Profiling of Concanavalin A-Binding Glycoproteins in Human Hepatic Stellate Cells Activated with Transforming Growth Factor-β1

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Abstract: Glycoproteins play important roles in maintaining normal cell functions depending on their glycosylations. Our previous study indicated that the abundance of glycoproteins recognized by concanavalin A (ConA) was increased in human hepatic stellate cells (HSCs) following activation by transforming growth factor-β1 (TGF-β1); however, little is known about the ConA-binding glycoproteins (CBGs) of HSCs. In this study, we employed a targeted glycoproteomics approach using lectin-magnetic particle conjugate-based liquid chromatography-tandem mass spectrometry to compare CBG profiles between LX-2 HSCs with and without activation by TGF-β1, with the aim of discovering novel CBGs and determining their possible roles in activated HSCs. A total of 54 and 77 proteins were identified in the quiescent and activated LX-2 cells, respectively. Of the proteins identified, 14.3% were glycoproteins and 73.3% were novel potential glycoproteins. Molecules involved in protein processing in the endoplasmic reticulum
(e.g., calreticulin) and calcium signaling (e.g., 1-phosphatidylinositol-4,5-bisphosphate phosphodiesterase β-2 [PLCB2]) were specifically identified in activated LX-2 cells. Additionally, PLCB2 expression was upregulated in the cytoplasm of the activated LX-2 cells, as well as in the hepatocytes and sinusoidal cells of liver cirrhosis tissues. In conclusion, the results of this study may aid future investigations to find new molecular mechanisms involved in HSC activation and antifibrotic therapeutic targets.

**Keywords:** ConA-binding glycoprotein; hepatic stellate cells; transforming growth factor-β1; mass spectrometry; peptides; KEGG pathway

### 1. Introduction

Liver fibrosis is a major complication of various chronic hepatic diseases, resulting from increased production and decreased degradation of the extracellular matrix. Activation and phenotypic transformation of hepatic stellate cells (HSCs) play a central role in the development and resolution of liver fibrosis and cirrhosis [1,2], and in the promotion of hepatocellular carcinoma [3]. Transforming growth factor-β1 (TGF-β1), as the key fibrogenic cytokine [4,5], activates HSCs via the TGFβ1/activin receptor-like kinase 1 (ALK1)/Smad1 pathway and stimulates collagen transcription in HSCs [6].

Glycosylation is the major posttranslational modification to the secretory and the membrane-anchored proteins in eukaryotic cells [7], which plays important roles in cell-cell adhesion, bacterial infection, viral attachment, ligand-receptor binding, and other key cellular processes [8]. In recent years, a growing number of studies have reported that protein glycosylations are frequently altered during physiological and pathological changes [9–11]. Many receptor–ligand interactions lead to alteration of protein-bound glycans, and numerous receptors are regulated by glycosylation to a lesser or greater degree; for example, Notch receptors are the recipient substrates of specific glycosyltransferases [12,13], which regulate the activation of Notch by its ligands and thus affect various processes during development. In HSCs, interleukin-1 and tumor necrosis factor-α have lectin-like activities [14] and recognize disialylated diantennary N-glycans bearing two Neu5Aca2,3 residues [15] and heparin [16,17], respectively. Our previous study also indicated that concanavalin A (ConA) and six other lectins showed stronger binding to the activated HSCs than to the quiescent HSCs [18].

Lectins are carbohydrate-binding proteins that discriminate terminal glycans based on subtle differences in their structures, which can be exploited in the laboratory to detect or isolate specific carbohydrate structures of glycoconjugates, including glycoproteins and glycolipids as well as glycosaminoglycans [19,20]. Along with the rapid improvement of cutting-edge separation technologies coupled with mass spectrometry in the fields of glycoproteomics and glycomics, glycoproteins can be enriched for analysis by hydrazide chemistry [21,22], lectin affinity [23,24], immunoaffinity [25,26], boric acid chemistry [27,28], size exclusion chromatography [29,30], hydrophilic interaction [31,32], and other methods. Lectin affinity chromatography is a unique method that can isolate glycoproteins/glycopeptides containing similar terminal glycan structures from complex samples.
In this study, we employed a targeted glycoproteomics approach using lectin-magnetic particle conjugate-based liquid chromatography-tandem mass spectrometry (LC-MS/MS) to compare the profiles of ConA-binding glycoproteins (CBGs) between human HSCs (LX-2 cell line) with and without activation by TGF-β1. The aim of this study was to determine whether any novel CBGs may be differentially expressed in the activated, compared with quiescent LX-2 cells, and consider the potential roles that these CBGs may play in the pathophysiology of liver fibrosis.

2. Results and Discussion

2.1. Isolation of Con A-Binding Glycoproteins (CBGs) from LX-2 Cells

ConA-magnetic particle conjugates (CMPCs) were used to isolate CBGs from total protein lysates of the activated and quiescent LX-2 cells. The total, unbound, and eluted proteins were resolved by SDS-PAGE and visualized by silver staining. The results showed that the bands of CBGs (Figure 1A, lanes 5 and 6) were complementary to those of the unbound proteins (lanes 3 and 4), and that the eluted CBGs were enriched to some degree from the total proteins (lanes 1 and 2) (Figure 1A).

**Figure 1.** SDS-PAGE analysis and MS identification of the isolated CBGs. (A) Proteins were resolved on 10% SDS gels and stained with silver staining. Lanes 1 and 2 showed total proteins, lanes 3 and 4 showed the unbound proteins, and lanes 5 and 6 showed the enriched CBGs from the quiescent and the activated LX-2 cells, respectively; (B) The Venn diagrams present the numbers of peptides and glycoproteins identified by MS. Left, the quiescent LX-2 cells; right, the activated LX-2 cells.
2.2. Identification of CBGs Isolated by CMPCs by LC-MS/MS

CBGs isolated by CMPCs from total proteins of the activated and quiescent LX-2 cells were identified by Agilent 6530 accurate-mass Q-TOF LC-MS/MS. A total of 118 and 152 unique peptides were identified, respectively, from the quiescent and activated LX-2 cells, and these peptides represented 54 and 77 proteins, respectively (Figure 1B,C). Then, the percentage amounts by which emPAI-based protein abundance estimates deviated from the actual measured values were calculated to permit the analysis and comparison of protein expression between the activated and quiescent LX-2 cells. As a result, four proteins (calreticulin [CALR], histone H4, galectin [LGALS]1, and isoform 1 of nucleoside diphosphate kinase A) were upregulated (fold-change > 2), and two proteins (14-3-3 protein ζ/δ and protein disulfide-isomerase) were downregulated in the activated LX-2 cells (fold-change < 0.5). In addition, 28 proteins (including 14-3-3 protein ε, protease serine 4 isoform B, and 14-3-3 protein γ) were specifically identified in the quiescent LX-2 cells and 51 proteins (including isoform 2 of procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2, POTE ankyrin domain family member F, and vimentin) were specifically identified in the activated LX-2 cells. In total, by mapping to the Uniprot database, 13 (12.4%) of the identified proteins were classified as unreviewed proteins, 15 proteins (14.3%) were classified as glycoproteins, and 77 proteins (73.3%) were classified as unknown proteins (Table 1). According to two online glycosylation site prediction servers (NetNGlyc 1.0 [33] and NetOGlyc 4.0 [34]), 62 of the 77 “unknown” proteins were predicted to have potential N-glycosylation sites, and 15 proteins were predicted to have potential O-glycosylation sites. There were still three proteins that were not identified as glycoproteins by the online servers (Table 1).

Lectins are defined as carbohydrate-binding proteins that are neither antibodies nor enzymes, which have a wide range of glycan-binding specificities, and are therefore suitable for the partial isolation and characterization of a glycome. ConA is a lectin originally extracted from the jack-bean Canavalia ensiformis, which binds specifically to internal and nonreducing terminal α-D-mannosyl (α-Man) and α-D-glucosyl groups (α-Glu) [35]. In this study, two possible consequences of the affinity attachment of CBGs to ConA were considered. First, total proteins were extracted under non-denaturing conditions; therefore, some non-target proteins that interacted with CBGs might have been pulled down together. Second, ConA is known to be a glycoprotein containing N-glycans linked to the 152th amino acid (Asn) for each sub-unit [36], and therefore, it was inevitable that cellular glycan-binding proteins with an affinity for the glycans of ConA might also have been collected by CMPCs. These two factors, together with the addition of the possible unusual O-glycosylations (e.g., O-mannosylation and O-GlcNAc) of the CBGs may explain the identification of some “non-glycoproteins” by MS (Table 1). To solve those two problems as effectively as possible, denaturing rinses were conducted several times with 0.1% (v/v) Tween® 20, and specific competitive elution was performed with 0.5 M methyl-α-D-mannose and 0.2 M methyl-α-D-glucose.
Table 1. Detailed information about the CBGs (ConA-binding glycoproteins) isolated by CMPCs (ConA-magnetic particle conjugates) from activated and quiescent LX-2 cells.

| No. | Accession | Description | Gene Name  | Search Score | Coverage % | A/Q Ratio | Known |
|-----|-----------|-------------|------------|--------------|------------|-----------|-------|
| 1   | IPI00003865 | Isoform 1 of Heat shock cognate 71 kDa protein | HSPA8      | 375          | 26.8       | Q, A      | P      |
| 2   | IPI00022434 | Putative uncharacterized protein ALB | ALB        | 260          | 6.5        | Q, A      | Y      |
| 3   | IPI00021263 | 14-3-3 protein zeta/delta | YWHAZ      | 164          | 15.1       | 0.16      | Q, A   |
| 4   | IPI00010796 | Protein disulfide-isomerase | P4HB       | 109          | 17.5       | 0.5       | Q, A   |
| 5   | IPI00011134 | Putative heat shock 70 kDa protein 7 | HSPA7      | 96           | 6.5        | Q, A      | P      |
| 6   | IPI00220327 | Keratin, type II cytoskeletal 1 | KRT1       | 92           | 3.4        | Q, A      | P      |
| 7   | IPI00009865 | Keratin, type I cytoskeletal 10 | KRT10      | 75           | 14.6       | Q, A      | P      |
| 8   | IPI00217966 | Isoform 1 of L-lactate dehydrogenase A chain | LDHA       | 68           | 4.5        | Q, A      | P      |
| 9   | IPI00021439 | Actin, cytoplasmic 1 | ACTB       | 65           | 17.9       | Q, A      | P      |
| 10  | IPI01969230 | LOC644914; H3F3B; LOC440926; H3F3A Histone H3;xx | Null       | 60           | 8.9        | —         | Q, A   |
| 11  | IPI00418471 | Vimentin  | VIM        | 56           | 2.1        | —         | Q, A   |
| 12  | IPI0020599  | Calreticulin | CALR       | 53           | 10.8       | 2.44      | Q, A   |
| 13  | IPI00183968 | tropomyosin alpha-3 chain isoform 1 | TPM3      | 53           | 7.4        | —         | Q, A   |
| 14  | IPI00218820 | Isoform 3 of Tropomyosin beta chain | TPM2      | 53           | 8.9        | —         | Q, A   |
| 15  | IPI00293665 | Keratin, type II cytoskeletal 6B | KRT6B      | 49           | 4.4        | —         | Q, A   |
| 16  | IPI00453473 | Histone H4 | HIST1H4H   | 36           | 29.1       | 3.13      | Q, A   |
| 17  | IPI00003269 | Beta-actin-like protein 2 | ACTBL2     | 32           | 6.6        | —         | Q, A   |
| 18  | IPI00216783 | ubiquitin carboxyl-terminal hydrolase 2 isoform b | USP2      | 32           | 2.3        | —         | Q, A   |
| 19  | IPI00219219 | Galectin-1 | LGALS1     | 29           | 8.9        | 2.8       | Q, A   |
| 20  | IPI0009636  | Membrane-spanning 4-domains subfamily A member 7 | MS4A7     | 28           | 7.1        | —         | Q, A   |
| 21  | IPI00375617 | Isoform 2 of Abhydrolase domain-containing protein 12B | ABHD12B   | 26           | 2.1        | —         | Q, A   |
| 22  | IPI00012048 | Isoform 1 of Nucleoside diphosphate kinase A | NME1-NME2 | 24           | 11.2       | 2.73      | Q, A   |
| 23  | IPI00005685 | Paraneoplastic antigen Ma1 | PNMA1      | 22           | 3.7        | —         | Q, A   |
| 24  | IPI00022774 | Transitional endoplasmic reticulum ATPase | VCP        | 22           | 3          | —         | Q, A   |
| 25  | IPI00470859 | Putative uncharacterized protein DKFZp686C04126 | MAN1B1    | 22           | 0.8        | —         | Q, A   |
| 26  | IPI00888712 | actin, beta-like 3 | POTEE      | 20           | 2.6        | —         | Q, A   |
Table 1. Cont.

| No.  | Accession   | Description                          | Gene Name  | Search Score | Coverage % | A/Q Ratio | Q or A  | Known |
|------|-------------|--------------------------------------|------------|--------------|------------|-----------|---------|--------|
| 27   | IPI00000816 | 14-3-3 protein epsilon               | YWHAE      | 116          | 14.5       | 0.01      | Q       | P      |
| 28   | IPI00385250 | Protease serine 4 isoform B          | PRSS3      | 75           | 9.6        | 0.01      | Q       | P      |
| 29   | IPI0013508  | Alpha-actinin-1                      | ACTN1      | 61           | 1.3        | 0.01      | Q       | P      |
| 30   | IPI00451401 | Triosephosphate isomerase            | TPI1       | 17           | 7.4        | 0.01      | Q       | P      |
| 31   | IPI00220642 | 14-3-3 protein gamma                 | YWHAG      | 58           | 15         | 0.01      | Q       | P      |
| 32   | IPI00465028 | TPI1 triosephosphate isomerase isoform 2 | TPI1P1    | 56           | 18.9       | 0.01      | Q       | P      |
| 33   | IPI00479722 | Proteasome activator complex subunit 1 | PSME1      | 56           | 4.4        | 0.01      | Q       | P      |
| 34   | IPI00453476 | 29 kDa protein                       | Null       | 37           | 15.7       | 0.01      | Q       | Null   |
| 35   | IPI0021304  | Keratin, type II cytoskeletal 2 epidermal | KRT2      | 33           | 4.5        | 0.01      | Q       | P      |
| 36   | IPI0008527  | 60S acidic ribosomal protein P1      | RPLP1      | 32           | 14         | 0.01      | Q       | P      |
| 37   | IPI0021428  | Actin, alpha skeletal muscle         | ACTA1      | 26           | 15.6       | 0.01      | Q       | P      |
| 38   | IPI00479743 | Isoform 1 of POTE ankyrin domain family member E | POTEKP   | 26           | 5.8        | 0.01      | Q       | P      |
| 39   | IPI00009791 | Isoform WB of Plasma membrane calcium-transporting ATPase 2 | ATP2B2  | 25           | 0.7        | 0.01      | Q       | P      |
| 40   | IPI0014845  | Isoform 1 of Dynein heavy chain 8, axonemal | DNAH8     | 25           | 0.9        | 0.01      | Q       | P      |
| 41   | IPI0086949  | LOC100129520, similar to hCG2044193 | Null       | 25           | 4.6        | 0.01      | Q       | Null   |
| 42   | IPI0002850  | Hepatic leukemia factor              | HLF        | 24           | 4.1        | 0.01      | Q       | P      |
| 43   | IPI0050020  | Parathymosin                         | PTMS       | 24           | 10.8       | 0.01      | Q       | P      |
| 44   | IPI0064885  | Zinc finger protein 3 homolog        | ZFP3       | 23           | 1.8        | 0.01      | Q       | P      |
| 45   | IPI00927540 | ECT2 Protein                         | ECT2       | 20           | 47.8       | 0.01      | Q       | P      |
| 46   | IPI00002349 | Nuclear fragile X mental retardation-interacting protein 2 | NUFIP2    | 19           | 2.6        | 0.01      | Q       | P      |
| 47   | IPI00218667 | Stathmin-2                           | STMN2      | 19           | 5          | 0.01      | Q       | P      |
| 48   | IPI0018146  | 14-3-3 protein theta                 | YWHAQ      | 18           | 9.8        | 0.01      | Q       | P      |
| 49   | IPI00156689 | Synaptic vesicle membrane protein VAT-1 homolog | VAT1     | 18           | 6.4        | 0.01      | Q       | P      |
| 50   | IPI00457114 | Isoform 1 of IQ motif and SEC7 domain-containing protein 1 | IQSEC1   | 18           | 3.7        | 0.01      | Q       | P      |
| 51   | IPI00797750 | 11 kDa protein                       | Null       | 17           | 16.7       | 0.01      | Q       | Null   |
| 52   | IPI0038247  | LOC100287408,hypothetical protein XP_002344194 | Null     | 17           | 8.8        | 0.01      | Q       | Null   |
| 53   | IPI00966637 | Putative uncharacterized protein SDAD1 | SDAD1     | 17           | 23.9       | 0.01      | Q       | P      |
Table 1. Cont.

| No. | Accession   | Description                                                                 | Gene Name                  | Search Score | Coverage % | A/Q Ratio a | Q or A b | Known c |
|-----|-------------|------------------------------------------------------------------------------|-----------------------------|--------------|------------|-------------|----------|---------|
| 54  | IPI00937642 | LOC100290701 hypothetical protein XP_002347764                             | Null                        | 15           | 5.6        | 0.01        | Q        | Null    |
| 55  | IPI00304925 | Heat shock 70 kDa protein 1A/1B                                            | HSPA1A; HSPA1B              | 85           | 9.2        | 100 A      | P N      |
| 56  | IPI00830052 | 62 kDa protein                                                               | Null                        | 85           | 10.5       | 100 A      | Null    |
| 57  | IPI0015614  | Isoform A of Trypsin-3                                                      | PRSS3                       | 78           | 6.6        | 100 A      | P N      |
| 58  | IPI00337495 | Isoform 2 of Procollagen-lysine,2-oxoglutarate 5-dioxygenase 2              | PLOD2                       | 73           | 1.8        | 100 A      | Y N      |
| 59  | IPI00739539 | POTE ankyrin domain family member F                                         | POTENE                       | 63           | 3.6        | 100 A      | P N      |
| 60  | IPI0000230  | tropomyosin alpha-1 chain isoform 2                                          | TPM1                        | 59           | 7.7        | 100 A      | P N      |
| 61  | IPI0013991  | Isoform 1 of Tropomyosin beta chain                                         | TPM2                        | 59           | 8.8        | 100 A      | P N      |
| 62  | IPI0026260  | Isoform 1 of Nucleoside diphosphate kinase B                                 | NME1-NME2                   | 59           | 25         | 100 A      | N        |
| 63  | IPI00879437 | Protein disulfide-isomerase A1                                              | P4HB                        | 54           | 16.5       | 100 A      | P N      |
| 64  | IPI00937995 | Actin-like protein (Fragment)                                                | ACTB                        | 50           | 19.4       | 100 A      | P N      |
| 65  | IPI0024320  | Putative RNA-binding protein 3                                               | RBM3                        | 40           | 20.4       | 100 A      | P N      |
| 66  | IPI00171611 | Histone H3.2                                                                 | HIST2H3A                    | 40           | 11.8       | 100 A      | P N      |
| 67  | IPI0078360  | SARNP 18 kDa protein                                                         | SARNP                       | 40           | 4.3        | 100 A      | P N      |
| 68  | IPI0078173  | cDNA FLJ39583 fis, clone SKMUS2004897, highly similar to ACTIN, ALPHA SKELETAL MUSCLE | ACTA1                       | 38           | 15.2       | 100 A      | P N      |
| 69  | IPI0045396  | Calumenin isoform 4                                                          | CALU                        | 34           | 2.2        | 100 A      | Y N      |
| 70  | IPI0016768  | L-lactate dehydrogenase A-like 6B                                           | LDHAL6B                     | 31           | 4.5        | 100 A      | P N      |
| 71  | IPI00784327 | Isoform 1 of 1-phosphatidylinositol-4,5-bisphosphate phosphodiesterase beta-2 | PLCB2                       | 30           | 1.9        | 100 A      | P N      |
| 72  | IPI0000874  | Peroxiredoxin-1                                                             | PRDX1                       | 29           | 10.1       | 100 A      | P N      |
| 73  | IPI00216817 | HEAT repeat containing 7B1                                                  | HEATR7B1                    | 29           | 1.3        | 100 A      | P N      |
| 74  | IPI00293975 | 16 kDa protein                                                               | Null                        | 29           | 6.2        | 100 A      | Null    |
| 75  | IPI00298622 | Intestinal-type alkaline phosphatase                                         | ALPI                        | 29           | 2.1        | 100 A      | Y N      |
| 76  | IPI00945706 | BOC Protein                                                                 | BOC                         | 29           | 14.3       | 100 A      | P N      |
| 77  | IPI00329801 | Annexin A5                                                                  | ANXA5                       | 26           | 2.8        | 100 A      | P N      |
| 78  | IPI0014424  | Elongation factor 1-alpha 2                                                 | EEF1A2                      | 25           | 3.9        | 100 A      | P N      |
| 79  | IPI00030282 | Isoform 1 of Filensin                                                        | BFSP1                       | 25           | 3.9        | 100 A      | P N      |
| 80  | IPI00910689 | Polysorase-2                                                                 | PRSS36                      | 25           | 3.2        | 100 A      | Y N      |
Table 1. Cont.

| No. | Accession | Description | Gene Name | Search Score | Coverage % | A/Q Ratio a | Q or A b | Known c |
|-----|------------|-------------|-----------|--------------|------------|-------------|----------|---------|
| 81  | IPI00023673 | Galectin-3-binding protein | LGALS3BP | 24 | 3.8 | 100 | A | Y* |
| 82  | IPI00414717 | Isoform 2 of Golgi apparatus protein 1 | GLG1 | 24 | 2.1 | 100 | A | Y* |
| 83  | IPI00007249 | Ectonucleotide pyrophosphatase/phosphodiesterase family member 4 | ENPP4 | 23 | 5.7 | 100 | A | Y* |
| 84  | IPI00008692 | Isoform 1 of Keratin, type I cuticular Ha6 | KRT36 | 23 | 4.9 | 100 | A | P* |
| 85  | IPI00290077 | Keratin, type I cytoskeletal 15 | KRT15 | 23 | 4.4 | 100 | A | P* |
| 86  | IPI00384497 | Protein-tyrosine phosphatase-like member B | PTPLB | 23 | 7.1 | 100 | A | Y* |
| 87  | IPI00908888 | cDNA FLJ57836, highly similar to Myb-binding protein 1A | MYBBP1A | 23 | 2.2 | 100 | A | P* |
| 88  | IPI00024658 | OTU domain-containing protein 7B | OTUD7B | 22 | 2.5 | 100 | A | P* |
| 89  | IPI00384972 | Isoform 1 of MLL1/MLL complex subunit KIAA1267 | KIAA1267 | 22 | 1.5 | 100 | A | P* |
| 90  | IPI00394814 | Serine protease 55 | PRSS55 | 22 | 4.8 | 100 | A | Y* |
| 91  | IPI00216704 | Isoform 2 of Spectrin beta chain, erythrocyte | SPTB I | 21 | 1.7 | 100 | A | P* |
| 92  | IPI00456575 | Centrosomal protein of 192 kDa | CEP192 | 20 | 1.1 | 100 | A | P* |
| 93  | IPI00552749 | DNAH8 478 kDa protein | DNAH8 | 20 | 1.7 | 100 | A | P* |
| 94  | IPI00748715 | SEPT9 protein (Fragment) | Null | 19 | 3.5 | 100 | A | Null |
| 95  | IPI00020035 | Protein NipSnap homolog 3B | NIPSNAP3B | 18 | 12.6 | 100 | A | P* |
| 96  | IPI00217899 | E3 ubiquitin-protein ligase RNF168 | RNF168 | 18 | 1.9 | 100 | A | P* |
| 97  | IPI00010293 | D(1A) dopamine receptor | DRD1 | 17 | 4 | 100 | A | Y* |
| 98  | IPI00478586 | Isoform 2 of Vacuolar protein sorting-associated protein 13A | VPS13A | 17 | 1.9 | 100 | A | P* |
| 99  | IPI00029137 | Conserved hypothetical protein | Null | 17 | 9.9 | 100 | A | Null |
| 100 | IPI00011500 | Isoform 1 of Testicular acid phosphatase | ACPT | 16 | 2.8 | 100 | A | Y* |
| 101 | IPI00299507 | Condensin complex subunit 2 | NCAPH | 16 | 0.9 | 100 | A | P* |
| 102 | IPI00009101 | Isoform 2 of Helicase SRCAP | SRCAP | 15 | 1 | 100 | A | P* |
| 103 | IPI00442299 | Isoform 1 of Neurexin-1-alpha | NRXXN1 | 15 | 3.3 | 100 | A | Y* |
| 104 | IPI00947233 | PCOLCE2 Protein | PCOLCE2 | 14 | 42.6 | 100 | A | P* |
| 105 | IPI00294052 | Single-stranded DNA-binding protein 2 | SSBP2 | 13 | 3.6 | 100 | A | P* |

a: Glycoproteins with an emPAI ratio of >2.0 or <0.5 are shown. “A/Q” represents the ratio of the emPAI value of a protein in the activated LX-2 cells vs. that in the quiescent LX-2 cells. The “A/Q” value of glycoproteins identified specifically in quiescent LX-2 was assigned as 0.01, while that of glycoproteins identified specifically in the activated LX-2 was assigned as 100; b: Data in column “Q or A” represent the CBG identified in the quiescent LX-2 (Q) or the activated LX-2 (A); c: “YN” represents the CBGs annotated as “N-linked glycosylated” in Swiss-Prot; “Y*” represents the CBGs annotated as “O-linked glycosylated” in Swiss-Prot; “PN” represents potential N-linked glycoproteins predicted by the software NetNGlyc 1.0 Server; “P*” represents potential O-linked glycoproteins predicted by the software NetOGlyc 4.0 Server; “N” represents proteins with no typical glycosylation site.
2.3. GO Classification of the Identified CBGs Using Blast2GO®

To investigate the major biological functions of CBGs in LX-2 cells, the commercially available software program Blast2GO was used to analyze the proteins that were identified in LX-2 cells for functional enrichment according to three grouping classifications: cellular components, biological processes, and molecular functions (Figure 2A). Of 105 identified CBGs, 98 had gene ontology (GO) annotations available. In terms of cellular components, 62 proteins (30.1%) were grouped as cell proteins, while 58 proteins (28.2%) were grouped as organelle proteins. Moreover, other protein groupings were found, with groups including: cell membrane (26, 12.6%), macromolecular complex (26, 12.6%), membrane-enclosed lumen (14, 6.8%), and extracellular region (10, 4.9%). In terms of biological processes, about 48 proteins (16.3%), 44 proteins (15.0%), 31 proteins (10.5%), 29 proteins (9.9%), and 27 proteins (9.2%) were included in the groups of cellular process, single-organism process, biological regulation, metabolic process, and response to stimulus, respectively. In terms of molecular function, proteins with binding ability formed the largest group (30, 43.9%) and other smaller groups identified included catalytic activity (14, 21.9%), structural molecule activity (11, 17.2%), and receptor activity (3, 4.7%). Detailed analysis of the binding ability group revealed that proteins with the ability to bind to proteins (21, 47.7%) and ions (8, 18.2%) comprised the most abundant subgroups. To determine whether there were any differences in the three aspects of GO annotations between activated and quiescent LX-2 cells, GO enrichment analysis was performed. Greater enrichment was found in the response to stimulus, detection of stimulus, activation of immune response, and gene silencing groups, while enrichment of the translation initiation and cell cycle process groups was much lesser in activated LX-2 cells than in the quiescent control cells (Figure 2B).

In our previous study, we found large differences in the distribution of glycoproteins bound among ConA and other lectins in LX-2 cells. The glycoproteins recognized by *Aleuria aurantia* lectin (AAL), *Erythrina cristagalli* lectin (ECA), and phytohemagglutinin (PHA-E) were mainly located on the cytoplasmic membrane and the perinuclear cytoplasm (i.e., the endoplasmic reticulum [ER] and Golgi complex), while ConA showed positive binding to the glycoproteins located in the whole cytoplasm and/or cytoplasmic membrane [18]. In the present study, 62 proteins (30.1%) were grouped as intracellular proteins and 58 (28.2%) as organelle proteins. Most of the proteins in the organelle proteins group were also included in the intracellular proteins group. It is known that the common protein-bound glycan adduct, N-linked core oligosaccharide, is composed of two N-acetylgalactosamine, nine mannose, and three glucose residues, and this oligosaccharide molecule is synthesized and processed in the ER [37]. Glycans are subjected to extensive modifications as glycoproteins mature and move through the ER via the Golgi complex to their final destinations inside and outside the cell [38]. Con A can specifically recognize the trimannoside core of the N-glycan [39], so N-glycosylated proteins that are still undergoing processing and sorting in the ER and Golgi can also be recognized by ConA, in addition to the mature membrane and secreted glycoproteins. For some organelle proteins, such as Golgi glycoprotein 1 (GLG1) (Table 1) and lysosomal enzymes, their synthesis and positioning also depend on the glycosylation associated with ER-Golgi trafficking [40,41]. In addition, O-glycosylations such as O-GlcNAc, which contribute to nutrient sensing and regulation of insulin signaling, cell cycle, calcium signaling pathway, and cellular stress response [42–44], may contain
multiple mannose residues that can also be recognized by ConA, since CBGs were distributed not only in the perinuclear cytoplasm of the LX-2 cells, but also in the whole cytoplasm and cell membrane.

**Figure 2.** The GO annotations of the identified CBGs in LX-2 cells. (A) Total CBGs identified in LX-2 cells were analyzed for functional enrichment according to the three grouping classifications of cellular component, biological process, and molecular function using the software Blast2GO®; (B) Gene ontology (GO) classification and comparison of enrichment of functional groups between the quiescent and the activated LX-2 cells was performed using WEGO software.
2.4. KEGG Pathway Analysis and Functional Protein Association Networks

In total, 91 of 105 identified CBGs had been annotated in DAVID Bioinformatics Resources version 6.7. These glycoproteins were mapped to 6 KEGG pathways with the thresholds of count of >2 and p value of <0.05 compared with the background signal of the human genome; the identified KEGG pathways included protein processing in the ER, calcium signaling pathway, cell cycle, glycolysis/gluconeogenesis, and others (Figure 3A,B, Table S1). Proteins involved in protein processing in the ER (e.g., CALR, protein disulfide-isomerase A1, and heat shock 70-kDa protein 1A/1B) and calcium signaling pathway (e.g., D[1A] dopamine receptor [DRD1] and 1-phosphatidylinositol-4,5-bisphosphate phosphodiesterase β-2) were specifically identified or upregulated in the activated LX-2 cells. In contrast, 14-3-3 protein family members (e.g., 14-3-3 ζ/δ, 14-3-3 ε, and 14-3-3 γ) involved in the cell cycle and the neurotrophin signaling pathway were specifically identified in the quiescent LX-2 cells.

Figure 3. KEGG pathway analysis and functional protein association networks. (A,B) show the CBGs mapped to the KEGG pathways of protein processing in the ER and calcium signaling. The CBGs involved in these networks are labeled with a red frame; (C,D) display the potential interactions among total CBGs and show the CBGs that were determined to show significant correlations by STRING analysis.
Figure 3. Cont.
A total of 90 matched CBGs were queried against the STRING *Homo sapiens* database to determine their functional relevance (Figure 3C). Through K-mean clustering analysis, associations among TPM1, TPM2, ACTB, DNAH8, and ACTA1; LGALS1, ANXA5, and LGALS3BP; P4HB and CALR; and HSPA1B and HSPA1A were specifically identified in the activated LX-2 cells, whereas associations among YWHAZ, YWHAE, YWHAG, and YWHAQ were specifically identified in the quiescent LX-2 cells (Figure 3D).

The aims of this study were not only to find novel CBGs that differentially expressed in the activated HSCs, but also to speculate the possible pathway networks associated with fibrogenesis in HSCs. Proteins involved in protein processing in ER and calcium signaling pathway were higher expressed in the activated HSCs (Figure 3A, B, and Table S1), which partially demonstrated that these pathways were activated in HSCs when stimulated by TGF-β1. Interestingly, the expression levels of galectin-1 (LGALS1) and galectin-3-binding protein (LGALS3BP) were upregulated or specifically identified in the activated LX-2 cells (Table 1). The functions of galectins have been reported to be...
involved in physiological and pathological processes of the liver [45,46]. A previous proteomics analysis of rat HSC proteins revealed that the production and secretion of LGALS1 is greatly increased in activated HSCs compared to that in quiescent HSCs [45]. LGALS3 expression was found to be induced in regenerative nodules of liver cirrhosis tissues and in hepatocellular carcinomas [47]. A further study demonstrated that both LGALS1 and LGALS3 activate mitogen-activated protein kinase (MAPK) pathways, presumably by forming cross-links with target molecules through their β-galactoside-containing glycoconjugates, leading to the proliferation of HSCs [48]. In addition, an increased concentration of cytoplasmic Ca2+ can also activate the Ca2+/calmodulin-dependent protein kinase (CaMKII)/MAPK signaling pathway [49]. Intracellular free Ca2+ is a crucial second messenger that plays various roles in regulating a wide range of cellular processes in different cells and tissues. To maintain a low Ca2+ concentration of 10–100 nM in the cytoplasm, Ca2+ is actively pumped from the cytoplasm to the extracellular space and ER, and sometimes into the mitochondria. Specific signals can trigger an increase in the cytoplasmic Ca2+ level up to 500–1000 nM by opening channels in the plasma membrane or ER [50]. DRD1 is a G protein-coupled receptor that stimulates adenylate cyclase and was found to inhibit Ca2+ from being pumped from the cytosol to the ER [51]. The plasma membrane Ca2+ ATPase (ATP2B) is a transport protein in the plasma membrane of cells that serves to remove Ca2+ from the cytosol [52]. 1-phosphatidylinositol-4,5-bisphosphate phosphodiesterase β-2 (PLCB2) participates in phosphatidylinositol 4,5-bisphosphate (PIP2) metabolism and indirectly promotes the release of Ca2+ from the ER to the cytosol [53]. According to our results, DRD1 and PLCB2 were specifically identified in the activated LX-2 cells, whereas ATP2B was specifically identified in the quiescent LX-2 cells, indicating that Ca2+ became more inclined to accumulation in the cytoplasm, rather than the extracellular space or the ER in the LX-2 cells activated with TGF-β1 (Figure 3B). Thus, it can be conjectured that increases in galectin expression and intracellular Ca2+ concentration might collectively activate the MAPK signaling pathway to realize the activation of HSC induced by TGF-β1. This view is different from the traditional view that platelet derived growth factor (PDGF) induced the activation of HSCs through MAPK signaling pathway [54] and TGF-β1 through TGFβ1/ALK1/Smad1 pathway, and might be therapeutic targets of liver fibrosis. Actually, a recent study suggested that the production of CTGF by TGF-β activated MAPK signaling in hepatic progenitor cells [55]. However, the detailed molecular mechanisms of these cytokines still need to be further studied.

2.5. Expression of PLCB2 in Activated LX-2 Cells and Human Liver Cirrhosis Tissue

To further validate and investigate the expression of CBGs and their relationship to liver fibrosis and cirrhosis, the increased expression of PLCB2, which was specifically identified in the activated LX-2 cells, was measured in LX-2 cells and human liver by western blotting and immunohistochemistry, respectively. The expression of PLCB2 was found to be distinctly increased in activated, compared with quiescent LX-2 cells (Figure 4A,B) and was also increased in liver cirrhosis tissues compared with normal para-carcinoma tissues (Figure 4C). PLCB2 was found to be mainly distributed in the cytoplasm of the activated LX-2 cells (Figure 4B), as well as that of hepatocytes and sinusoidal cells in liver tissues (Figure 4C). These results were consistent with the MS analysis and
provided further evidence indicating a possible vital function of PLCB2 in the development of liver fibrosis and cirrhosis.

**Figure 4.** Expression of PLCB2 in the activated LX-2 cells and human liver cirrhosis tissue. (A) Western blots showed that the expression of PLCB2 was significantly increased in the activated, compared with quiescent LX-2 cells; (B) Immunohistochemistry to validate and investigate the expression and location of PLCB2 in the quiescent and the activated LX-2 cells. The images were shown on the same scale for the quiescent and activated LX-2 cells (images were obtained using a 40× objective lens); (C) Immunohistochemistry to investigate the expression and location of PLCB2 in liver cirrhosis tissues and the normal para-carcinoma tissues. The images were taken using 20×, 40×, and 100× objective lenses, respectively.

In this study, 62 proteins (30.1%) were intracellular proteins and 26 (12.6%) were located on the cell membrane, in accordance with the result that ConA showed strong binding to the cytoplasm and/or
membrane of LX-2 cells in our previous study [18]. The activation of HSCs is a complex and persistent response to chronic liver injury. In the initial stages, HSCs receive paracrine stimulation from cytokines secreted by neighboring cells such as Kupffer cells, hepatocytes, and leukocytes. In the persistent stages, autocrine cytokines (e.g., TGF-β and connective tissue growth factor) persistently stimulate HSCs so as to regulate and maintain their activation [56,57]. In this study, a greater number of CBGs were identified from the activated LX-2 cells than from the quiescent LX-2 cells. In addition, glycoproteins involved in the biological processes of response to stimulus, detection of stimulus, and activation of immune response were upregulated in the activated LX-2 cells. For example, PLCB2 was validated to be upregulated in the cytoplasm of the activated LX-2 cells, as well as in the hepatocytes and sinusoidal cells of liver cirrhosis tissues. Based upon the above findings, CBGs might play important roles in signal conduction and response to stimulus, and be associated with the formation and development of liver fibrosis and cirrhosis.

3. Experimental Section

3.1. Materials

Dulbecco’s modified Eagle’s medium (DMEM) and fetal bovine serum (FBS) were purchased from Invitrogen (Carlsbad, CA, USA). TGF-β1 was purchased from R&D systems (Minneapolis, MN, USA). Con A was purchased from Vector Laboratories (Burlingame, CA, USA). DTT and iodoacetamide were purchased from GE Healthcare (Little Chalfont, Buckinghamshire, UK). Mouse anti-PLCB2 monoclonal antibody, urea, ammonia bicarbonate (NH₄HCO₃), sodium periodate (NaIO₄), HPLC-grade acetonitrile (ACN) and trifluoroacetic acid (TFA), protease inhibitor cocktail, and Bradford assay reagent were purchased from Sigma-Aldrich (St. Louis, MO, USA). Trypsin was purchased from Promega (Madison, WI, USA). Peptide-N-glycosidase F (PNGase F) was purchased from New England Biolabs (Ipswich, MA, USA). Bovine serum albumin (BSA) was purchased from Merck (Darmstadt, Germany). T-PER® reagent was purchased from Pierce Biotechnology (Rockford, IL, USA). Other chemical reagents were obtained from commercial suppliers and used without further purification. All of the solutions were prepared with ultra-pure water obtained from a Milli-Q® 50 SP Reagent Water System (Millipore, Bradford, MA, USA).

Amicon Ultra-0.5 10-kDa devices were obtained from Millipore. C18 SepPak® columns were from Waters (Milford, CT, USA). The ZHWY- 2101C Oscillator was purchased from ZhiCheng Corporation, China. The SpeedVac™ vacuum concentrator was obtained from Thermo Scientific (Waltham, MA, USA). Nanospray CHIP-LC-MS/MS 6530 mass spectrometers equipped with HP 1200 solvent delivery systems were purchased from Agilent (Santa Clara, CA, USA).

3.2. Cell Culture and Stimulation

The LX-2 cells were a gift from Dr. Friedman of the Mount Sinai School of Medicine, New York, NY, USA. Cells were cultured and stimulated as described previously [18,58]. About 2 × 10⁵ LX-2 cells were seeded and rested in high-glucose DMEM supplemented with 10% (v/v) FBS for 24 h. After 24 h of starvation, cells were stimulated by adding 2 ng/mL TGF-β1 for 24 h. The control (quiescent) groups were not treated with TGF-β1.
3.3. Non-Denaturing Extraction of Total Proteins

Total protein was extracted from LX-2 cells using non-denaturing T-PER reagent as described previously [18]. The adherent cells were washed twice with ice-cold 1× PBS (0.01 M phosphate buffer containing 0.15 M NaCl, pH 7.4) to remove culture medium. The cells were incubated for 15 min with T-PER reagent mixed with protease inhibitors (10 μL/mL T-PER reagent) and then homogenization was achieved by triturating cells with a pipette. Following centrifugation at 10,000×g for 10 min, the supernatants were either immediately collected for use or stored at −80 °C. The protein concentration in the supernatants was determined by Bradford assay.

3.4. Isolation of Glycoproteins from LX-2 Cells Using ConA-Magnetic Particle Conjugates (CMPCs)

A subset of glycoproteins binding to ConA were selectively isolated by CMPCs as described previously [59,60]. Briefly, 2 mg of total proteins from LX-2 cells were diluted into 600 µL of binding buffer (0.1 M Tris-HCl, 150 mM NaCl, 1 mM CaCl₂, 1 mM MgCl₂, and 1 mM MnCl₂, pH 7.4) supplemented with 1% (v/v) proteinase inhibitor. The homemade ConA-magnetic particle conjugates (CMPCs) were rinsed three times with binding buffer and then incubated with proteins at room temperature for 3 h under gentle shaking. The unbound proteins were removed by washing three times with the rinsing buffer (binding buffer with the addition of 0.1% (v/v) Tween 20) thoroughly. Then, the glycoproteins bound to CMPCs were eluted with 300 µL of the elution buffer (binding buffer supplemented with 0.5 M methyl-α-D-mannose, 0.2 M methyl-α-D-glucose, and 1% (v/v) proteinase inhibitor) at room temperature for 1 h under gentle shaking, and the supernatants were collected.

3.5. Identification of Peptides by LC-MS/MS

The glycoproteins isolated by CMPCs were concentrated and desalted by a size-exclusion spin filter (Amicon Ultra-0.5 10 K device), with a molecular mass cutoff of 10 kDa. The obtained glycoproteins were denatured in 8 M urea, then deoxidated with 10 mM DTT and carboxyamidomethylated with 20 mM iodoacetamide. Subsequently, trypsin was added at a 1:100 (w/w) ratio of enzyme to protein and the samples were incubated overnight at 37 °C. Glacial acetic acid (5 µL, pH 2.0) was added to stop the reaction, samples were centrifuged, and the supernatants were collected and lyophilized. Then, the N-linked glycans of the glycopeptides were released by incubation with PNGase F overnight at 37 °C. The peptides were desalted using C18 SepPak columns and lyophilized. Finally, the peptides were resolubilized in 0.1% (v/v) formic acid and analyzed using nanospray CHIP-LC-MS/MS 6530 mass spectrometers equipped with HP 1200 solvent delivery systems.

3.6. Label-Free Relative Quantification of Identified Glycoproteins by emPAI

Label-free quantification methods are popular because of their convenience and accuracy. Mascot search engine software (Matrix Science, Boston, MA, USA) includes a semi-quantification method, emPAI, which is a convenient tool for relative quantification [61]. To compare the activated versus quiescent LX-2 glycoproteomes, we calculated normalized emPAI values for each protein (see Formula (1)), and estimated the fold-change in the activated versus quiescent LX-2 cells. When a
protein was not identified in the quiescent LX-2 cells, we arbitrarily assigned the fold-change as 100 to avoid dividing by zero.

\[
\text{normalized emPAI of a protein} = \frac{\text{emPAI of that protein}}{\text{sum of all emPAIs in a sample}} \times 100 \quad (1)
\]

### 3.7. Data Mining and Bioinformatics

Ontology analysis was performed according to the standard procedure of Blast2GO [62] to gain insights into functional groupings within the set of identified glycoproteins in three aspects of GO. The differences in the GO terms of glycoproteins between the activated and quiescent LX-2 cells were also discovered by analysis using WEGO [63]. Biological pathways that were enriched in the identified glycoproteins originated from the KEGG human pathway database [64]. To validate the results of Blast2GO and KEGG, an independent functional enrichment analysis was performed using DAVID (Database for Annotation, Visualization and Integrated Discovery) [65]. Finally, to determine the functional relevance of the identified glycoproteins, we performed an analysis of protein association networks using STRING 9.05 [66].

### 3.8. SDS-PAGE and Western Blotting

For SDS-PAGE, protein samples were mixed with 5× loading buffer and boiled for 4 min at 100 °C, and then separated on a 10% polyacrylamide resolving gel and a 3% stacking gel. Molecular mass standards (Thermo Scientific) were run for each gel. Gels were then stained directly with alkaline silver. For western blotting, 30 μg of proteins were separated by 10% SDS-PAGE and then transferred to PVDF membranes (Millipore). After blocking for 2 h at room temperature with 5% (w/v) skimmed milk powder in TBS (100 mM Tris-HCl, 150 mM NaCl, pH 7.6), the membrane was incubated overnight at 4 °C with mouse monoclonal antibodies against PLCB2 in antibody dilution buffer (5% (w/v) skimmed milk in TBST (0.05% (v/v) Tween 20 in TBS, pH 7.6)). The membranes were washed three times with TBST, and then incubated with alkaline phosphatase-conjugated secondary antibodies (1:1000 (v/v) dilution in antibody dilution buffer) for 1 h at room temperature. The membranes were washed three times with TBST and detected using a 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium salt substrate kit. β-Actin was used as an internal control.

### 3.9. Immunohistochemistry

Immunohistochemistry was performed as described previously [67]. Briefly, 2 × 10^5 LX-2 cells were seeded in 6-well culture plates containing sterile coverslips. The adherent cells were fixed with 4% (w/v) paraformaldehyde and permeabilized in ice-cold 1× PBS supplemented with 1% (v/v) Triton™ X-100 at 4 °C for 10 min and rinsed twice in 1× PBS. The permeabilized cells were then treated with hydrogen peroxide (H₂O₂) (3% (v/v) in methanol) for 20 min to block endogenous peroxidase. Prior to incubation with anti-PLCB2, cells were blocked with blocking solution (10% (w/v) BSA in 1× PBS) for 1 h at room temperature. Then, cells were incubated with PLCB2 antibody diluted at 1:1000 in blocking solution overnight at 4 °C. Then, cells were incubated with alkaline phosphatase-conjugated secondary antibodies in blocking solution for 1 h at room temperature, and horseradish peroxidase colorimetric development solution was applied according to the manufacturer’s
instructions. Finally, the cells were further stained with hematoxylin and histological images were obtained.

4. Conclusions

The profile of CBGs in human HSCs (LX-2 cell line) activated with TGF-β1 was compared with that of quiescent HSCs by employing a glycoproteomics approach using a CMPCs coupled MS technique. Novel CBGs and the signaling pathways/networks in which they were involved were discovered and partially validated to be connected with the activation of HSCs. The results of this study may provide useful information to aid future investigation to identify new molecular mechanisms for HSC activation and antifibrotic therapeutic targets.

Supplementary Materials

Supplementary materials can be accessed at: http://www.mdpi.com/1420-3049/19/12/19845/s1.

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Author Contributions

Y.Q. carried out enrichment of CBGs by CMPCs, generated the graphs for proteomic data and wrote the manuscript; Y.Z. participated in the culture and stimulation of LX-2 cells; G.Y. carried out MS identification; T.M. performed the preparation of CMPCs; L.J. participated in data analysis; C.H. participated in the revision of the manuscript; and Z.L. participated in the design of the project, coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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**Sample Availability**: Samples of the compounds upon request are available from the authors.