Article

Differential Gene Transcription of Extracellular Matrix Components in Response to In Vivo Corneal Crosslinking (CXL) in Rabbit Corneas

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Purpose: We studied changes in gene transcription after corneal crosslinking (CXL) in the rabbit cornea in vivo and identified potential molecular signaling pathways.

Methods: A total of 15 corneas of eight male New-Zealand-White rabbits were de-epithelialized and equally divided into five groups. Group 1 served as an untreated control. Groups 2 to 5 were soaked with 0.1% riboflavin for 20 minutes, which in Groups 3 to 5 was followed by UV-A irradiation at a fluence of 5.4 J/cm². Ultraviolet A (UVA) irradiation was delivered at 3 mW/cm² for 30 minutes (Group 3, standard CXL protocol), 9 mW/cm² for 10 minutes (Group 4, accelerated), and 18 mW/cm² for 5 minutes (Group 5, accelerated). At 1 week after treatment, corneal buttons were obtained; mRNA was extracted and subjected to cDNA sequencing (RNA-seq).

Results: A total of 297 differentially transcribed genes were identified after CXL treatment. CXL downregulated extracellular matrix components (collagen types 1A1, 1A2, 6A2, 11A1, keratocan, fibromodulin) and upregulated glycan biosynthesis and proteoglycan glycosylation (GALNT 3, 7, and 8, B3GALT2). Also, CXL activated pathways related to protein crosslinking (transglutaminase 2 and 6). In 9.1% of the significantly different genes, CXL at 3 mW/cm² (Group 1) induced a more distinct change in gene transcription than the accelerated CXL protocols, which induced a lower biomechanical stiffening effect.

Conclusions: Several target genes have been identified that might be related to the biomechanical stability and shape of the cornea. Stiffening-dependent differential gene transcription suggests the activation of mechano-sensitive pathways.

Translational Relevance: A better understanding of the molecular mechanisms behind CXL will permit an optimization and individualization of the clinical treatment protocol.

Introduction

Until recently, corneal ectasia could not be treated and typically required corneal transplantation, involving the risks of infection, protracted wound healing, and rejection. In 1997, Spoerl et al.1 proposed a new technique to increase the biomechanical stiffness of the cornea: corneal crosslinking (CXL). The treatment involves de-epithelialization of the cornea, soaking the corneal stroma with a chromophore (Vitamin B2, riboflavin), and ultraviolet A (UVA) irradiation with 3 mW/cm² for an additional 30 minutes. Multiple studies have shown that CXL successfully stops keratoconus progression and also arrests postsurgical corneal ectasia.3 Since its introduction, a number of modifications of the original treatment protocol have been proposed, aiming at increasing its efficacy, shortening treatment duration,
and reducing the risk of postoperative complications. The most widely used modified treatment protocol is accelerated CXL, using a higher irradiance in combination with a shorter irradiation time. However, several studies showed a reduced treatment efficacy. In clinical settings, a shallower demarcation line and minor corneal flattening was reported.

Efficiency. In clinical settings, a shallower demarcation line and minor corneal flattening was reported. Experimental settings, such as a higher tensile elastic modulus and a lower dry-weight after enzymatic digestion were found. Further modified treatment protocols include iontophoresis-assisted, trans-epithelial, hypo-osmolar, pulsed, contact lens-assisted, and customized CXL. All modified protocols share limited success: the increase in corneal stiffness is lower compared to that of the standard CXL treatment. A reason why it is difficult to optimize CXL is that its working principle is poorly understood. Although most mechanical strengthening would be expected if bonds were formed between collagen lamellae, x-ray scattering experiments indicate that bonds are formed rather at the collagen fibril surface and in the protein network surrounding the collagen. Also, the corneal swelling capacity is reduced strongly after CXL, suggesting that proteoglycans and glycosaminoglycans are involved.

Clinical trials currently are performed to address the question whether CXL has the potential for primary refractive corrections of myopia and hyperopia. A better understanding of the basic mechanisms behind CXL would allow better adaptation of the protocol for different therapies, but also to identify its limitations.

One might speculate that the arrest of keratocorneal progression induced by CXL implies long-term and permanent changes on transcriptional, translational, and/or posttranslational levels. This hypothesis is supported by the fact that the increase in corneal stiffness after CXL lasts potentially longer than the actual collagen turnover in the corneal tissue and that significant—sometimes even progressive—corneal flattening is observed after CXL treatment. There are different mechanisms of how CXL may change gene transcription: the generation of large amounts of reactive oxygen species (ROS) may activate signaling pathways with the potential of reintroducing homeostasis. Another mechanism may involve mechanotransduction, which means the process of converting mechanical signals into biochemical responses. Mechanical signals may result from dynamically acting forces, but also from remodeling of the extracellular matrix (ECM), leading to changes in cell adhesion and cell–cell contact that finally determine the mechanical interaction with the surrounding matrix.

Different mechanisms of action have been identified for mechanotransduction: certain ion-channels open in response to increased tension in the plasma membrane (observed during osmotic changes), proteins can unfold domains upon tension that reveal cryptic binding, and phosphorylation may increase upon stretching. These immediate changes may activate signaling pathways and/or gene transcription within minutes to hours.

The purpose of this study was to analyze the corneal transcriptome before and after CXL treatment to identify differentially transcribed candidate genes that potentially affect corneal stiffness.

**Methods**

Eight New Zealand White rabbits (2.5 kg weight) were purchased from Charles River Laboratories (Saint-Germaine-Nuelles, France). All experiments were approved by the local ethical committee and adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Visual Research.

**CXL Treatment Protocol**

Rabbits were anesthetized with a subcutaneous injection of ketamine (Ketalar; Pfizer AG, Zurich, Switzerland) and xylazine (Rompun 2%, 20 mg ml⁻¹; Bayer, Basel, Switzerland). A total of 15 eyes then were assigned to one of five treatment groups (n = 3 per group). The corneas of all groups were de-epithelialized. Group 1 served as untreated control. Groups 2 to 5 corneas additionally received 0.1% riboflavin instillation during 20 minutes, using a suction ring. Group 2 served as riboflavin control. Group 3 corneas subsequently were irradiated with 3 mW/cm² during 30 minutes, Group 4 corneas with 9 mW/cm² during 10 minutes, and Group 5 corneas with 18 mW/cm² during 5 minutes. Riboflavin was not renewed during UV irradiation. Three different irradiances were included to study the effect of different degrees of biomechanical stiffening. Directly after treatment, antibiotic ointment (Ofloxacin, Floxal 0.3%; Bausch & Lomb, Zug, Switzerland) was administered prophylactically onto the cornea and repeated twice daily (until epithelial closure on postoperative days 3–4) to avoid infections. In addition, buprenorphin (Temgesic) was administered...
subcutaneously twice daily at 50 μg/kg until epithelial closure.

**Sample Preparation**

One week after CXL treatment the rabbits were sacrificed (intravenous 120 mg/kg, Pentothal; Ospedalita AG, Hünenberg, Switzerland) and the corneas obtained with a trephine (8 mm diameter). The corneal tissue was immersed in RLT lysis buffer + 1% β-mercaptoethanol and homogenized, first with scissors and then with a tissue disruptor (Qiagen GmbH, Hilden, Germany). Afterwards, samples were frozen in liquid nitrogen and stored at −80°C.

Then, mRNA of the entire cornea, including epithelial, keratocyte, and endothelial cells, was extracted using an RNeasy kit (Qiagen) according to the manufacturer’s instructions. mRNA quantity and quality were assessed with a spectrophotometer (Qubit; Life Technologies, Carlsbad, CA, USA) and the Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), respectively.

**Differential Gene Transcription**

Equal amounts of mRNA (300 ng) were reverse transcribed, then cDNA sequencing (RNAseq) was performed with the HighSeq 2500 system (Illumina, San Diego, CA, USA) using the TruSeq stranded mRNA protocol with 100 single-end reads. The sequencing quality was controlled with FastQC v.0.11.2 leading to a Phred quality score of >28 corresponding to a 1/1000 chance of errors. TopHat v2.0.13 software was used for mapping against the reference genome. The alignment percentage was not optimal (~65%), probably due to low sequencing quality of the rabbit genome. As a consequence, multiple-mapping reads were not considered in the counts. Here, counts corresponded to the total number of reads aligning to a genomic feature. Biological quality control and summarization were done with RSeQC v2.4 and PicardTools v1.92 software, respectively. Only genes with a count above 10 in at least three samples were included for further analysis. The normalization and differential transcription analysis was performed with the R/Bioconductor package edgeR v.3.10.5, for the genes annotated in the reference genome.

**Statistical Analysis**

Differentially transcribed genes were determined for each individual treatment group using a General Linear Model (GLM), a negative binomial distribution and a quasi-likelihood test. Ten pairwise comparisons (edgeR, GLM, quasi-likelihood F test) of the experimental groups were analyzed (Table 1). Instead of correcting the P values of the differentially transcribed genes with the Bonferroni method for multiple testing error, a different approach was chosen selecting significant genes according to the response of the whole set of CXL and control conditions. For this purpose, a composite null hypothesis, H0, was created summarizing the five most important comparisons. The condition CXL at 18 mW/cm² was excluded in this selection process, as its treatment efficacy is smallest, as shown experimentally⁹–¹¹ and clinically⁵–⁸ and, hence, its meaningfulness is lower than the other comparisons.

\[
H_0 = H_{\text{virgin}=3\text{mW}} \cap H_{\text{virgin}=9\text{mW}} \cap H_{\text{ribo}=3\text{mW}} \cap H_{\text{ribo}=9\text{mW}} \\
\sim H_{\text{virgin}=\text{ribo}}
\]

and hence:

\[
H_1 = \sim H_0 \\
= \sim H_{\text{virgin}=3\text{mW}} \& \sim H_{\text{virgin}=9\text{mW}} \& \sim H_{\text{ribo}=3\text{mW}} \& \sim H_{\text{ribo}=9\text{mW}} \& H_{\text{virgin}=\text{ribo}}
\]

where \(H_1\) is the composite null hypothesis. \(H_{x>y}\) represents an individual null hypothesis, that is there is no difference between \(x\) and \(y\). \(\sim H_{x>y}\) represent a rejected null hypothesis, that is there is a difference between \(x\) and \(y\). Each comparison between CXL (at 3 or 9 mW/cm²) and control (virgin or riboflavin) is expected to be significant. In contrast, the comparison between the two control conditions is expected not to be significant. A given gene then will be considered significant, if \(H_1\) is true. With a confidence interval of 95%, the probability for a false positive in one comparison is:

\[
P_i = (P_{\text{virgin}=9\text{mW}} \cdot P_{\text{ribo}=3\text{mW}}) \cdot (P_{\text{virgin}=3\text{mW}}) \cdot (P_{\text{ribo}=9\text{mW}}) \cdot (P_{\text{ribo}=\text{virgin}})
\]

The probability of \(P_{\text{ribo}=\text{virgin}}\) cannot be calculated exactly, as it is the power of the test. However, assuming that the power is 1, we have neglected this term resulting in \(P_i \leq 0.05^4\). Applied to the entire set of \(n = 9335\) analyzed genes, the probability of having at least one false-positive can be calculated:

\[
P_{\text{cumulative}} = 1 - (1 - P_i)^n \leq 0.0567
\]

This \(P\) value, \(P_{\text{cumulative}}\), is comparable to the standard significance level. An alternative correc-
tion for multiple testing is the Bonferroni method, which, however, can be applied only to one group at a time. The above-described whole-data-set approach is superior, as it accounts for the reproducibility of the CXL effect before correcting for multiple testing. Figure 1 illustrates that with Bonferroni correction, lower statistical significance (19 significantly different genes) can be reached than

Table 1. Differential Gene Transcription was Computed for a Total of 10 Comparisons Between Different Treatment and Control Groups

| Comparison Between Groups | # Significant Genes, at 5% FDR | # Downregulated, FC ≥ 2 | # Upregulated, FC ≥ 2 |
|---------------------------|-------------------------------|-------------------------|-----------------------|
| Riboflavin vs. virgin     | 2                             | 1                       | 1                     |
| CXL 3 mW 30 min vs. virgin| 504                           | 201                     | 303                   |
| CXL 9 mW 10 min vs. virgin| 18                            | 10                      | 8                     |
| CXL 18 mW 5 min vs. virgin| 4                             | 0                       | 4                     |
| CXL 3 mW 30 min vs. riboflavin| 862                         | 341                     | 521                   |
| CXL 9 mW 10 min vs. riboflavin| 36                           | 19                      | 17                    |
| CXL 18 mW 5 min vs. riboflavin| 1                             | 1                       | 0                     |
| CXL 9 mW 10 min vs. CXL 3 mW 30 min| 161                        | 93                      | 68                    |
| CXL 18 mW 5 min vs. CXL 3 mW 30 min| 165                        | 88                      | 77                    |
| CXL 18 mW 5 min vs. CXL 9 mW 10 min| 0                            | 0                       | 0                     |

Figure 1. Comparison of different approaches to correct for multiple statistical testing. (A) Bonferroni method resulting in 19 significantly differently transcribed genes. (B) Whole-data-set method developed in this manuscript resulting in 297 significantly differently transcribed genes.
with the whole-data-set approach (297 significantly different genes).

**Filter for Stiffening Dependent Gene Transcription**

The resulting list of significantly transcribed genes then was subjected to filtering to determine genes that are transcribed differentially in a stiffening-dependent manner. The following criteria Filter\(_{\text{(stiffening)}}\) was imposed:

\[
C_1 = [(\log FC_{\text{virgin}} - 3\text{mW} > 0) \& (\log FC_{\text{virgin}} - 9\text{mW} > 0) \\
\& (\log FC_{\text{virgin}} - 18\text{mW} > 0) \& (\log FC_{\text{riboflavin}} - 3\text{mW} > 0) \\
\& (\log FC_{\text{riboflavin}} - 9\text{mW} > 0) \& (\log FC_{\text{riboflavin}} - 18\text{mW} > 0) \\
\& (\log FC_{3\text{mW}} - 9\text{mW} < 0) \& (\log FC_{3\text{mW}} - 18\text{mW} < 0) \\
\& (\log FC_{9\text{mW}} - 18\text{mW} < 0)]
\]

\[
C_2 = [(\log FC_{\text{virgin}} - 3\text{mW} < 0) \& (\log FC_{\text{virgin}} - 9\text{mW} < 0) \\
\& (\log FC_{\text{virgin}} - 18\text{mW} < 0) \& (\log FC_{\text{riboflavin}} - 3\text{mW} < 0) \\
\& (\log FC_{\text{riboflavin}} - 9\text{mW} < 0) \& (\log FC_{\text{riboflavin}} - 18\text{mW} < 0) \\
\& (\log FC_{3\text{mW}} - 9\text{mW} > 0) \& (\log FC_{3\text{mW}} - 18\text{mW} > 0) \\
\& (\log FC_{9\text{mW}} - 18\text{mW} > 0)]
\]

\[
\text{Filter}_{\text{(stiffening)}} = H_1 \& \sim H_{3\text{mW}=9\text{mW}} \& \sim H_{3\text{mW}=18\text{mW}} \\
\& (C_1 \mid C_2)
\]

where logFC is the fold-change in log2 scale between the different tested conditions; & and | represent the logical operators AND and OR, respectively.

**Correlation Analysis**

The Pearson’s linear correlation coefficient among all treatment conditions was calculated for selected differentially transcribed genes using Matlab software (Mathworks, Bern, Switzerland) to investigate mutual gene interactions. The online tool DAVID\textsuperscript{32,33} Bioinformatics Resources (Version 6.8) was used to extract related signaling pathways.

**Results**

**Differential Gene Transcription**

From a total of 9335 transcripts, 297 were significantly differentially transcribed between the two clinically efficient CXL conditions (at 3 and 9 mW/cm\(^2\)) and controls (virgin and riboflavin). Of these differentially transcribed genes, 9.1% (27 genes) were significantly stiffening-dependent, as per the definition above.

Most of the 297 differently transcribed genes were related to signaling (42), disulfide bonding (34), nucleotide binding (26), ATP binding (21), hydrolase (19), transferase (17), secreted (14), DNA binding (14), extracellular matrix (8), DNA replication (8), immunoglobulin domain (6), helicase (5), tyrosine protein kinase (5), collagen (3), DNA repair (3), and DNA damage (3). Figure 2 presents a subset of pathways and genes that are likely involved in corneal mechanical properties.

**Stiffening-dependent and -independent Differentially Transcribed Genes**

Table 2 and Supplementary Table S1 present genes that were significantly differentially transcribed in a stiffening-dependent and stiffening-independent manner, respectively. Several genes of either subset have been reported previously to show an altered gene expression in keratoconus (references provided in the Tables).

Figure 3 shows the change in normalized counts of selected genes for the different treatment and control conditions: Enzymatic crosslinking by transglutaminases 2 and 6 was increased significantly after CXL (Figs. 3A, 3B). Also, the expression of polypeptide N-acetylgalactosaminyltransferase 3 and β-1,3-galactosyltransferase 2, both related to the glycosylation of proteoglycans, was increased in crosslinked corneas (Figs. 3C, 3D). The only collagen type that was significantly upregulated after CXL was type IV, which forms part of the basement membrane. All other collagen types (I, VI, XI) were downregulated (Figs. 3E–H). Downregulation also was observed in noncollagenous ECM components, including thrombospondin 4 and keratocan (Figs. 3I, 3J). At the same time, enzymatic glycolysis by means of enolase 1 and transketolase was reduced in crosslinked corneas (Figs. 3K, 3L).

**Most Affected Signaling Pathways after CXL Treatment**

Table 3 presents the two most affected pathways. Seven genes of the ECM receptor interaction pathway and 19 genes of the glycan biosynthesis and metabolism pathway were significantly differentially transcribed.

**Correlation analysis**

Figure 4 shows genes that strongly correlated (\(r_{\text{Pearson}} > 0.8, P > 0.05\)) with thrombospondin 4, a matricellular protein that is involved in tissue
remodeling. Among its highest correlated genes were structural extracellular matrix components, including collagen (types I, II, VI, XI), keratocan, and fibromodulin.

**Discussion**

We analyzed differential gene transcription induced by CXL treatment and observed a significant remodeling of the ECM, including changes in collagen synthesis, glycan biosynthesis, and proteoglycan glycosylation.

Fibrillar collagen types I and XI were downregulated after CXL, while the epithelial basement membrane constituting collagen type IV was upregulated. Decreased collagen types I and XI transcription potentially results from a reduced collagen degradation after CXL, while increased collagen type IV may be attributed to the recent re-epithelialization and continuing epithelial remodeling.

The activity of enzymes related to glycosylation (enolase 1, transketolase) and, hence, to ECM degradation, was decreased after CXL treatment. Previously, enolase 1 and transketolase overexpression had been reported in context with increased ECM degradation and cancer invasion. Interestingly, a reduced expression of enolase, transketolase, and the protease inhibitor α2-macroglobulin-like 1 has been reported in keratoconus, which, however, was not able to prevent corneal ectasia.

In contrast, other genes were inversely differentially transcribed after CXL treatment when compared to...
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Table 2. Genes that Were Significantly Differently Transcribed in a Stiffening-Dependent Manner

| Gene                  | External Name | Chromosome | Description                                                                 |
|-----------------------|---------------|------------|-----------------------------------------------------------------------------|
| ENSOCUG00000006901    | ANKRD1        | 18         | Ankyrin repeat domain-containing protein 1                                  |
| ENSOCUG00000011970    | TAGLN         | 1          | Transgeline                                                                 |
| ENSOCUG00000008236    | LPL           | 15         | Lipoprotein lipase-like precursor                                            |
| ENSOCUG00000026419    | DHFR          | 11         | Dihydrofolate Reductase                                                     |
| ENSOCUG0000003636     | TGM2          | 4          | Transglutaminase 2                                                          |
| ENSOCUG0000002632     | KRT7          | 4          | Keratin 7, type II                                                          |
| ENSOCUG00000012542    | CACNA2D3      | 9          | Calcium channel, voltage-dependent, alpha 2/delta subunit 3                 |
| ENSOCUG0000014740     | SLC37A2       | 1          | Solute carrier family 37 (glucose-6-phosphate transporter), member 2         |
| ENSOCUG0000002272     | MYH7B         | 4          | Myosin, heavy chain 7B, cardiac muscle, beta                                |
| ENSOCUG0000017128     | NMU           | 15         | Neuromedin U                                                                |
| ENSOCUG0000015001     | CYB5R2        | 1          | Cytochrome b5 reductase 2                                                   |
| ENSOCUG0000011919     | SEMA3A        | 10         | Sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3A |
| ENSOCUG0000002632     | KRT7          | 4          | Keratin 7, type II                                                          |
| ENSOCUG00000012542    | CACNA2D3      | 9          | Calcium channel, voltage-dependent, alpha 2/delta subunit 3                 |
| ENSOCUG0000014740     | SLC37A2       | 1          | Solute carrier family 37 (glucose-6-phosphate transporter), member 2         |

Keratoconus: collagen type I, keratan, and thrombospondin 4 were downregulated after CXL, but upregulated in keratoconus. These ECM components potentially may be involved in extracellular remodeling resulting from the increased corneal stiffness after CXL. Thrombospondin 4 has been identified previously as a mechano-sensing molecule in the cardiac contractile response to mechanical stress showing upregulation in response to hypertension. After CXL-treatment, the mechanical stress resistance increases and, as a consequence, the tissue strain decreases, which may have led to the downregulation of thrombospondin 4. In the same line, in keratoconus, where increased tissue strain in the cone region is observed, an overexpression of thrombospondin 4 has been reported. Further potential mechano-sensitive genes may be involved in the molecular signaling after CXL treatment (see Table 2), which in turn could modify the transcription of nonmechano-sensitive genes (see Supplementary Table S1).
One of the identified stiffening-independent mechanisms of CXL was the increase in enzymatic proteoglycan glycosylation and glycan biosynthesis (Table 3). \( \beta_1,3 \)-galactosyltransferase 2 is involved in the N-acetyl-D-glucosamine sugar addition on the keratan sulfate proteoglycan. A deficiency in a similar enzyme, \( \beta_1–4 \) galactosyltransferase 7, has been associated with Ehlers-Danlos syndrome, 49 which manifests in joint hyperelasticity and previously also has been reported in context with corneal curvature abnormalities, including keratoconus, keratoglobus, and cornea plana. 50,51 These pathologies likely arise from an alteration of corneal stiffness. Other conditions that affect corneal stiffness include diabetes and aging, in which nonenzymatic glycation is increased. 52,53 In contrast with increased enzymatic glycosylation (as observed after CXL), increased nonenzymatic glycation is a random process that makes it less specific in ECM crosslinking.

### Table 2. Extended

| Virgin | Riboflavin 3 mW | 9 mW | 18 mW | Virgin | Riboflavin 3 mW | 9 mW | 18 mW |
|--------|----------------|------|------|--------|----------------|------|------|
| 0.00   | 0.00           | 10.67| 0.6667| 0.3333 | 0.00           | 0.00 | 4.51 |
| 0.00   | 0.00           | 7.67 | 1.6667| 1.3333 | 0.00           | 0.00 | 1.53 |
| 1.33   | 1.33           | 53.33| 10.3333| 7.6667 | 0.58           | 0.58 | 18.45 |
| 82.33  | 88.67          | 0.67 | 6.3333| 14.3333| 65.65          | 73.66| 0.58 |
| 17.00  | 14.33          | 393.67| 104.0000| 36.0000| 8.54           | 12.86| 151.96|
|        |                |      |      |        |                |      |      |
| 16.33  | 21.67          | 308.33| 99.3333| 91.6667| 7.37           | 19.14| 26.50 |
| 7.67   | 4.67           | 0.67 | 2.0000| 2.0000 | 1.53           | 2.08 | 0.58 |
|        |                |      |      |        |                |      |      |
| 5.00   | 3.33           | 23.67| 9.0000| 9.3333 | 1.00           | 0.58 | 15.89 |
|        |                |      |      |        |                |      |      |
| 3.33   | 4.00           | 0.33 | 1.3333| 1.6667 | 1.53           | 1.00 | 0.58 |
| 4.67   | 6.00           | 0.33 | 1.6667| 3.0000 | 1.53           | 3.00 | 0.58 |
| 91.33  | 127.33         | 9.00 | 32.3333| 64.3333| 33.98          | 44.06| 2.00 |
| 27.00  | 36.33          | 142.00| 78.6667| 73.6667| 7.55           | 9.87 | 25.24 |
|        |                |      |      |        |                |      |      |
| 17.00  | 13.67          | 77.33| 35.3333| 27.6667| 9.54           | 5.86 | 12.10 |
| 393.67 | 447.00         | 47.67| 170.0000| 199.6667| 59.00          | 55.56| 7.23 |
| 5.67   | 5.00           | 27.33| 12.0000| 9.0000 | 2.08           | 3.46 | 13.61 |
| 13.33  | 10.00          | 46.33| 26.6667| 23.3333| 4.93           | 1.00 | 8.39 |
| 9.67   | 7.67           | 34.67| 16.0000| 13.3333| 1.53           | 2.08 | 6.81 |
|        |                |      |      |        |                |      |      |
| 381.33 | 322.00         | 1126.67| 680.6667| 675.6667| 119.78         | 133.63| 174.95|
| 73.67  | 111.00         | 19.67| 39.3333| 59.6667| 24.66          | 55.22| 1.53 |
| 17.00  | 21.67          | 5.67 | 10.0000| 10.6667| 6.00           | 3.79 | 1.53 |
| 14.00  | 14.00          | 45.00| 23.0000| 20.3333| 3.00           | 5.29 | 5.29 |
| 114.33 | 118.33         | 45.33| 65.0000| 66.3333| 16.29          | 18.45| 6.66 |
| 87.67  | 101.67         | 35.33| 54.6667| 56.6667| 9.29           | 14.01| 16.04 |
| 91.33  | 103.00         | 31.33| 59.6667| 63.3333| 26.63          | 18.52| 3.51 |
|        |                |      |      |        |                |      |      |
| 10,385.67| 9981.00        | 3718.33| 6365.3333| 7070.0000| 1039.99        | 395.81| 361.28|
| 42.00  | 46.33          | 17.33| 28.0000| 34.3333| 9.17           | 11.55| 4.16 |
| 27.33  | 31.00          | 11.33| 17.6667| 24.0000| 7.51           | 4.36 | 3.06 |
|        |                |      |      |        |                |      |      |
|        |                |      |      |        |                |      |      |

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Although the 18 mW/cm² condition was excluded to identify the significantly differentially transcribed genes between crosslinked and control corneas, its expression levels either were in a similar absolute range as the 3 and 9 mW/cm² conditions, or did confirm the gradient between the 3 and 9 mW/cm² conditions. This can be considered as an additional quality control, but at the same time emphasizes the fact that CXL protocols differ on the molecular level in an irradiance/time dependent way.

Table 2. Extended

| log10 (cum_P Value) | Cum_logFC | Remark |
|---------------------|-----------|--------|
| 8.41 inf            |           | Increased expression in keratoconus⁴¹ |
| 11.03 inf           |           | Increased expression in vitro after CXL treatment;⁴³ catalyzes covalent crosslinking e-(g-glutamyl) lysine bonds |
| 6.31 4.16           |           | Increased expression in keratoconus⁵⁴ |
| 7.22 −3.59          |           | |
| 7.53 3.51           |           | |
| 8.25 3.13           |           | |
| 8.29 −1.99          |           | |
| 7.13 1.75           |           | Involved in glycogenolysis and gluconeogenesis; channels excess sugar phosphates to glycolysis in the pentose phosphate pathway |
| 7.03 −1.72          |           | |
| 7.01 −1.68          |           | |
| 9.07 −1.63          |           | |
| 8.83 1.63           |           | |
| 6.18 1.61           |           | |
| 8.95 −1.60          |           | Decreased in keratoconus;⁴⁵ inhibitor of several proteases |
| 5.83 1.59           |           | |
| 8.64 1.46           |           | |
| 7.63 1.30           |           | |
| 8.70 1.23           |           | Involved in gluconeogenesis⁵⁵ |
| 6.10 −1.22          |           | |
| 7.89 −1.14          |           | |
| 7.64 1.07           |           | |
| 10.51 −0.98         |           | |
| 7.20 −0.95          |           | |
| 7.33 −0.92          |           | |
| 7.40 −0.83          |           | Decreased expression in keratoconus epithelium;⁴² involved in glycosaminoglycan metabolism; disulfide as acceptor |
| 7.46 −0.73          |           | |
| 7.29 −0.72          |           | |

In absence of an animal model of keratoconus, we used healthy corneas in the experimental groups. It remains to be investigated, if the identified pathways differ in keratoconic corneas. Also, more studies are needed to fully understand the interaction between gene transcription and phenotypic response after CXL. Although it would have been interesting to validate the significantly transcribed genes on the proteomic level, this aspect was out of scope of this study given the high number of...
Figure 3. Changes in the normalized counts of transcription for selected genes: (A, B) related to enzymatic crosslinking, (C, D) related to proteoglycan glycosylation, (E–H) structural ECM components, (I, J) other ECM components, and (K, L) related to ECM degradation.
identified genes. A further limitation was that we could not separate the differentially transcribed genes according to their origin (keratocytes, epithelial and endothelial cells). Therefore, the results presented here describe the overall response of ECM relevant differential transcription. Future studies may address the individual contribution of keratocytes and epithelial cells, as well as potential effects on wound healing.

In summary, several target genes potentially related to the biomechanical stability and shape of the cornea were identified. Our findings suggest that corneal stiffening after CXL likely results from a decreased ECM degradation in combination with an increased enzymatic glycosylation, and hence, an altered proteoglycan interaction with collagen fibrils. A proteoglycan-based stiffening after CXL also would be in line with previous findings from x-ray scattering.18

### Table 3. Significantly Differentially Transcribed Genes of the Two Strongest Affected Pathways 1 Week After CXL Treatment

| Ensembl ID                        | Gene Name                                      | Gene | Cum_logFC | log10 (cum_P Value) |
|-----------------------------------|------------------------------------------------|------|-----------|---------------------|
| **ECM receptor interaction**      |                                                 |      |           |                     |
| ENSOCUG00000012881                | Collagen type I alpha 1 chain                  | COL1A1 | −2.53     | 2.22                |
| ENSOCUG00000009244                | Thrombospondin 4                               | THBS4 | −2.39     | 3.79                |
| ENSOCUG00000013367                | Collagen type XI alpha 1 chain                 | COL11A1 | −2.33     | 2.9                 |
| ENSOCUG00000012264                | Collagen type I alpha 2 chain                  | COL1A2 | −2.18     | 2.23                |
| ENSOCUG00000000409                | Collagen type VI alpha 2 chain                 | COL6A2 | −2.06     | 1.78                |
| ENSOCUG00000017726                | Integrin subunit alpha 11                      | ITGA11 | −2.03     | 11.21               |
| ENSOCUG00000013276                | Collagen type IV alpha 2 chain                 | COL4A2 | 2.01      | 2.95                |
| **Glycan biosynthesis and metabolism** |                                                 |      |           |                     |
| ENSOCUG00000005127                | Dihydrofolate reductase                        | DHFR  | −3.59     | 7.22                |
| ENSOCUG00000001596                | Beta-1,3-galactosyltransferase 2               | B3GALT2 | 2.42     | 3.3                 |
| ENSOCUG00000009557                | Polypeptide N-acetylatedosaminyltransferase 8  | GALNT8 | 2.29      | 6.9                 |
| ENSOCUG00000009957                | Tyrosine aminotransferase                      | TAT   | 1.58      | 3.89                |
| ENSOCUG0000002336                | Bone marrow stromal cell antigen 1             | BST1   | −1.01     | 8.57                |
| ENSOCUG0000001419                | Cysteine sulfonic acid deacetylase             | CSAD   | −0.98     | 10.51               |
| ENSOCUG00000011080                | Polypeptide N-acetylatedosaminyltransferase 7  | GALNT7 | 0.94      | 3.57                |
| ENSOCUG0000000356                | Glucosylceramide beta                          | GBA    | 0.93      | 5.23                |
| ENSOCUG00000010086                | Enolase 1                                      | ENO1   | −0.85     | 5.15                |
| ENSOCUG0000008667                | Thymidylate synthetase                         | TYMS   | −0.85     | 9.52                |
| ENSOCUG00000000006                | Inositol polyphosphate-1-phosphatase           | INPP1  | 0.83      | 5.33                |
| ENSOCUG00000015904                | Transketolase                                  | TKT    | −0.83     | 7.4                 |
| ENSOCUG00000004762                | Synaptojanin 2                                 | SYNJ2  | −0.8      | 5.45                |
| ENSOCUG00000010823                | Phosphoribosylformylglycinamidine synthase     | PFAS   | −0.77     | 3.86                |
| ENSOCUG00000003862                | Deoxyuridine triphosphatase                    | DUT    | −0.72     | 7.29                |
| ENSOCUG00000013372                | Ribonucleotide reductase catalytic subunit M1   | RRM1   | −0.69     | 9.62                |
| ENSOCUG00000004957                | Polypeptide N-acetylatedosaminyltransferase 3  | GALNT3 | 0.54      | 2.24                |
| ENSOCUG00000004221                | Tyrosinase related protein 1                   | TYRP1  | 0.28      | 7.33                |
| ENSOCUG000000028025                | Ethanolamine kinase 2                          | ETNK2  | 0.24      | 4.35                |
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