Heparan sulfate is necessary for the early formation of nascent bronectin and collagen I bris at matrix assembly sites

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Fibronectin FN), an essential component of the extracellular matrix ECM), is assembled *vi* a cell-mediated process in which integrin receptors bind secreted FN and mediate its polymerization into bris that extend between cells, ultimately forming an insoluble matrix. Our previous work using mutant Chinese hamster ovary CHO) cells identi ed the glycosaminoglycan heparan sulfate HS) and its binding to FN as essential for the formation of insoluble FN bris. In this study, we investigated the contributions of HS at an early stage of the assembly process using knockdown of exostosin-1 EXT1), one of the glycosyltransferases required for HS chain synthesis. NIH 3T3 broblasts with decreased EXT1 expression exhibited a signi cant reduction in both FN and type I collagen in the insoluble matrix. We show that FN bril formation is initiated at matrix assembly sites, and while these sites were formed by cells with EXT1 knockdown, their growth was stunted compared with wild-type cells. The most severe defect observed was in the polymerization of nascent FN bris, which was reduced 2.5-fold upon EXT1 knockdown. This defect was rescued by the addition of exogenous soluble heparin chains long enough to simultaneously bind multiple FN molecules. The activity of soluble heparin in this process indicates that nascent bril formation depends on HS more so than on the protein component of a speci c HS proteoglycan. Together, our results suggest that heparin or HS is necessary for concentrating and localizing FN molecules at sites of early bril assembly.

Assembly of a bronectin (FN) extracellular matrix (ECM) is essential for embryogenesis and the development, repair, regeneration, and homeostasis of all tissues. Disrupted or disordered bronectin assembly occurs in many diseases and is key to the progression of brosis and scarring. FN binding to integrin receptors, especially 5β1 integrin, is required for assembly (1, 2). Fibril formation also depends on FN self-association, mediated by its N-terminal assembly domain (3). FN has many other binding partners, including the glycosaminoglycans (GAGs) heparan sulfate and heparin that interact with its main heparin-binding domain (HepII) (4). A dose-dependent loss of FN matrix was detected when GAG chain addition to proteins was blocked with xylosides (5) further implicating GAGs in matrix assembly.

Heparan sulfate (HS)-modi ed proteoglycans (HSPGs) are found within the ECM and at the cell surface. HS chains are elongated onto core proteins in the Golgi apparatus by a complex of glycosyltransferases, exostosin-1 (EXT1) and exostosin-2 (EXT2) (6–10). Screening of a mutagen-treated Chinese hamster ovary (CHO) cell library identi ed CHO-677 cells, which show signi cantly reduced EXT1 mRNA levels and GlcA/GlcNAc transferase activities compared with wild-type CHO cells. The mutation eliminates HS synthesis (11) and causes a severe de ciency in assembly of FN (12, 13) presumably due to decreased EXT1 expression although the speci c mutation has not been determined. Mutations in EXT1 have been linked to skeletal abnormalities and osteochondromas (14). Conditional knockout of EXT1 in limb bud mesenchyme causes defects in growth and differentiation of cartilage condensations, including an abnormal perichondrium with dispersed FN matrix (15). It seems clear that HS plays a role in FN matrix assembly and organization both *in vivo* and in cell culture.

Our previous work using CHO cells identi ed a role for HS in conversion of FN bris into a stabilized, detergent-insoluble form (13). Since insolubility is the nal step in FN assembly, this nding shows that HS functions late in assembly. We also observed higher levels of detergent-soluble FN in lysates from CHO cells treated with heparin. This observation suggests that HS may have another role in assembly, in an earlier step. To determine how HS promotes FN brillogenesis in cells that normally produce and assemble FN, we performed siRNA-mediated knockdown of EXT1 in NIH 3T3 broblasts and investigated the effects of loss of HS on the progression of FN matrix assembly. We analyzed and quanti ed matrix assembly sites (the initial sites of FN bril formation), nascent FN bris, and stable FN matrix in mock treated *versus* EXT1 knockdown broblasts. Here we show that decreasing EXT1 mRNA expression in broblasts reduces HS production causing defects in the initial stages of FN brillogenesis. Furthermore, the role of HS/heparin is chain length dependent. We propose that in addition to its role in forming insoluble FN bris, HS binding to FN is also involved in early steps of assembly where

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it promotes FN-FN interactions by concentrating FN near sites of matrix assembly initiation.

Results

Reduced HS with EXT1 knockdown in NIH 3T3 cells

To establish the role of HS in assembly of an FN matrix, we used NIH 3T3 broblasts, which synthesize and secrete high levels of FN and assemble it into a dense brillar matrix. NIH 3T3 broblasts were treated with an siRNA SMARTpool targeting the EXT1 transcript. Analysis of RNA harvested 48 h and 120 h posttransfection shows that, compared with mock-transfected cells, EXT1 mRNA was signi cantly decreased with siRNA knockdown by approximately 60 at 48 h and 40 at 120 h as determined by qPCR (Fig. 1*A*). Since EXT1 mRNA levels appear to begin recovering at 120 h, all experiments were conducted within 4 days of siRNA treatment. FN transcript levels were measured in parallel and did not show a signi cant change (Fig. 1*A*). In addition, there was no signi - cant difference in 5 integrin levels between mock and EXT1 knockdown cells (data not shown). Therefore, any difference in FN matrix assembly is not due to loss of FN or its primary receptor.

EXT1 is required for synthesis of HS so we evaluated HS levels by immunofluorescence staining. HS fluorescence was signi cantly reduced, ∼tenfold, for cell surface HS detected with an anti-HS monoclonal antibody (10E4) in EXT1 knockdown cells compared with mock-treated cells (Fig. 1*B*). A similar difference in anti-HS staining was observed between wild-type CHO cells and HS-de cient CHO-677 cells (Fig. 1*C*). These results con rm that siRNA knockdown of EXT1 signi cantly reduced HS expression.

EXT1 knockdown cells assemble less FN matrix

To test if decreased EXT1 and HS lead to a decrease in FN matrix assembly, matrix levels were assessed by immunofluorescence at 8 h, 24 h, and 48 h postseeding. Mock-treated cells assembled more FN matrix than EXT1 knockdown cells at all time points. Mean fluorescence intensities of FN matrix were signi cantly lower in EXT1 knockdown cells in comparison to the mock-transfected cells (Fig. 2*A*).

Nascent FN brils are soluble in deoxycholate (DOC) detergent but are ultimately converted to a DOC-insoluble form, which serves as a foundational matrix for deposition of other ECM proteins (1). Analysis of DOC-insoluble matrix provides a quantitative measure of matrix assembly. Immunoblots of DOC-insoluble FN showed dramatically higher FN levels in mock-treated compared with EXT1 knockdown cells at 8, 24, and 48 h (Fig. 2*B*). Quanti cation of DOC-insoluble FN normalized to GAPDH (Fig. S1) shows that FN levels in EXT1 knockdown cells never reach the level detected in mock cells even at the latest time point indicating that decreased matrix is not due to a simple difference in the rate of FN assembly. We conclude that HS facilitates assembly of stable matrix and, in its absence, assembly is defective. In support of this conclusion, we found ∼2.3-fold higher FN in the conditioned medium of EXT1 knockdown cells compared with mock-transfected cells suggesting that, while similar levels of FN are produced (as shown by qPCR in Fig. 1*A*), less FN is assembled in the absence of EXT1.

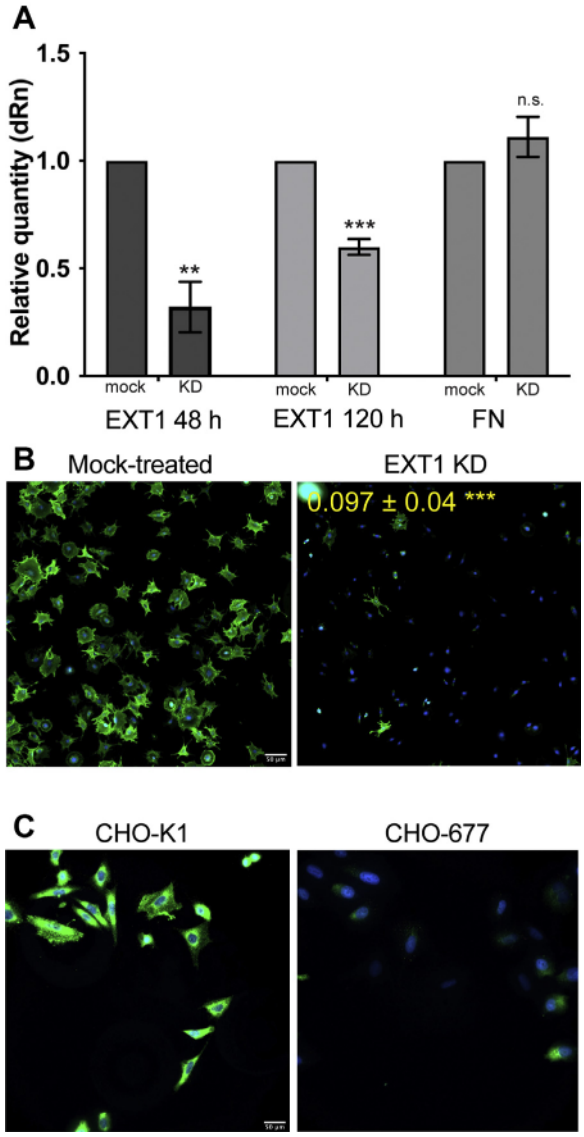

Figure 1 Reduced HS with EXT1 knockdown in broblasts *A*, RNA was isolated from EXT1 siRNA-treated (KD) and mock-transfected NIH 3T3 - broblasts at 48 and 120 h after transfection. Expression of EXT-1 and FN (at 48 h) was assessed by qPCR. Each sample was normalized to GAPDH, and fold-change was calculated relative to mock-treated cells. Error bars represent SEM. $^{**}p$ 0.01, $^{***}p$ 0.001, n.s. = not signi cant (three independent experiments). *B*, mock-treated and EXT-1 knockdown cells were grown on FN-coated coverslips for 4 h and then xed and stained with anti-HS antibody (*green*) and DAPI (*blue*). Scale = 50 m. Representative images are shown for each condition. $^{***}p$ 0.001. *C*, wild-type Chinese hamster ovary cells (CHO-K1) and HS-de cient CHO-677 mutant cells grown on FN-coated coverslips for 4 h were xed and stained with anti-HS antibody (*green*) and DAPI (*blue*). Scale = 50 m. Representative images are shown for each cell type.

The genetic bone disorder, hereditary multiple exostoses (HME), is caused by mutations in EXT1 and is characterized by multiple benign bony outgrowths (16). Type I collagen, the major protein component of bones, is critical for proper bone