, melanoma cells, keratinocytes and hair follicle stem cells) has been reported to cause a similar inhibition of HA synthesis.[12,127,224] Therefore, I assume a reduction of HA levels, which might be causal for the reduced proliferative potential seen upon mannose treatment. It is discussed but not completely understood how mannose decreases UDP-HexNAc and HA synthesis. Because of its fast effect, an alteration in transcription is excluded as well as transcriptional regulation of HBP enzymes as neither the mRNA level of GFAT-1 nor of glucosamine-6-phosphate deaminase (GNPDA) enzymes changed.[127] Moreover, it is not due to osmotic pressure or an increase of energy metabolites as glucose, galactose and fructose, which are related isomers, could not present a similar effect. Also, no direct cell toxicity of mannose was observed.[127] Instead, it is supposed that mannose induces a backward flux of the HBP through altered GNPDA function (Figure 26). GNPDA is an enzyme that can regulate the HPB in both directions: Fru6P to GlcN6P towards UDP-GlcNAc but also GlcN6P backwards to Fru6P depending on the need of hexosamine concentration.[225] Indeed, mannose-6-phosphate regulates GNPDA increasing the GlcN6P to Fru6P reaction which leads to a reduction in UDP-GlcNAc level and therefore HA production.[225,226] As HA is also relevant for cell proliferation, a decrease of HA level due to mannose treatment might explain the decline of proliferation potential in my experiments. To assure such a reducing effect of HA level in my experiments, ELISA measurements could be performed. In the future, GNPDA activity should be measured upon mannose supplementation *in vitro*. If further study confirms the effect on GNPDA, mannose analogues might be used one

day as therapeutic strategies to suppress HA production, and thereby proliferation, in cancer.[227]

Another possible target of mannose might be GFAT (Figure 26). The team of *Jokela* showed that Man treatment reduced UDP-GlcNAc level but adding exogenous GlcN completely abrogated this result. As exogenous GlcN enters the pathway as GlcN6P, it seems that Man treatment affects the pathway above the conversion of Fru6P to GlcN6P by GFAT.[127] That this enzyme is important in the synthesis of UDP-GlcNAc shows the depletion of GFAT-1 which caused a decrease in UDP-GlcNAc level and HA synthesis.[227] Then again, a gain-of-function of GFAT-1 enhances UDP-GlcNAc level as shown for *C. elegans* as well as for mammalian cells.[2,90] This indicates its role in regulating the concentration of UDP-GlcNAc. As GFAT-1 is a key player in HBP it is likely that it can be modulated by exogenous supplementation, here Man.

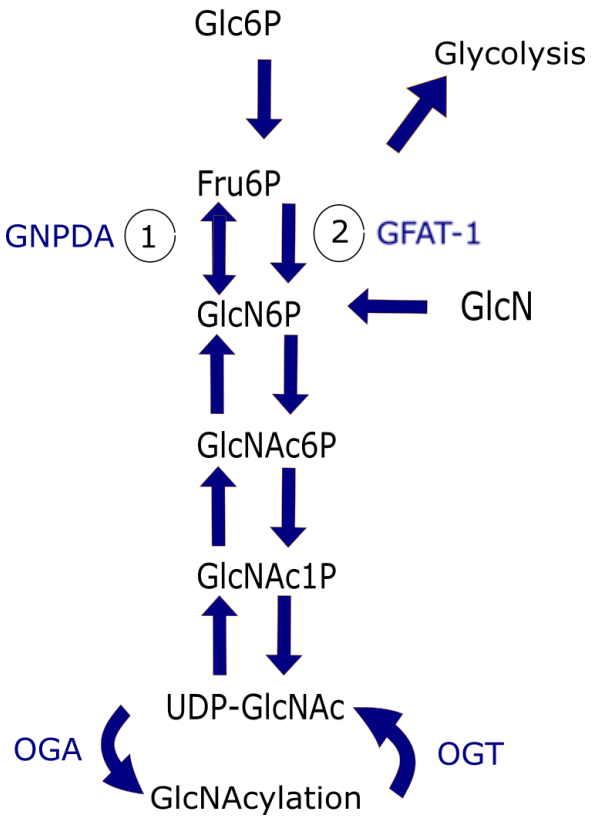


Figure 26: Possible targets for mannose causing a backward flux and a reduction of UDP-GlcNAc

1. Mannose could regulate GNPDA to convert hexosamines to fructose-6-phosphate which reduces UDP-GlcNAc level.

2. Mannose could regulate GFAT-1 so that the conversion of fructose-6-phosphate to D-glucosamine-6-phosphate is lowered/inhibited. Modified from Denzel MS et al. 2014.[2]

It is also possible that mannose acts as a competitive substrate in the HA synthesis, like 4-methylumbelliferone (4-MU), a coumarin derivative.[112] It affects HA production negatively by being a competitive substrate for UDP-glucuronosyltransferase (UGT). UGT binds UDP to glucuronic acid, leading to UDP-GlcUA.[228] Although UDP-GlcNAc is mainly produced by the HBP, also UGT can bind UDP to N-acetylglucosamine leading to UDP-GlcNAc (Figure *27*A).[229] These two, UDP-GlcUA and UDP-GlcNAc, are needed for HA synthesis. Now, 4-MU can bind to glucuronic acid instead of UDP and can therefore lead to a reduction in UDP-GlcUA concentration and also in HA production (Figure 27B).[229]

There are three imaginable ways for mannose treatment to interact and reduce UDP-GlcNAc levels (Figure 27C). As discussed above, mannose treatment is likely to interact in the HA synthesis. First, mannose could compete with UDP to bind N-acetylglucosamine which reduces the concentration of UDP-GlcNAc. Second, mannose could bind GlcUA decreasing the UDP-GlcUA level. Both can cause an imbalance between UDP-GlcNAc and UDP-GlcUA like 4-MU treatment does, so that the organism needs to react to remedy this imbalance. This could be done by shifting the higher proportion of UDP-GlcNAc in relation to UDP-GlcUA which results in less UDP-GlcNAc. Third, mannose could interfere with UGT consequently reducing/inhibiting the conversion from N-acetyl-glucosamine to UDP-GlcNAc and from glucuronic acid to UDP-GlcUA. Thus, the substrate supply for HA production is reduced. To investigate which of these effects occurs upon mannose treatment, the levels of UDP-GlcNAc and UDP-GlcUA should be determined in future experiments.

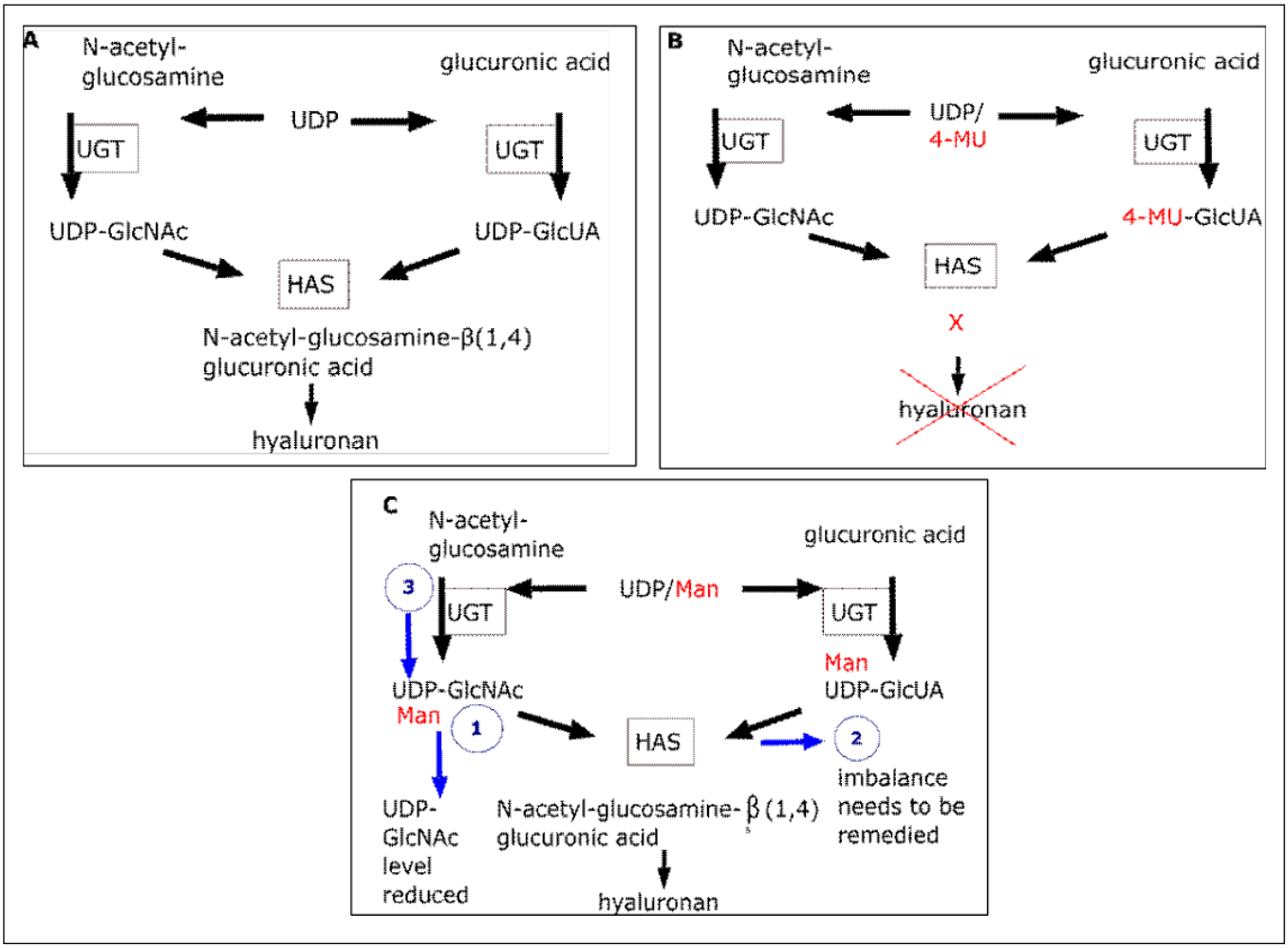


Figure 27: Possible targets of mannose in the HA synthesis

A *This scheme shows the physiological HA synthesis whereas hyaluronan synthetases use UDP-GlcNAc and UDP-GlcUA to produce hyaluronan.*

B *This scheme shows the postulated mechanism of 4-MU interfering in the HA synthesis.*

C *Schematic figure of possible targets of mannose that could lead to reduced UDP-GlcNAc and HA levels. 1. Mannose could compete with UDP to bind to GlcNAc. Consequently, it would cause a lower UDP-GlcNAc level. 2. Mannose could bind to GlcUA leading to less UDP-GlcUA. To remedy this imbalance between UDP-GlcUA and UDP-GlcNAc the level of UDP-GlcNAc would decrease to shunt UDP to GlcUA. 3. Mannose could affect UGT so that less/no conversion to UDP-GlcNAc or UDP-GlcUA would take place.*

Modified from Nagy N et al. 2015.[229]

In conclusion, mannose treatment decreases proliferative potential of primary keratinocytes as it likely inhibits HA synthesis probably due to backward flux of the HBP. How it actually works at a mechanistic level remains a question for further research.

In summary, the three sugar treatments presented different outcomes on proliferation of keratinocytes in colony formations assays. First, HBP activation by GlcNAc supplementation had no effect on proliferation in keratinocytes. In regard to the reducing effect on keratinocyte proliferation caused by the other two sugars, Glc and Man, there might even be a protective effect by HBP activation that maintains a normal proliferation rate. It remains unclear how ECM gets affected so that research needs to be continued. Second, Glc treatment showed a mild decrease on proliferation. Focusing on the link between HBP and diabetes, where keratinocytes show also less proliferation, this might explain the findings. Yet, consequently, one would also consider a diabetic-like effect of GlcNAc treatment. As hypothesized in the first part of the project, there could be a different way GlcNAc gets shunted into the HBP that could explain the difference. Third, Man treatment inhibits proliferation. It is assumed that it decreases UDP-HexNAc and HA levels which would present the opposite of activating HBP. In fact, it states the importance of high UDP-HexNAc levels for proliferation ability.

5.3 Outlook

Now, as we try to understand the mechanisms behind the findings, further experiments could be considered. I would like to mention several aspects for further research.

My experiments in fibroblasts showed only a slight change in the concentrations of GlcNAc, UDP-HexNAc and HA upon GlcNAc supplementation which might explain why we did not see changes in migration speed of fibroblasts in wound healing assays. Considering the idea of a possible maximum of an advantageous UDP-GlcNAc level that cannot be enhanced further by GlcNAc supplementation, we could adapt the glucose concentration in the experiments. *Lamers et al*. saw a reduction of around 40% in migration speed in fibroblasts of rats by 25 mM D-glucose.[13] My experiments did not support the idea of increasing this inhibitory effect. Yet, my cells were cultivated in a hyperglycemic state in high glucose medium (25 mM D-glucose) and additionally treated by 50 mM GlcNAc. Now, we could repeat the wound healing assays in low glucose medium (5 mM D-glucose) and add 50 mM GlcNAc to investigate how HBP activation in lower glucose level affects cell migration. If there is slowed migration, this could support the idea of a UDP-GlcNAc maximum so that a high UDP-GlcNAc level is just not more advantageous. On the other hand, a higher UDP-GlcNAc level could have a protective function when migration is reduced by 25 mM D-glucose environment but not by additional 50 mM GlcNAc supplementation.

Furthermore, we could take a closer look on alterations of cell-cell and cell-matrix adhesions caused by GlcNAc supplementation. As described in 5.1.5., the interplay of loosening and strengthening these adhesions is essential for cell migration and consequently for effective

wound healing. Increased O-GlcNAcylation might lead to higher tension in adhesion. To unravel the effect of GlcNAc supplementation on adhesions, we could perform adhesion tests using CytoTox 96 like described in *Grose et al.,* in the protocol of *Chen* or in the review of *Khalili and Ahmad*.[150,151,230] As elevated phosphorylated $p125^{FAK}$ is indispensable for cytoskeletal protein modifications concerning cell migration,[184] it would be worth to test its concentration and expression in my experiments to depict an alteration eventually caused by elevated UDP-GlcNAc concentration.

Next, we could compare the effect of HBP activation on wound healing by GlcNAc versus glucose supplementation. This could test if GlcNAc really goes into the HBP and increases its flux as it is seen for excessive glucose. In addition, we could also use binding probe assays. To examine the global change of O-GlcNAcylation, we could perform Western blot analysis using anti-O-GlcNAc antibody (RL2) and molecular analyses.[231] O-GlcNAc modifications are regulated by the activity of OGT and OGA so that it remains in an adequate balance. Chronic disruption of this homeostatic balance increases the prevalence of diabetes, cancer, and Alzheimer's disease.[99,185,187,188] This emphasizes the narrow range of O-GlcNAcylation for optimal physiological conditions. One could investigate the wound closure rate using 10, 15, 20 and 25 mM GlcNAc supplementation to confirm a positive effect and to narrow down the best range of UDP-GlcNAc and O-GlcNAc level.

Another aspect can lead to impaired wound healing. It was observed that an imbalance of signals towards a prolonged inflammation leads to chronic wounds.[80] As this complex process of wound healing requires regulators, we could take a closer look on the cytokines. One of the main cytokine associated to such persistence is TNF-α.[232] Others elevated cytokines in chronic wounds are IL-1β, TNF-α, MMP, fibronectin, collagenase and gelatinases A and B. EGF, FGF, TGF-β, PDGF and VEGF and probably IL-6 and G-CSF diminish on the other hand.[83–85] To examine if GlcNAc supplementation causes changes in cytokine and kinase concentration, it would be of interest to measure IL-1β, TNF-α, MMPs, fibronectin, IL-8, IL-6 and G-CSF in control and treated group. O-GlcNAc can modify and also regulate kinases.[233–236] There could be a switch in a regulator that explains the slowing effect on keratinocyte migration. ELISA, multiplex assays, Cytokine Bead Array (Biosciences) or flow-based Cytokine Capture Assays (Miltenyi Biotec) can investigate cytokine secretion.

GlcNAc, which is an intermediate of the HBP, was used for HBP activation. If its HBP activation could cause cell damage cannot be excluded. Therefore, it would be interesting to test for oxidative stress. We could measure the reactive oxygen species production as well as the

apoptotic rate during wound healing assays with GlcNAc supplementation to exclude that the reduced migration seen in keratinocytes is due to elevated oxidative stress.

The experiments on cell proliferation performed with fibroblasts and keratinocytes showed no effect by GlcNAc treatment, a slight reducing effect by Glc treatment, and an inhibitory effect by Man treatment. Previous research revealed a positive effect on cell proliferation of cancer cells when O-GlcNAc modifications were high. My experiments, though, did not lead to a higher proliferative potential in keratinocytes by GlcNAc treatment, yet we did not measure O-GlcNAcylation. To get a better understanding on O-GlcNAc modulation, we should measure O-GlcNAcylation before and after different GlcNAc treatment. We could also compare in which way GlcNAc supplementation changes the O-GlcNAcylation in cancer tissue compared to keratinocytes used in my experiments to understand if any advantage for proliferation occurs.

Also, the fact that 50 mM GlcNAc treatment did not increase proliferation might indicate the importance of a narrow range of UDP-GlcNAc level for efficient cell function. The team of *Mavrogonatou and Kletsas* stated a dose dependency effect for glucosamine sulfate on cell function of nucleus pulposus cells. Whereas 1 mM glucosamine did not affect viability, proliferation rate or DNA synthesis, 10 mM glucosamine affected proliferation negatively.[237] They concluded that hyperosmotic stress caused the difference. We could test more doses between 10 and 50 mM GlcNAc to determine when the switch between no effect and negative effect happens. We could further add D-Arginine as a compound with a similar osmolarity to take a closer look at possible hyperosmotic stress. In the next steps, the focus would lay on differences in cellular function, in ECM and in signaling.

One of the potential reasons why GlcNAc supplementation slows cell migration in keratinocytes is a diabetic-like state. In this context, it would be interesting to take biopsies of diabetic patients of non-damaged skin and wound areas as well as biopsies of healthy skin of control to compare GlcNAc and HA levels. If GlcNAc levels are elevated in patients' skin, it would confirm its involvement in diabetic wound healing processes. Especially, if an elevated GlcNAc pool is found in biopsies of wound areas in diabetic patients.

Hyperglycemia in diabetes diminishes keratinocyte proliferation. In my experiments, the same trend is shown for Glc treatment. For further investigation of proliferation, we could compare Ki67 as proliferation marker in control group and keratinocytes treated with 50 mM GlcNAc and

50 mM Glc. If this treatment inhibits proliferation, we would expect a reduction of Ki67 level. One could try to establish the best condition for this in further steps.

Man treatment inhibited proliferation. As the complete mechanism is not yet understood, we could test whether and how this supplementation decreases UDP-HexNAc levels and how it stops cell proliferation. A decrease of HA production seems likely. Metabolic flux analysis could detect if Man treatment really affects metabolite turnover here. Also, possible targets are GNPDA and GFAT which lead to a backward flux of the HBP. Man treatment might alter these enzymes on which biochemical structure analysis and *in vitro* activity assays could deliver more information.

All in all, my project underlines the significance of the HBP for effective cellular function including migration and proliferation, which are required for wound healing. In the future, more experiments should be performed to unravel its complete effect and to possibly identify targets for clinical application. One of them could be a medium with viscous feature. My experiments showed that HMW dextran had a similar positive effect on wound healing as HA treatment. As both treatments are characterized by a comparable viscosity, further research should also consider viscosity as a possible medical strategy for chronic wound treatment.

6. References

1 Chiaradonna F, Ricciardiello F, Palorini R. The Nutrient-Sensing Hexosamine Biosynthetic Pathway as the Hub of Cancer Metabolic Rewiring. *Cells* 2018; **7.** **https://doi.org/**10.3390/cells7060053.

2 Denzel MS, Storm NJ, Gutschmidt A, et al. Hexosamine pathway metabolites enhance protein quality control and prolong life. *Cell* 2014; **156:** 1167–78. https://doi.org/10.1016/j.cell.2014.01.061.

3 Horn M, Denzel SI, Srinivasan B, et al. Hexosamine Pathway Activation Improves Protein Homeostasis through the Integrated Stress Response. *iScience* 2020; **23:** 100887. https://doi.org/10.1016/j.isci.2020.100887.

4 D'Agostino A, Stellavato A, Busico T, et al. In vitro analysis of the effects on wound healing of high- and low-molecular weight chains of hyaluronan and their hybrid H-HA/L-HA complexes. *BMC Cell Biol* 2015; **16:** 19. https://doi.org/10.1186/s12860-015-0064-6.

5 David-Raoudi M, Tranchepain F, Deschrevel B, et al. Differential effects of hyaluronan and its fragments on fibroblasts: relation to wound healing. *Wound Repair Regen* 2008; **16:** 274–87. https://doi.org/10.1111/j.1524-475X.2007.00342.x.

6 McDonald J, Hascall VC. Hyaluronan minireview series. *J Biol Chem* 2002; **277:** 4575–79. https://doi.org/10.1074/jbc.R100064200.

7 Lee JY, Spicer AP. Hyaluronan: a multifunctional, megaDalton, stealth molecule. *Curr Opin Cell Biol* 2000; **12:** 581–86. https://doi.org/10.1016/S0955-0674(00)00135-6.

8 Poulsen JH, Jensen IM, Petersen U. D-3Hglucosamine labelling of epidermal and dermal glycosaminoglycans in cultured human skin. *J Clin Chem Clin Biochem* 1988; **26:** 123–33.

9 Nussbaum SR, Carter MJ, Fife CE, et al. An Economic Evaluation of the Impact, Cost, and Medicare Policy Implications of Chronic Nonhealing Wounds. *Value Health* 2018; **21:** 27–32. https://doi.org/10.1016/j.jval.2017.07.007.

10 Olsson M, Järbrink K, Divakar U, et al. The humanistic and economic burden of chronic wounds: A systematic review. *Wound Repair Regen* 2018. https://doi.org/10.1111/wrr.12683.

11 Bateman JF, Boot-Handford RP, Lamandé SR. Genetic diseases of connective tissues: cellular and extracellular effects of ECM mutations. *Nat Rev Genet* 2009; **10:** 173–83. https://doi.org/10.1038/nrg2520.

12 Allmeroth K. Metabolic control of hair follcile stem cell fate decisions. PhD thesis. Köln, Deutschland, 2020.

13 Lamers ML, Almeida MES, Vicente-Manzanares M, Horwitz AF, Santos MF. High glucose-mediated oxidative stress impairs cell migration. *PLoS ONE* 2011; **6:** e22865. https://doi.org/10.1371/journal.pone.0022865.

14 Arda O, Göksügür N, Tüzün Y. Basic histological structure and functions of facial skin. *Clin Dermatol* 2014; **32:** 3–13. https://doi.org/10.1016/j.clindermatol.2013.05.021.

15 Venus M, Waterman J, McNab I. Basic physiology of the skin. *Surgery (Oxford)* 2011; **29:** 471–74. https://doi.org/10.1016/j.mpsur.2011.06.010.

16 Schittek B, Paulmann M, Senyürek I, Steffen H. The role of antimicrobial peptides in human skin and in skin infectious diseases. *Infect Disord Drug Targets* 2008; **8:** 135–43.

17 Clemens TL, Adams JS, Henderson SL, Holick MF. Increased skin pigment reduces the capacity of skin to synthesise vitamin D3. *Lancet* 1982; **1:** 74–76.
18 Mostafa WZ, Hegazy RA. Vitamin D and the skin: Focus on a complex relationship: A review. *J Adv Res* 2015; **6:** 793–804. https://doi.org/10.1016/j.jare.2014.01.011.
19 Lüllmann-Rauch R, Paulsen F. Taschenlehrbuch Histologie. 10 Tabellen. Stuttgart: Thieme, 2012.
20 Gartner LP, Hiatt JL. Color Textbook of Histology. s.l.: Elsevier Health Care - Lehrbücher, 2006.
21 Aumüller G. Anatomie. Stuttgart: Thieme, 2014.
22 Elias PM. Structure and function of the stratum corneum extracellular matrix. *J Invest Dermatol* 2012; **132:** 2131–33. https://doi.org/10.1038/jid.2012.246.
23 Ayala-Fontánez N, Soler DC, McCormick TS. Current knowledge on psoriasis and autoimmune diseases. *Psoriasis (Auckl)* 2016; **6:** 7–32. https://doi.org/10.2147/PTT.S64950.
24 Rendon A, Schäkel K. Psoriasis Pathogenesis and Treatment. *Int J Mol Sci* 2019; **20. https://doi.org/**10.3390/ijms20061475.
25 Lai-Cheong JE, McGrath JA. Structure and function of skin, hair and nails. *Medicine* 2009; **37:** 223–26. https://doi.org/10.1016/j.mpmed.2009.03.002.
26 Tobin DJ. Biochemistry of human skin--our brain on the outside. *Chem Soc Rev* 2006; **35:** 52–67. https://doi.org/10.1039/b505793k.
27 Gwashu A. Protective properties of rooibos (Aspalathus linearis) flavonoids on the prevention of skin cancer. https://scholar.sun.ac.za/handle/10019.1/98536 (accessed Mar 01, 2022).
28 Oikarinen A. The aging of skin: chronoaging versus photoaging. *Photodermatol Photoimmunol Photomed* 1990; **7:** 3–4.
29 Lazarus GS, Cooper DM, Knighton DR, Percoraro RE, Rodeheaver G, Robson MC. Definitions and guidelines for assessment of wounds and evaluation of healing. *Wound Repair Regen* 1994; **2:** 165–70. https://doi.org/10.1046/j.1524-475X.1994.20305.x.
30 Singer AJ, Clark RA. Cutaneous wound healing. *N Engl J Med* 1999; **341:** 738–46. https://doi.org/10.1056/NEJM199909023411006.
31 Friedl P, Gilmour D. Collective cell migration in morphogenesis, regeneration and cancer. *Nat Rev Mol Cell Biol* 2009; **10:** 445–57. https://doi.org/10.1038/nrm2720.
32 Rittié L, Sachs DL, Orringer JS, Voorhees JJ, Fisher GJ. Eccrine sweat glands are major contributors to reepithelialization of human wounds. *Am J Pathol* 2013; **182:** 163–71. https://doi.org/10.1016/j.ajpath.2012.09.019.
33 Eming SA, Martin P, Tomic-Canic M. Wound repair and regeneration: mechanisms, signaling, and translation. *Sci Transl Med* 2014; **6:** 265sr6. https://doi.org/10.1126/scitranslmed.3009337.
34 Dallon J, Sherratt J, Maini P, Ferguson M. Biological implications of a discrete mathematical model for collagen deposition and alignment in dermal wound repair. *IMA J Math Appl Med Biol* 2000; **17:** 379–93.
35 Sorg H, Tilkorn DJ, Hager S, Hauser J, Mirastschijski U. Skin Wound Healing: An Update on the Current Knowledge and Concepts. *Eur Surg Res* 2017; **58:** 81–94. https://doi.org/10.1159/000454919.
36 Tomasek JJ, Gabbiani G, Hinz B, Chaponnier C, Brown RA. Myofibroblasts and mechano-regulation of connective tissue remodelling. *Nat Rev Mol Cell Biol* 2002; **3:** 349–63. https://doi.org/10.1038/nrm809.

37 Rodrigues M, Kosaric N, Bonham CA, Gurtner GC. Wound Healing: A Cellular Perspective. *Physiol Rev* 2019; **99:** 665–706. https://doi.org/10.1152/physrev.00067.2017.
38 Furie B, Furie BC. Mechanisms of thrombus formation. *N Engl J Med* 2008; **359:** 938–49. https://doi.org/10.1056/NEJMra0801082.
39 Schecter AD, Spirn B, Rossikhina M, et al. Release of active tissue factor by human arterial smooth muscle cells. *Circ Res* 2000; **87:** 126–32.
40 Butenas S. Tissue factor structure and function. *Scientifica (Cairo)* 2012; **2012:** 964862. https://doi.org/10.6064/2012/964862.
41 Diegelmann RF, Evans MC. Wound healing: an overview of acute, fibrotic and delayed healing. *Front Biosci* 2004; **9:** 283–89.
42 Clark RA. Fibrin and wound healing. *Ann N Y Acad Sci* 2001; **936:** 355–67.
43 Kim WJ, Gittes GK, Longaker MT. Signal transduction in wound pharmacology. *Arch Pharm Res* 1998; **21:** 487–95.
44 Hall M-C, Young DA, Waters JG, et al. The comparative role of activator protein 1 and Smad factors in the regulation of Timp-1 and MMP-1 gene expression by transforming growth factor-beta 1. *J Biol Chem* 2003; **278:** 10304–13. https://doi.org/10.1074/jbc.M212334200.
45 Myers SR, Leigh IM, Navsaria H. Epidermal repair results from activation of follicular and epidermal progenitor keratinocytes mediated by a growth factor cascade. *Wound Repair Regen* 2007; **15:** 693–701. https://doi.org/10.1111/j.1524-475X.2007.00297.x.
46 Jorch SK, Kubes P. An emerging role for neutrophil extracellular traps in noninfectious disease. *Nat Med* 2017; **23:** 279–87. https://doi.org/10.1038/nm.4294.
47 Jacinto A, Martinez-Arias A, Martin P. Mechanisms of epithelial fusion and repair. *Nat Cell Biol* 2001; **3:** E117-23. https://doi.org/10.1038/35074643.
48 Rittié L. Cellular mechanisms of skin repair in humans and other mammals. *J Cell Commun Signal* 2016; **10:** 103–20. https://doi.org/10.1007/s12079-016-0330-1.
49 Santoro MM, Gaudino G. Cellular and molecular facets of keratinocyte reepithelization during wound healing. *Exp Cell Res* 2005; **304:** 274–86. https://doi.org/10.1016/j.yexcr.2004.10.033.
50 Cotsarelis G. Epithelial stem cells: a folliculocentric view. *J Invest Dermatol* 2006; **126:** 1459–68. https://doi.org/10.1038/sj.jid.5700376.
51 Liu Y, Lyle S, Yang Z, Cotsarelis G. Keratin 15 promoter targets putative epithelial stem cells in the hair follicle bulge. *J Invest Dermatol* 2003; **121:** 963–68. https://doi.org/10.1046/j.1523-1747.2003.12600.x.
52 Blanpain C, Fuchs E. Stem cell plasticity. Plasticity of epithelial stem cells in tissue regeneration. *Science* 2014; **344:** 1242281. https://doi.org/10.1126/science.1242281.
53 Safferling K, Sütterlin T, Westphal K, et al. Wound healing revised: a novel reepithelialization mechanism revealed by in vitro and in silico models. *J Cell Biol* 2013; **203:** 691–709. https://doi.org/10.1083/jcb.201212020.
54 Bornstein P, Sage E. Matricellular proteins: extracellular modulators of cell function. *Curr Opin Cell Biol* 2002; **14:** 608–16. https://doi.org/10.1016/S0955-0674(02)00361-7.
55 Salonurmi T, Parikka M, Kontusaari S, et al. Overexpression of TIMP-1 under the MMP-9 promoter interferes with wound healing in transgenic mice. *Cell Tissue Res* 2004; **315:** 27–37. https://doi.org/10.1007/s00441-003-0814-1.
56 Pilcher BK, Dumin JA, Sudbeck BD, Krane SM, Welgus HG, Parks WC. The activity of collagenase-1 is required for keratinocyte migration on a type I collagen matrix. *J Cell Biol* 1997; **137:** 1445–57. https://doi.org/10.1083/jcb.137.6.1445.

57 Krampert M, Bloch W, Sasaki T, et al. Activities of the matrix metalloproteinase stromelysin-2 (MMP-10) in matrix degradation and keratinocyte organization in wounded skin. *Mol Biol Cell* 2004; **15:** 5242–54. https://doi.org/10.1091/mbc.e04-02-0109.
58 Artuc M, Hermes B, Steckelings UM, Grützkau A, Henz BM. Mast cells and their mediators in cutaneous wound healing--active participants or innocent bystanders? *Exp Dermatol* 1999; **8:** 1–16.
59 Ng MFY. The role of mast cells in wound healing. *Int Wound J* 2010; **7:** 55–61. https://doi.org/10.1111/j.1742-481X.2009.00651.x.
60 Theoharides TC, Alysandratos K-D, Angelidou A, et al. Mast cells and inflammation. *Biochim Biophys Acta* 2012; **1822:** 21–33. https://doi.org/10.1016/j.bbadis.2010.12.014.
61 Trautmann A, Toksoy A, Engelhardt E, Brcker E-B, Gillitzer R. Mast cell involvement in normal human skin wound healing: expression of monocyte chemoattractant protein-1 is correlated with recruitment of mast cells which synthesize interleukin-4in vivo. *J. Pathol.* 2000; **190:** 100–06. https://doi.org/10.1002/(SICI)1096-9896(200001)190:1<100::AID-PATH496>3.0.CO;2-Q.
62 Weller K, Foitzik K, Paus R, Syska W, Maurer M. Mast cells are required for normal healing of skin wounds in mice. *FASEB J* 2006; **20:** 2366–68. https://doi.org/10.1096/fj.06-5837fje.
63 Garbuzenko E, Nagler A, Pickholtz D, et al. Human mast cells stimulate fibroblast proliferation, collagen synthesis and lattice contraction: a direct role for mast cells in skin fibrosis. *Clin Exp Allergy* 2002; **32:** 237–46. https://doi.org/10.1046/j.1365-2222.2002.01293.x.
64 Abe M, Kurosawa M, Ishikawa O, Miyachi Y. Effect of mast cell-derived mediators and mast cell-related neutral proteases on human dermal fibroblast proliferation and type I collagen production. *J Allergy Clin Immunol* 2000; **106:** S78-84. https://doi.org/10.1067/mai.2000.106058.
65 Younan G, Suber F, Xing W, et al. The inflammatory response after an epidermal burn depends on the activities of mouse mast cell proteases 4 and 5. *J Immunol* 2010; **185:** 7681–90. https://doi.org/10.4049/jimmunol.1002803.
66 Dvorak AM. Mast cell-derived mediators of enhanced microvascular permeability, vascular permeability factor/vascular endothelial growth factor, histamine, and serotonin, cause leakage of macromolecules through a new endothelial cell permeability organelle, the vesiculo-vacuolar organelle. *Chem Immunol Allergy* 2005; **85:** 185–204. https://doi.org/10.1159/000086517.
67 Diegelmann RF, Cohen IK, Kaplan AM. The role of macrophages in wound repair: a review. *Plast Reconstr Surg* 1981; **68:** 107–13.
68 Koh TJ, DiPietro LA. Inflammation and wound healing: the role of the macrophage. *Expert Rev Mol Med* 2011; **13:** e23. https://doi.org/10.1017/S1462399411001943.
69 Sorokin L. The impact of the extracellular matrix on inflammation. *Nat Rev Immunol* 2010; **10:** 712–23. https://doi.org/10.1038/nri2852.
70 Gurtner GC, Werner S, Barrandon Y, Longaker MT. Wound repair and regeneration. *Nature* 2008; **453:** 314–21. https://doi.org/10.1038/nature07039.
71 Tandara AA, Mustoe TA. Oxygen in wound healing--more than a nutrient. *World J Surg* 2004; **28:** 294–300. https://doi.org/10.1007/s00268-003-7400-2.
72 Eilken HM, Adams RH. Dynamics of endothelial cell behavior in sprouting angiogenesis. *Curr Opin Cell Biol* 2010; **22:** 617–25. https://doi.org/10.1016/j.ceb.2010.08.010.
73 Liu L, Marti GP, Wei X, et al. Age-dependent impairment of HIF-1alpha expression in diabetic mice: Correction with electroporation-facilitated gene therapy increases wound

healing, angiogenesis, and circulating angiogenic cells. *J Cell Physiol* 2008; **217:** 319–27. https://doi.org/10.1002/jcp.21503.
74 Willenborg S, Lucas T, van Loo G, et al. CCR2 recruits an inflammatory macrophage subpopulation critical for angiogenesis in tissue repair. *Blood* 2012; **120:** 613–25. https://doi.org/10.1182/blood-2012-01-403386.
75 Broughton G, Janis JE, Attinger CE. The basic science of wound healing. *Plast Reconstr Surg* 2006; **117:** 12S-34S. https://doi.org/10.1097/01.prs.0000225430.42531.c2.
76 Okonkwo UA, DiPietro LA. Diabetes and Wound Angiogenesis. *Int J Mol Sci* 2017; **18. https://doi.org/**10.3390/ijms18071419.
77 Caley MP, Martins VLC, O'Toole EA. Metalloproteinases and Wound Healing. *Adv Wound Care (New Rochelle)* 2015; **4:** 225–34. https://doi.org/10.1089/wound.2014.0581.
78 Visse R, Nagase H. Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry. *Circ Res* 2003; **92:** 827–39. https://doi.org/10.1161/01.RES.0000070112.80711.3D.
79 Hornstra IK, Birge S, Starcher B, Bailey AJ, Mecham RP, Shapiro SD. Lysyl oxidase is required for vascular and diaphragmatic development in mice. *J Biol Chem* 2003; **278:** 14387–93. https://doi.org/10.1074/jbc.M210144200.
80 Eming SA, Hammerschmidt M, Krieg T, Roers A. Interrelation of immunity and tissue repair or regeneration. *Semin Cell Dev Biol* 2009; **20:** 517–27. https://doi.org/10.1016/j.semcdb.2009.04.009.
81 Barrientos S, Stojadinovic O, Golinko MS, Brem H, Tomic-Canic M. Growth factors and cytokines in wound healing. *Wound Repair Regen* 2008; **16:** 585–601. https://doi.org/10.1111/j.1524-475X.2008.00410.x.
82 Beidler SK, Douillet CD, Berndt DF, Keagy BA, Rich PB, Marston WA. Inflammatory cytokine levels in chronic venous insufficiency ulcer tissue before and after compression therapy. *J Vasc Surg* 2009; **49:** 1013–20. https://doi.org/10.1016/j.jvs.2008.11.049.
83 Tarnuzzer RW, Schultz GS. Biochemical analysis of acute and chronic wound environments. *Wound Repair Regen* 1996; **4:** 321–25. https://doi.org/10.1046/j.1524-475X.1996.40307.x.
84 Eming SA, Koch M, Krieger A, et al. Differential proteomic analysis distinguishes tissue repair biomarker signatures in wound exudates obtained from normal healing and chronic wounds. *J Proteome Res* 2010; **9:** 4758–66. https://doi.org/10.1021/pr100456d.
85 Yager DR, Zhang L-Y, Liang H-X, Diegelmann RF, Cohen IK. Wound Fluids from Human Pressure Ulcers Contain Elevated Matrix Metalloproteinase Levels and Activity Compared to Surgical Wound Fluids. *J Invest Dermatol* 1996; **107:** 743–48. https://doi.org/10.1111/1523-1747.ep12365637.
86 Guest JF, Fuller GW, Vowden P. Cohort study evaluating the burden of wounds to the UK's National Health Service in 2017/2018: update from 2012/2013. *BMJ Open* 2020; **10:** e045253. https://doi.org/10.1136/bmjopen-2020-045253.
87 Ghosh S, Blumenthal HJ, Davidson E, Roseman S. Glucosamine metabolism. V. Enzymatic synthesis of glucosamine 6-phosphate. *J Biol Chem* 1960; **235:** 1265–73.
88 Kato N, Dasgupta R, Smartt CT, Christensen BM. Glucosamine:fructose-6-phosphate aminotransferase: gene characterization, chitin biosynthesis and peritrophic matrix formation in Aedes aegypti. *Insect Mol Biol* 2002; **11:** 207–16.
89 Assrir N, Richez C, Durand P, et al. Mapping the UDP-N-acetylglucosamine regulatory site of human glucosamine-6P synthase by saturation-transfer difference NMR and site-directed mutagenesis. *Biochimie* 2014; **97:** 39–48. https://doi.org/10.1016/j.biochi.2013.09.011.

90 Ruegenberg S, Horn M, Pichlo C, Allmeroth K, Baumann U, Denzel MS. Loss of GFAT-1 feedback regulation activates the hexosamine pathway that modulates protein homeostasis. *Nat Commun* 2020; **11:** 687. https://doi.org/10.1038/s41467-020-14524-5.
91 Eguchi S, Oshiro N, Miyamoto T, et al. AMP-activated protein kinase phosphorylates glutamine : fructose-6-phosphate amidotransferase 1 at Ser243 to modulate its enzymatic activity. *Genes Cells* 2009; **14:** 179–89. https://doi.org/10.1111/j.1365-2443.2008.01260.x.
92 Schjoldager KT-BG, Clausen H. Site-specific protein O-glycosylation modulates proprotein processing - deciphering specific functions of the large polypeptide GalNAc-transferase gene family. *Biochim Biophys Acta* 2012; **1820:** 2079–94. https://doi.org/10.1016/j.bbagen.2012.09.014.
93 Liu F-C, Su C-R, Wu T-Y, et al. Efficient H-NMR quantitation and investigation of N-acetyl-d-glucosamine (GlcNAc) and N,N'-diacetylchitobiose (GlcNAc)(2) from chitin. *Int J Mol Sci* 2011; **12:** 5828–43. https://doi.org/10.3390/ijms12095828.
94 Held M, Meerbach E, Hinderlich S, Reutter W, Schütte C. Conformational Studies of UDP-GlcNAc in Environments of Increasing Complexity. In: Hansmann U, Meinke J, Mohanty S, Zimmermann O, eds. From Computational Biophysics to Systems Biology, 2007: 145–48.
95 Wang ZV, Deng Y, Gao N, et al. Spliced X-box binding protein 1 couples the unfolded protein response to hexosamine biosynthetic pathway. *Cell* 2014; **156:** 1179–92. https://doi.org/10.1016/j.cell.2014.01.014.
96 Roth J, Zuber C, Park S, et al. Protein N-glycosylation, protein folding, and protein quality control. *Mol Cells* 2010; **30:** 497–506. https://doi.org/10.1007/s10059-010-0159-z.
97 Denzel MS, Antebi A. Hexosamine pathway and (ER) protein quality control. *Curr Opin Cell Biol* 2015; **33:** 14–18. https://doi.org/10.1016/j.ceb.2014.10.001.
98 Parodi AJ. Role of N-oligosaccharide endoplasmic reticulum processing reactions in glycoprotein folding and degradation. *Biochem J* 2000; **348:** 1–13.
99 Hart GW, Slawson C, Ramirez-Correa G, Lagerlof O. Cross talk between O-GlcNAcylation and phosphorylation: roles in signaling, transcription, and chronic disease. *Annu Rev Biochem* 2011; **80:** 825–58. https://doi.org/10.1146/annurev-biochem-060608-102511.
100 Walter P, Ron D. The unfolded protein response: from stress pathway to homeostatic regulation. *Science* 2011; **334:** 1081–86. https://doi.org/10.1126/science.1209038.
101 Alberts B. Molecular biology of the cell. New York, NY: Garland Science, 2002.
102 Hynes RO. The extracellular matrix: not just pretty fibrils. *Science* 2009; **326:** 1216–19. https://doi.org/10.1126/science.1176009.
103 Alberts B, Bray D, Hopkin K, et al. Essential cell biology. New York: Garland, 2004.
104 Mishra MK, ed. Encyclopedia of biomedical polymers and polymeric biomaterials. Boca Raton: CRC Press/Taylor & Francis Group, 2015.
105 unknown. A Concise Overview: Structure and Functions of Proteoglycans. https://biologywise.com/structure-function-of-proteoglycans (accessed Feb 20, 2022).
106 Rees MD, Hawkins CL, Davies MJ. Hypochlorite and superoxide radicals can act synergistically to induce fragmentation of hyaluronan and chondroitin sulphates. *Biochem J* 2004; **381:** 175–84. https://doi.org/10.1042/BJ20040148.
107 Varki A, Cummings RD, Esko JD, et al., eds. Essentials of Glycobiology. Cold Spring Harbor (NY), 2009.
108 Toole BP. Hyaluronan: from extracellular glue to pericellular cue. *Nat Rev Cancer* 2004; **4:** 528–39. https://doi.org/10.1038/nrc1391.

109 Juhlin L. Hyaluronan in skin. *J Intern Med* 1997; **242:** 61–66. https://doi.org/10.1046/j.1365-2796.1997.00175.x.
110 Tammi R, Ripellino JA, Margolis RU, Tammi M. Localization of epidermal hyaluronic acid using the hyaluronate binding region of cartilage proteoglycan as a specific probe. *J Invest Dermatol* 1988; **90:** 412–14. https://doi.org/10.1111/1523-1747.ep12456530.
111 Fraser JR, Laurent TC, Laurent UB. Hyaluronan: its nature, distribution, functions and turnover. *J Intern Med* 1997; **242:** 27–33. https://doi.org/10.1046/j.1365-2796.1997.00170.x.
112 Steen EH, Short WD, Li H, et al. Skin-specific knockdown of hyaluronan in mice by an optimized topical 4-methylumbelliferone formulation. *Drug Deliv* 2021; **28:** 422–32. https://doi.org/10.1080/10717544.2021.1886376.
113 Slevin M, Krupinski J, Gaffney J, et al. Hyaluronan-mediated angiogenesis in vascular disease: uncovering RHAMM and CD44 receptor signaling pathways. *Matrix Biol* 2007; **26:** 58–68. https://doi.org/10.1016/j.matbio.2006.08.261.
114 Hardingham TE, Fosang AJ. Proteoglycans: many forms and many functions. *FASEB J* 1992; **6:** 861–70.
115 Schiraldi C, Cimini D, Rosa M de. Production of chondroitin sulfate and chondroitin. *Appl Microbiol Biotechnol* 2010; **87:** 1209–20. https://doi.org/10.1007/s00253-010-2677-1.
116 Kumar S, Wong PF, Leaper DJ. Intra-peritoneal prophylactic agents for preventing adhesions and adhesive intestinal obstruction after non-gynaecological abdominal surgery. *Cochrane Database Syst Rev* 2009: CD005080. https://doi.org/10.1002/14651858.CD005080.pub2.
117 Voigt J, Driver VR. Hyaluronic acid derivatives and their healing effect on burns, epithelial surgical wounds, and chronic wounds: a systematic review and meta-analysis of randomized controlled trials. *Wound Repair Regen* 2012; **20:** 317–31. https://doi.org/10.1111/j.1524-475X.2012.00777.x.
118 Taylor KR, Trowbridge JM, Rudisill JA, Termeer CC, Simon JC, Gallo RL. Hyaluronan fragments stimulate endothelial recognition of injury through TLR4. *J Biol Chem* 2004; **279:** 17079–84. https://doi.org/10.1074/jbc.M310859200.
119 Turley EA, Noble PW, Bourguignon LYW. Signaling properties of hyaluronan receptors. *J Biol Chem* 2002; **277:** 4589–92. https://doi.org/10.1074/jbc.R100038200.
120 Dechert TA, Ducale AE, Ward SI, Yager DR. Hyaluronan in human acute and chronic dermal wounds. *Wound Repair Regen* 2006; **14:** 252–58. https://doi.org/10.1111/j.1743-6109.2006.00119.x.
121 Maneiro E, Andres MC de, Fernández-Sueiro JL, Galdo F, Blanco FJ. The biological action of hyaluronan on human osteoartritic articular chondrocytes: the importance of molecular weight. *Clin Exp Rheumatol* 2004; **22:** 307–12.
122 Stern R, Asari AA, Sugahara KN. Hyaluronan fragments: an information-rich system. *Eur J Cell Biol* 2006; **85:** 699–715. https://doi.org/10.1016/j.ejcb.2006.05.009.
123 Berthiaume F, Maguire TJ, Yarmush ML. Tissue engineering and regenerative medicine: history, progress, and challenges. *Annu Rev Chem Biomol Eng* 2011; **2:** 403–30. https://doi.org/10.1146/annurev-chembioeng-061010-114257.
124 Badylak SF, Weiss DJ, Caplan A, Macchiarini P. Engineered whole organs and complex tissues. *Lancet* 2012; **379:** 943–52. https://doi.org/10.1016/S0140-6736(12)60073-7.

125 Iozzo RV, Schaefer L. Proteoglycan form and function: A comprehensive nomenclature of proteoglycans. *Matrix Biol* 2015; **42:** 11–55. https://doi.org/10.1016/j.matbio.2015.02.003.
126 Schaefer L, Schaefer RM. Proteoglycans: from structural compounds to signaling molecules. *Cell Tissue Res* 2010; **339:** 237–46. https://doi.org/10.1007/s00441-009-0821-y.
127 Jokela TA, Jauhiainen M, Auriola S, et al. Mannose inhibits hyaluronan synthesis by down-regulation of the cellular pool of UDP-N-acetylhexosamines. *J Biol Chem* 2008; **283:** 7666–73. https://doi.org/10.1074/jbc.M706001200.
128 Vigetti D, Deleonibus S, Moretto P, et al. Role of UDP-N-acetylglucosamine (GlcNAc) and O-GlcNAcylation of hyaluronan synthase 2 in the control of chondroitin sulfate and hyaluronan synthesis. *J Biol Chem* 2012; **287:** 35544–55. https://doi.org/10.1074/jbc.M112.402347.
129 Jokela TA, Makkonen KM, Oikari S, et al. Cellular content of UDP-N-acetylhexosamines controls hyaluronan synthase 2 expression and correlates with O-linked N-acetylglucosamine modification of transcription factors YY1 and SP1. *J Biol Chem* 2011; **286:** 33632–40. https://doi.org/10.1074/jbc.M111.265637.
130 Stojadinovic O, Pastar I, Nusbaum AG, Vukelic S, Krzyzanowska A, Tomic-Canic M. Deregulation of epidermal stem cell niche contributes to pathogenesis of nonhealing venous ulcers. *Wound Repair Regen* 2014; **22:** 220–27. https://doi.org/10.1111/wrr.12142.
131 Todaro GJ, Green H. Quantitative studies of the growth of mouse embryo cells in culture and their development into established lines. *Journal of Cell Biology* 1963; **17:** 299–313. https://doi.org/10.1083/jcb.17.2.299.
132 Boukamp P, Petrussevska RT, Breitkreutz D, Hornung J, Markham A, Fusenig NE. Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. *Journal of Cell Biology* 1988; **106:** 761–71. https://doi.org/10.1083/jcb.106.3.761.
133 Olschläger V, Schrader A, Hockertz S. Comparison of primary human fibroblasts and keratinocytes with immortalized cell lines regarding their sensitivity to sodium dodecyl sulfate in a neutral red uptake cytotoxicity assay. *Arzneimittelforschung* 2009; **59:** 146–52. https://doi.org/10.1055/s-0031-1296378.
134 Klimaj SD, Licon Munoz Y, Del Toro K, Hines WC. A high-throughput imaging and quantification pipeline for the EVOS imaging platform. *PLoS ONE* 2020; **15:** e0236397. https://doi.org/10.1371/journal.pone.0236397.
135 unknown. 25 Culture-Inserts 2 Well for self-insertion. https://ibidi.com/removable-chambers/25-25-culture-inserts-2-well-for-self-insertion.html (accessed Aug 23, 2020).
136 Jensen KB, Driskell RR, Watt FM. Assaying proliferation and differentiation capacity of stem cells using disaggregated adult mouse epidermis. *Nat Protoc* 2010; **5:** 898–911. https://doi.org/10.1038/nprot.2010.39.
137 Chacón-Martínez CA, Klose M, Niemann C, Glauche I, Wickström SA. Hair follicle stem cell cultures reveal self-organizing plasticity of stem cells and their progeny. *EMBO J* 2017; **36:** 151–64. https://doi.org/10.15252/embj.201694902.
138 Reitinger S, Lepperdinger G. Hyaluronan, a ready choice to fuel regeneration: a mini-review. *Gerontology* 2013; **59:** 71–76. https://doi.org/10.1159/000342200.
139 Bouché C, Serdy S, Kahn CR, Goldfine AB. The cellular fate of glucose and its relevance in type 2 diabetes. *Endocr Rev* 2004; **25:** 807–30. https://doi.org/10.1210/er.2003-0026.

140 Hart GW, Housley MP, Slawson C. Cycling of O-linked beta-N-acetylglucosamine on nucleocytoplasmic proteins. *Nature* 2007; **446:** 1017–22. https://doi.org/10.1038/nature05815.
141 Love DC, Hanover JA. The hexosamine signaling pathway: deciphering the "O-GlcNAc code". *Sci STKE* 2005; **2005:** re13. https://doi.org/10.1126/stke.3122005re13.
142 Hascall VC, Wang A, Tammi M, et al. The dynamic metabolism of hyaluronan regulates the cytosolic concentration of UDP-GlcNAc. *Matrix Biol* 2014; **35:** 14–17. https://doi.org/10.1016/j.matbio.2014.01.014.
143 Sakai S, Yasuda R, Sayo T, Ishikawa O, Inoue S. Hyaluronan exists in the normal stratum corneum. *J Invest Dermatol* 2000; **114:** 1184–87. https://doi.org/10.1046/j.1523-1747.2000.00992.x.
144 Poujade M, Grasland-Mongrain E, Hertzog A, et al. Collective migration of an epithelial monolayer in response to a model wound. *Proc Natl Acad Sci U S A* 2007; **104:** 15988–93. https://doi.org/10.1073/pnas.0705062104.
145 Tremel A, Cai A, Tirtaatmadja N, et al. Cell migration and proliferation during monolayer formation and wound healing. *Chemical Engineering Science* 2009; **64:** 247–53. https://doi.org/10.1016/j.ces.2008.10.008.
146 Lutolf MP, Hubbell JA. Synthetic biomaterials as instructive extracellular microenvironments for morphogenesis in tissue engineering. *Nat Biotechnol* 2005; **23:** 47–55. https://doi.org/10.1038/nbt1055.
147 Ridley AJ. Cell Migration: Integrating Signals from Front to Back. *Science* 2003; **302:** 1704–09. https://doi.org/10.1126/science.1092053.
148 Brem H, Tomic-Canic M. Cellular and molecular basis of wound healing in diabetes. *J Clin Invest* 2007; **117:** 1219–22. https://doi.org/10.1172/JCI32169.
149 Grinnell F, Petroll WM. Cell motility and mechanics in three-dimensional collagen matrices. *Annu Rev Cell Dev Biol* 2010; **26:** 335–61. https://doi.org/10.1146/annurev.cellbio.042308.113318.
150 Chen Y. Cell Adhesion Assay. *BIO-PROTOCOL* 2012; **2.** **https://doi.org/**10.21769/BioProtoc.98.
151 Khalili AA, Ahmad MR. A Review of Cell Adhesion Studies for Biomedical and Biological Applications. *Int J Mol Sci* 2015; **16:** 18149–84. https://doi.org/10.3390/ijms160818149.
152 Andrews KL, Houdek MT, Kiemele LJ. Wound management of chronic diabetic foot ulcers: from the basics to regenerative medicine. *Prosthet Orthot Int* 2015; **39:** 29–39. https://doi.org/10.1177/0309364614534296.
153 Baltzis D, Eleftheriadou I, Veves A. Pathogenesis and treatment of impaired wound healing in diabetes mellitus: new insights. *Adv Ther* 2014; **31:** 817–36. https://doi.org/10.1007/s12325-014-0140-x.
154 Harrison B, Khansa I, Janis JE. Evidence-Based Strategies to Reduce Postoperative Complications in Plastic Surgery. *Plast Reconstr Surg* 2016; **137:** 351–60. https://doi.org/10.1097/PRS.0000000000001882.
155 Loots MA, Lamme EN, Zeegelaar J, Mekkes JR, Bos JD, Middelkoop E. Differences in cellular infiltrate and extracellular matrix of chronic diabetic and venous ulcers versus acute wounds. *J Invest Dermatol* 1998; **111:** 850–57. https://doi.org/10.1046/j.1523-1747.1998.00381.x.
156 Lobmann R, Ambrosch A, Schultz G, Waldmann K, Schiweck S, Lehnert H. Expression of matrix-metalloproteinases and their inhibitors in the wounds of diabetic and non-

diabetic patients. *Diabetologia* 2002; **45:** 1011–16. https://doi.org/10.1007/s00125-002-0868-8.
157 Galkowska H, Wojewodzka U, Olszewski WL. Chemokines, cytokines, and growth factors in keratinocytes and dermal endothelial cells in the margin of chronic diabetic foot ulcers. *Wound Repair Regen* 2006; **14:** 558–65. https://doi.org/10.1111/j.1743-6109.2006.00155.x.
158 Galiano RD, Tepper OM, Pelo CR, et al. Topical vascular endothelial growth factor accelerates diabetic wound healing through increased angiogenesis and by mobilizing and recruiting bone marrow-derived cells. *Am J Pathol* 2004; **164:** 1935–47. https://doi.org/10.1016/S0002-9440(10)63754-6.
159 Falanga V. Wound healing and its impairment in the diabetic foot. *Lancet* 2005; **366:** 1736–43. https://doi.org/10.1016/S0140-6736(05)67700-8.
160 Goren I, Linke A, Müller E, Pfeilschifter J, Frank S. The suppressor of cytokine signaling-3 is upregulated in impaired skin repair: implications for keratinocyte proliferation. *J Invest Dermatol* 2006; **126:** 477–85. https://doi.org/10.1038/sj.jid.5700063.
161 Gibran NS, Jang YC, Isik FF, et al. Diminished neuropeptide levels contribute to the impaired cutaneous healing response associated with diabetes mellitus. *J Surg Res* 2002; **108:** 122–28.
162 Maruyama K, Asai J, Ii M, Thorne T, Losordo DW, D'Amore PA. Decreased macrophage number and activation lead to reduced lymphatic vessel formation and contribute to impaired diabetic wound healing. *Am J Pathol* 2007; **170:** 1178–91. https://doi.org/10.2353/ajpath.2007.060018.
163 Brem H, Stojadinovic O, Diegelmann RF, et al. Molecular markers in patients with chronic wounds to guide surgical debridement. *Mol Med* 2007; **13:** 30–39. https://doi.org/10.2119/2006-00054.Brem.
164 Stojadinovic O, Brem H, Vouthounis C, et al. Molecular Pathogenesis of Chronic Wounds : The Role of β-Catenin and c-myc in the Inhibition of Epithelialization and Wound Healing. *Am J Pathol* 2005; **167:** 59–69.
165 Slawson C, Copeland RJ, Hart GW. O-GlcNAc signaling: a metabolic link between diabetes and cancer? *Trends Biochem Sci* 2010; **35:** 547–55. https://doi.org/10.1016/j.tibs.2010.04.005.
166 McClain DA. Hexosamines as mediators of nutrient sensing and regulation in diabetes. *J Diabetes Complicat* 2002; **16:** 72–80.
167 McClain DA, Crook ED. Hexosamines and insulin resistance. *Diabetes* 1996; **45:** 1003–09.
168 Buse MG. Hexosamines, insulin resistance, and the complications of diabetes: current status. *Am J Physiol Endocrinol Metab* 2006; **290:** E1-E8. https://doi.org/10.1152/ajpendo.00329.2005.
169 Konrad RJ, Janowski KM, Kudlow JE. Glucose and streptozotocin stimulate p135 O-glycosylation in pancreatic islets. *Biochem Biophys Res Commun* 2000; **267:** 26–32. https://doi.org/10.1006/bbrc.1999.1895.
170 Liu K, Paterson AJ, Chin E, Kudlow JE. Glucose stimulates protein modification by O-linked GlcNAc in pancreatic beta cells: Linkage of O-linked GlcNAc to beta cell death. *J Biol Chem* 2000; **97:** 2820–25. https://doi.org/10.1073/pnas.97.6.2820.
171 Brownlee M. Biochemistry and molecular cell biology of diabetic complications. *Nature* 2001; **414:** 813–20. https://doi.org/10.1038/414813a.
172 Akimoto Y, Kawakami H, Yamamoto K, Munetomo E, Hida T, Hirano H. Elevated Expression of O-GlcNAc–Modified Proteins and O-GlcNAc Transferase in Corneas of

Diabetic Goto-Kakizaki Rats. *Invest. Ophthalmol. Vis. Sci.* 2003; **44:** 3802. https://doi.org/10.1167/iovs.03-0227.
173 Banerjee PS, Lagerlöf O, Hart GW. Roles of O-GlcNAc in chronic diseases of aging. *Mol Aspects Med* 2016; **51:** 1–15. https://doi.org/10.1016/j.mam.2016.05.005.
174 Runager K, Bektas M, Berkowitz P, Rubenstein DS. Targeting O-glycosyltransferase (OGT) to promote healing of diabetic skin wounds. *J Biol Chem* 2014; **289:** 5462–66. https://doi.org/10.1074/jbc.M113.513952.
175 Seiler WO, Stahelin HB, Zolliker R, Kallenberger A, Luscher NJ. Impaired Migration of Epidermal Cells from Decubitus Ulcers in Cell Cultures: A Cause of Protracted Wound Healing? *American Journal of Clinical Pathology* 1989; **92:** 430–34. https://doi.org/10.1093/ajcp/92.4.430.
176 Cao Z, Said N, Amin S, et al. Galectins-3 and -7, but not galectin-1, play a role in re-epithelialization of wounds. *J Biol Chem* 2002; **277:** 42299–305. https://doi.org/10.1074/jbc.M200981200.
177 Liu W, Hsu DK, Chen H-Y, et al. Galectin-3 regulates intracellular trafficking of EGFR through Alix and promotes keratinocyte migration. *J Invest Dermatol* 2012; **132:** 2828–37. https://doi.org/10.1038/jid.2012.211.
178 Huang S-M, Wu C-S, Chiu M-H, Yang H-J, Chen G-S, Lan C-CE. High-glucose environment induced intracellular O-GlcNAc glycosylation and reduced galectin-7 expression in keratinocytes: Implications on impaired diabetic wound healing. *J Dermatol Sci* 2017; **87:** 168–75. https://doi.org/10.1016/j.jdermsci.2017.04.014.
179 Zachara NE, Cheung WD, Hart GW. Nucleocytoplasmic glycosylation, O-GlcNAc: identification and site mapping. *Methods Mol Biol* 2004; **284:** 175–94. https://doi.org/10.1385/1-59259-816-1:175.
180 Gill SE, Parks WC. Metalloproteinases and their inhibitors: regulators of wound healing. *Int J Biochem Cell Biol* 2008; **40:** 1334–47. https://doi.org/10.1016/j.biocel.2007.10.024.
181 Lan C-CE, Liu I-H, Fang A-H, Wen C-H, Wu C-S. Hyperglycaemic conditions decrease cultured keratinocyte mobility: implications for impaired wound healing in patients with diabetes. *Br J Dermatol* 2008; **159:** 1103–15. https://doi.org/10.1111/j.1365-2133.2008.08789.x.
182 Agren MS, Mirastschijski U, Karlsmark T, Saarialho-Kere UK. Topical synthetic inhibitor of matrix metalloproteinases delays epidermal regeneration of human wounds. *Exp Dermatol* 2001; **10:** 337–48.
183 Hanks SK, Polte TR. Signaling through focal adhesion kinase. *Bioessays* 1997; **19:** 137–45. https://doi.org/10.1002/bies.950190208.
184 Akasaka T, van Leeuwen RL, Yoshinaga IG, Mihm MC, Byers HR. Focal adhesion kinase (p125FAK) expression correlates with motility of human melanoma cell lines. *J Invest Dermatol* 1995; **105:** 104–08.
185 Zhang Z, Tan EP, VandenHull NJ, Peterson KR, Slawson C. O-GlcNAcase Expression is Sensitive to Changes in O-GlcNAc Homeostasis. *Front Endocrinol (Lausanne)* 2014; **5:** 206. https://doi.org/10.3389/fendo.2014.00206.
186 Stephen HM, Adams TM, Wells L. Regulating the Regulators: Mechanisms of Substrate Selection of the O-GlcNAc cycling enzymes OGT and OGA. https://www.physiciansweekly.com/regulating-the-regulators-mechanisms-of-substrate-selection-of-the-o-glcnac-cycling-enzymes-ogt-and-oga?pagetype=diabetes-primary-care (accessed Mar 02, 2022).

187 Solomon SS, Majumdar G, Martinez-Hernandez A, Raghow R. A critical role of Sp1 transcription factor in regulating gene expression in response to insulin and other hormones. *Life Sci* 2008; **83:** 305–12. https://doi.org/10.1016/j.lfs.2008.06.024.
188 Kudlow JE. Post-translational modification by O-GlcNAc: another way to change protein function. *J Cell Biochem* 2006; **98:** 1062–75. https://doi.org/10.1002/jcb.20926.
189 Weimer J, Sokolsky O, Bezzo N, Lee I. Towards Assurance Cases for Resilient Control Systems. In: 2014 IEEE International Conference on Cyber-Physical Systems, Networks, and Applications. IEEE, 2014 - 2014: 1–6.
190 Farlex Partner Medical Dictionary. "viscosity.". https://medical-dictionary.thefreedictionary.com/viscosity (accessed Aug 02, 2020).
191 Chen WY, Abatangelo G. Functions of hyaluronan in wound repair. *Wound Repair Regen* 1999; **7:** 79–89. https://doi.org/10.1046/j.1524-475X.1999.00079.x.
192 Fallacara A, Baldini E, Manfredini S, Vertuani S. Hyaluronic Acid in the Third Millennium. *Polymers (Basel)* 2018; **10. https://doi.org/**10.3390/polym10070701.
193 Bourguignon LYW, Ramez M, Gilad E, et al. Hyaluronan-CD44 interaction stimulates keratinocyte differentiation, lamellar body formation/secretion, and permeability barrier homeostasis. *J Invest Dermatol* 2006; **126:** 1356–65. https://doi.org/10.1038/sj.jid.5700260.
194 Laurent TC, Laurent UB, Fraser JR. The structure and function of hyaluronan: An overview. *Immunol Cell Biol* 1996; **74:** A1-7. https://doi.org/10.1038/icb.1996.32.
195 Saghazadeh S, Rinoldi C, Schot M, et al. Drug delivery systems and materials for wound healing applications. *Adv Drug Deliv Rev* 2018; **127:** 138–66. https://doi.org/10.1016/j.addr.2018.04.008.
196 Kim MK, Kwak HW, Kim HH, et al. Surface modification of silk fibroin nanofibrous mat with dextran for wound dressing. *Fibers Polym* 2014; **15:** 1137–45. https://doi.org/10.1007/s12221-014-1137-2.
197 Minh NN, Obara H, Shimokasa K, Zhu J. Tensile behavior and extensional viscosity of bile. *Biorheology* 2019; **56:** 237–52. https://doi.org/10.3233/BIR-190216.
198 Beigi Harchegani A, Rahmani H, Tahmasbpour E, Shahriary A. Hyperviscous Semen Causes Poor Sperm Quality and Male Infertility through Induction of Oxidative Stress. *Curr Urol* 2019; **13:** 1–6. https://doi.org/10.1159/000499302.
199 Kaviani M, Geramizadeh B, Rahsaz M, Marzban S. Considerations in the improvement of human epidermal keratinocyte culture in vitro. *Exp Clin Transplant* 2015; **13 Suppl 1:** 366–70.
200 O'Donnell N, Zachara NE, Hart GW, Marth JD. Ogt-Dependent X-Chromosome-Linked Protein Glycosylation Is a Requisite Modification in Somatic Cell Function and Embryo Viability. *Mol Cell Biol* 2004; **24:** 1680–90. https://doi.org/10.1128/MCB.24.4.1680-1690.2004.
201 Shafi R, Iyer SP, Ellies LG, et al. The O-GlcNAc transferase gene resides on the X chromosome and is essential for embryonic stem cell viability and mouse ontogeny. *Proc Natl Acad Sci U S A* 2000; **97:** 5735–39. https://doi.org/10.1073/pnas.100471497.
202 Tan EP, Duncan FE, Slawson C. The sweet side of the cell cycle. *Biochem Soc Trans* 2017; **45:** 313–22. https://doi.org/10.1042/BST20160145.
203 Gu Y, Mi W, Ge Y, et al. GlcNAcylation plays an essential role in breast cancer metastasis. *Cancer Res* 2010; **70:** 6344–51. https://doi.org/10.1158/0008-5472.CAN-09-1887.

204 Mi W, Gu Y, Han C, et al. O-GlcNAcylation is a novel regulator of lung and colon cancer malignancy. *Biochim Biophys Acta* 2011; **1812:** 514–19. https://doi.org/10.1016/j.bbadis.2011.01.009.
205 Lynch TP, Ferrer CM, Jackson SR, Shahriari KS, Vosseller K, Reginato MJ. Critical role of O-Linked β-N-acetylglucosamine transferase in prostate cancer invasion, angiogenesis, and metastasis. *J Biol Chem* 2012; **287:** 11070–81. https://doi.org/10.1074/jbc.M111.302547.
206 Shi Y, Tomic J, Wen F, et al. Aberrant O-GlcNAcylation characterizes chronic lymphocytic leukemia. *Leukemia* 2010; **24:** 1588–98. https://doi.org/10.1038/leu.2010.152.
207 Ferrer CM, Sodi VL, Reginato MJ. O-GlcNAcylation in Cancer Biology: Linking Metabolism and Signaling. *Journal of Molecular Biology* 2016; **428:** 3282–94. https://doi.org/10.1016/j.jmb.2016.05.028.
208 Nie H, Ju H, Fan J, et al. O-GlcNAcylation of PGK1 coordinates glycolysis and TCA cycle to promote tumor growth. *Nat Commun* 2020; **11:** 36. https://doi.org/10.1038/s41467-019-13601-8.
209 Oikari S, Kettunen T, Tiainen S, et al. UDP-sugar accumulation drives hyaluronan synthesis in breast cancer. *Matrix Biol* 2018; **67:** 63–74. https://doi.org/10.1016/j.matbio.2017.12.015.
210 Wice BM, Trugnan G, Pinto M, et al. The intracellular accumulation of UDP-N-acetylhexosamines is concomitant with the inability of human colon cancer cells to differentiate. *J Biol Chem* 1985; **260:** 139–46.
211 Boehmelt G, Wakeham A, Elia A, et al. Decreased UDP-GlcNAc levels abrogate proliferation control in EMeg32-deficient cells. *EMBO J* 2000; **19:** 5092–104. https://doi.org/10.1093/emboj/19.19.5092.
212 DeBerardinis RJ. Is cancer a disease of abnormal cellular metabolism? New angles on an old idea. *Genet Med* 2008; **10:** 767–77. https://doi.org/10.1097/GIM.0b013e31818b0d9b.
213 Vander Heiden MG, Cantley LC, Thompson CB. Understanding the Warburg effect: the metabolic requirements of cell proliferation. *Science* 2009; **324:** 1029–33. https://doi.org/10.1126/science.1160809.
214 Warburg O. THE METABOLISM OF TUMORS IN THE BODY. *The Journal of General Physiology* 1927; **8:** 519–30. https://doi.org/10.1085/jgp.8.6.519.
215 Martinez-Outschoorn UE, Peiris-Pagés M, Pestell RG, Sotgia F, Lisanti MP. Cancer metabolism: a therapeutic perspective. *Nat Rev Clin Oncol* 2017; **14:** 11–31. https://doi.org/10.1038/nrclinonc.2017.1.
216 Boroughs LK, DeBerardinis RJ. Metabolic pathways promoting cancer cell survival and growth. *Nat Cell Biol* 2015; **17:** 351–59. https://doi.org/10.1038/ncb3124.
217 Sakai S, Endo Y, Ozawa N, et al. Characteristics of the epidermis and stratum corneum of hairless mice with experimentally induced diabetes mellitus. *J Invest Dermatol* 2003; **120:** 79–85. https://doi.org/10.1046/j.1523-1747.2003.12006.x.
218 Spravchikov N, Sizyakov G, Gartsbein M, Accili D, Tennenbaum T, Wertheimer E. Glucose Effects on Skin Keratinocytes: Implications for Diabetes Skin Complications. *Diabetes* 2001; **50:** 1627–35. https://doi.org/10.2337/diabetes.50.7.1627.
219 Hehenberger K, Heilborn JD, Brismar K, Hansson A. Inhibited proliferation of fibroblasts derived from chronic diabetic wounds and normal dermal fibroblasts treated with high glucose is associated with increased formation of l-lactate. *Wound Repair Regen* 1998; **6:** 135–41. https://doi.org/10.1046/j.1524-475x.1998.60207.x.

220 Graier WF, Grubenthal I, Dittrich P, Wascher TC, Kostner GM. Intracellular mechanism of highd-glucose-induced modulation of vascular cell proliferation. *Eur J Pharmacol* 1995; **294:** 221–29. https://doi.org/10.1016/0014-2999(95)00534-x.
221 Higuchi C, Sanaka T, Sato T, et al. The effect of glucose on the proliferation of peritoneal fibroblasts. *Adv Perit Dial* 1997; **13:** 253–56.
222 Hayashi JN, Ito H, Kanayasu T, et al. Effects of glucose on migration, proliferation and tube formation by vascular endothelial cells. *Virchows Arch , B, Cell Pathol* 1991; **60:** 245–52. https://doi.org/10.1007/bf02899553.
223 Wolf G, Sharma K, Chen Y, Ericksen M, Ziyadeh FN. High glucose-induced proliferation in mesangial cells is reversed by autocrine TGF-beta. *Kidney Int* 1992; **42:** 647–56. https://doi.org/10.1038/ki.1992.330.
224 Karvinen S, Pasonen-Seppänen S, Hyttinen JMT, et al. Keratinocyte growth factor stimulates migration and hyaluronan synthesis in the epidermis by activation of keratinocyte hyaluronan synthases 2 and 3. *J Biol Chem* 2003; **278:** 49495–504. https://doi.org/10.1074/jbc.M310445200.
225 Arreola R, Valderrama B, Morante ML, Horjales E. Two mammalian glucosamine-6-phosphate deaminases: a structural and genetic study. *FEBS Lett* 2003; **551:** 63–70. https://doi.org/10.1016/s0014-5793(03)00896-2.
226 Cayli A, Hirschmann F, Wirth M, Hauser H, Wagner R. Cell lines with reduced UDP-N-acetylhexosamine pool in the presence of ammonium. *Biotechnol Bioeng* 1999; **65:** 192–200.
227 Oikari S, Makkonen K, Deen AJ, et al. Hexosamine biosynthesis in keratinocytes: roles of GFAT and GNPDA enzymes in the maintenance of UDP-GlcNAc content and hyaluronan synthesis. *Glycobiology* 2016; **26:** 710–22. https://doi.org/10.1093/glycob/cww019.
228 Kultti A, Li X, Jiang P, Thompson CB, Frost GI, Shepard HM. Therapeutic targeting of hyaluronan in the tumor stroma. *Cancers (Basel)* 2012; **4:** 873–903. https://doi.org/10.3390/cancers4030873.
229 Nagy N, Kuipers HF, Frymoyer AR, et al. 4-methylumbelliferone treatment and hyaluronan inhibition as a therapeutic strategy in inflammation, autoimmunity, and cancer. *Front Immunol* 2015; **6:** 123. https://doi.org/10.3389/fimmu.2015.00123.
230 Grose R, Hutter C, Bloch W, et al. A crucial role of beta 1 integrins for keratinocyte migration in vitro and during cutaneous wound repair. *Development* 2002; **129:** 2303–15.
231 Snow CM. Monoclonal antibodies identify a group of nuclear pore complex glycoproteins. *J Cell Biol* 1987; **104:** 1143–56. https://doi.org/10.1083/jcb.104.5.1143.
232 Naguib G, Al-Mashat H, Desta T, Graves DT. Diabetes prolongs the inflammatory response to a bacterial stimulus through cytokine dysregulation. *J Invest Dermatol* 2004; **123:** 87–92. https://doi.org/10.1111/j.0022-202X.2004.22711.x.
233 Robles-Flores M, Meléndez L, García W, et al. Posttranslational modifications on protein kinase c isozymes. Effects of epinephrine and phorbol esters. *Biochim Biophys Acta* 2008; **1783:** 695–712. https://doi.org/10.1016/j.bbamcr.2007.07.011.
234 Kang E-S, Han D, Park J, et al. O-GlcNAc modulation at Akt1 Ser473 correlates with apoptosis of murine pancreatic beta cells. *Exp Cell Res* 2008; **314:** 2238–48. https://doi.org/10.1016/j.yexcr.2008.04.014.
235 Luo B, Soesanto Y, McClain DA. Protein modification by O-linked GlcNAc reduces angiogenesis by inhibiting Akt activity in endothelial cells. *Arterioscler Thromb Vasc Biol* 2008; **28:** 651–57. https://doi.org/10.1161/ATVBAHA.107.159533.

236 Gandy JC, Rountree AE, Bijur GN. Akt1 is dynamically modified with O-GlcNAc following treatments with PUGNAc and insulin-like growth factor-1. *FEBS Lett* 2006; **580:** 3051–58. https://doi.org/10.1016/j.febslet.2006.04.051.

237 Mavrogonatou E, Kletsas D. The effect of glucosamine sulfate on the proliferative potential and glycosaminoglycan synthesis of nucleus pulposus intervertebral disc cells. *Spine* 2013; **38:** 308–14. https://doi.org/10.1097/BRS.0b013e31826a0a8d.

7. Adnexa

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8. Vorabveröffentlichungen von Ergebnissen

Es ist keine Vorabveröffentlichung erfolgt.