Changes in the Chondrocyte and Extracellular Matrix Proteome during Post-natal Mouse Cartilage Development*

Richard Wilson‡¶¶, Emma L. Norris¶¶, Bent Brachvogel**‡‡, Constanza Angelucci‡, Snezana Zivkovic‡, Lavinia Gordon‡, Bianca C. Bernardo‡¶¶, Jacek Stermann‡‡, Kiyotoshi Sekiguchi¶¶, Jeffrey J. Gorman¶¶, and John F. Bateman‡‡¶¶

Skeletal growth by endochondral ossification involves tightly coordinated chondrocyte differentiation that creates reserve, proliferating, prehypertrophic, and hypertrophic cartilage zones in the growth plate. Many human skeletal disorders result from mutations in cartilage extracellular matrix (ECM) components that compromise both ECM architecture and chondrocyte function. Understanding normal cartilage development, composition, and structure is therefore vital to unravel these disease mechanisms. To study this intricate process in vivo by proteomics, we analyzed mouse femoral head cartilage at developmental stages enriched in either immature chondrocytes or maturing/hypertrophic chondrocytes (postnatal days 3 and 21, respectively). Using LTQ-Orbitrap tandem mass spectrometry, we identified 703 cartilage proteins. Differentially abundant proteins (q < 0.01) included prototypic markers for both early and late chondrocyte differentiation (epiphycan and collagen X, respectively) and novel ECM and cell adhesion proteins with no previously described roles in cartilage development (tenascin X, vitrin, Urb, emilin-1, and the sushi repeat-containing proteins SRPX and SRPX2). Meta-analysis of cartilage development in vivo and an in vitro chondrocyte culture model (Wilson, R., Diseberg, A. F., Gordon, L., Zivkovic, S., Tatarczuch, L., Mackie, E. J., Gorman, J. J., and Bateman, J. F. (2010) Comprehensive profiling of cartilage extracellular matrix formation and maturation using sequential extraction and label-free quantitative proteomics. Mol. Cell. Proteomics 9, 1296–1313) identified components involved in both systems, such as Urb, and components with specific roles in vivo, including vitrin and CILP-2 (cartilage intermediate layer protein-2). Immunolocalization of Urb, vitrin, and CILP-2 indicated specific roles at different maturation stages. In addition to ECM-related changes, we provide the first biochemical evidence of changing endoplasmic reticulum function during cartilage development. Although the multifunctional chaperone BiP was not differentially expressed, enzymes and chaperones required specifically for collagen biosynthesis, such as the prolyl 3-hydroxylase 1, cartilage-associated protein, and peptidyl prolyl cis-trans isomerase B complex, were down-regulated during maturation. Conversely, the luminal proteins calumenin, reticulocalbin-1, and reticulocalbin-2 were significantly increased, signifying a shift toward calcium binding functions. This first proteomic analysis of cartilage development in vivo reveals the breadth of protein expression changes during chondrocyte maturation and ECM remodeling in the mouse femoral head. Molecular & Cellular Proteomics 11: 10.1074/mcp.M111.014159, 1–18, 2012.

Cartilage is a unique tissue characterized by an abundant extracellular matrix (ECM) and a single cell type, the chondrocyte. However, the permanent hyaline cartilage, which provides the articulating surfaces of long bones and vertebrae, and the transient growth plate cartilage responsible for endochondral bone growth are uniform in neither cellular phenotype nor protein composition. In articular cartilage, the chondrocytes form morphologically distinct regions comprising a superficial region of flattened cells, a sparsely populated middle layer, and a deep zone of hypertrophic chondrocytes embedded in calcified cartilage at the chondro-osseous junc-

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1 The abbreviations used are: ECM, extracellular matrix; LTQ, linear trap quadrupole; FDR, false discovery rate; GdnHCl, guanidinium hydrochloride; DAVID, Database for Annotation, Visualization and Integrated Discovery; GO, gene ontological; Pn, post-natal day n; ER, endoplasmic reticulum; COMP, cartilage oligomeric matrix protein; TGF, transforming growth factor.
In mature articular cartilage, these chondrocytes divide infrequently. In contrast, the active division and expansion of chondrocytes in growth plate cartilage is the primary mechanism for growth of the axial and appendicular skeletal elements (see Fig. 1). Growth plate chondrocytes enter the maturation process from a pool of reserve zone cells in the epiphyseal cartilage most distal to the chondro-osseous junction. These small round cells differentiate into discoid proliferating chondrocytes that align into columns and dictate the axis of bone growth. The chondrocytes then enter a postmitotic prehypertrophic phase and expand in volume to form fully differentiated hypertrophic chondrocytes that provide a niche for vascular invasion and remodeling of the cartilage into bone (1).

In humans, disruption of chondrocyte maturation in the growth plate results in inherited skeletal dysplasias ranging in severity from mild dwarfism (e.g. metaphyseal chondrodysplasia, Schmid type) and early onset osteoarthritis (e.g. multiple epiphyseal dysplasia) to perinatal lethality (e.g. achondrogenesis). Although individually rare, these skeletal dysplasias collectively affect 2–5 per 10,000 live born (reviewed in Ref. 2). Mutations underlying skeletal dysplasias frequently compromise the precise assembly and interaction of cartilage ECM components, highlighting the critical role of ECM networks in chondrocyte differentiation, organization, and survival (3). In addition to a loss of ECM integrity, endoplasmic reticulum (ER) stress and activation of the unfolded protein response contribute to the pathology (4).

Microarray analysis of microdissected mouse cartilage zones has generated differential mRNA expression profiles of chondrocyte subpopulations (5–7). However, proteomics level analysis of cartilage development is challenging because of the limited available tissue and dominance of poorly soluble matrix components (8). Novel methods developed for proteomic analysis of growth plate and articular cartilage (9–11), in particular using solubility-based tissue fractionation, have improved coverage of both the intracellular and extracellular cartilage proteome using both two-dimensional electrophoresis and capillary HPLC-tandem MS (12, 13). In this study we used mouse femoral head cartilage to identify novel proteins associated with chondrocyte differentiation and cartilage development in vivo. Developmental stages with clear differences in chondrocyte differentiation states were selected based on histomorphology and FACS. At post-natal day 3 (P3), femoral head cartilage is populated predominantly by immature reserve chondrocytes, which at 3 weeks (P21) are largely replaced by maturing prehypertrophic and hypertrophic cells.

Proteomic analysis of P3 and P21 cartilage extracts using label-free quantitative mass spectrometry identified 703 nonredundant proteins with high confidence. Using the beta-binomial distribution to model the spectral count data, we identified 146 significant differentially expressed proteins (q < 0.01). To extract functional information from the group of differentially expressed proteins, enriched gene ontological (GO) terms were identified using the Database for Annotation, Visualization and Integrated Discovery (DAVID). Many of the differentially expressed proteins were novel in the context of cartilage development, including ECM components, cell adhesion proteins, and a group of calcium-binding ER lumenal proteins, the reticulocalbins. The results of our proteomic analysis were validated by immunohistochemistry analysis of selected ECM components, revealing distinct regional expression patterns associated with zones of chondrocyte proliferation, maturation, and hypertrophy.

**EXPERIMENTAL PROCEDURES**

**Cartilage Dissection and Chondrocyte Preparation for Fluorescence-activated Cell Sorting**—Femoral head cartilage was obtained from 3- and 21-day post-natal C57/B16 mice by dislocation of the hip joint, fracture at the femoral neck, and removal of the ligamentum teres at the insertion site. The dissected cartilage was rinsed in PBS, frozen on dry ice, and stored at −80 °C in batches of eight P3 hips and six P21 hips, equivalent to ~10 mg of wet weight of tissue. Dissected femoral heads from P3 and P21 mice were also used to characterize the distribution of novel cartilage proteins by immunohistochemistry.

To analyze P3 and P21 chondrocytes by FACS, femoral heads were incubated for 2 h at 37 °C in DMEM containing 5% FCS and 2 mg/ml bacterial collagenase (Worthington Biochemicals). Chondrocytes were resuspended in PBS containing 5% FCS (6 × 10⁶ cells/ml in 50 ml) and incubated with primary conjugated antibodies specific for CD24a and CD200 (Becton Dickinson) for 15 min on ice. The cells were subsequently washed and co-stained with 7-aminoactinomycin D for dead cell detection, followed by flow cytometry (FACScantoll). The corresponding IgG isotypes (Becton Dickinson) were used as negative controls.

**Protein Extraction**—The P3 and P21 femoral head cartilage (three independent batches of tissue per developmental stage) was pulverized using a liquid nitrogen-cooled tissue grinder and transferred to Eppendorf tubes. Sequential protein extracts were prepared using a nondenaturing buffer (1 M NaCl in 100 mM Tris acetate, pH 8.0) followed by a chaotropic buffer (4 M GdnHCl, 65 mM DTT, 10 mM EDTA in 50 mM sodium acetate, pH 5.8) as described (13). The guanidine-extracted proteins were further partitioned by molecular mass cut-off filtration through a 100-kDa cut-off ultra centrifugal column (Amicon). Protein extracts were precipitated with 9 volumes of ethanol, and protein pellets washed twice in 70% (v/v) ethanol and resuspended in 150 μl of solubilization buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, and 30 mM Tris, pH 8.0. Protein concentrations were estimated using the Bradford assay (Pierce).

**SDS-PAGE and Immunoblotting**—Cartilage extracts were analyzed by SDS-PAGE to assess reproducibility and relative levels of cartilage proteins in each of the femoral head cartilage fractions. Aliquots of each extract equivalent to 2% of the total protein yield were heated in Laemmli buffer containing 50 mM dithiothreitol for 15 min at 65 °C and resolved through 4–12% acrylamide Bis-Tris NuPAGE gels (Invitrogen), and proteins were visualized by silver staining as described (14).

**Protein Reduction, Alkylation, and In-solution Trypsin Digestion**—Protein samples for LC-MS/MS analysis were sequentially reduced and alkylated under nitrogen by incubation in 10 mM dithiothreitol or Tris (Promega) overnight at ~20 °C in 1 ml of methanol. The trypsin-protein precipitates were washed once with chilled methanol, dried, and reconstituted in 100 mM ammonium bicarbonate, followed by trypsinization at 37 °C for 1 h.
37 °C for 5 h, with the addition of 1 μg of trypsin after 2 h. Digests were terminated by freezing on dry ice.

**Orbitrap Mass Spectrometry**—Each of the three fractions per sample was analyzed in duplicate by LC-MS/MS using an UltiMate 3000 HPLC system (Dionex) or a Tempo NanoLC system (Eksigent) in line with an LTQ-Orbitrap XL (ThermoScientific Scientific). Aliquots of triplicate peptides equivalent to 25% of the in-solution digests were loaded onto a 0.3 × 5-mm C18 trap column (Dionex) at 20 μl/min in 98% solvent A (0.1% (v/v) formic acid) and 2% solvent B (80% (v/v) acetonitrile, 0.1% (v/v) formic acid) for 5 min and subsequently backflushed onto a pre-equilibrated analytical column (Vydac Everest C18 300 Å, 150 μm × 150 mm; Alltech) using a flow rate of 1 μl/min. Peptides were separated at 40 °C using three linear gradient segments (2–12% solvent B over 6 min, 12–50% solvent B over 65 min, and then 50–100% solvent B over 15 min), holding at 100% solvent B for a further 15 min before returning to 2% solvent B over 5 min.

The LTQ-Orbitrap was fitted with a dynamic nanoelectrospray ion source (Proxeon) containing a 30-μm inner diameter uncoated silica emitter (New Objective). The LTQ-Orbitrap XL was controlled using Xcalibur 2.0 software (Thermo Electron) and operated in data-dependent acquisition mode whereby the survey scan was acquired in the Orbitrap with a resolving power set to 60,000 (at 400 z), MS/MS spectra were concurrently acquired in the LTQ mass analyzer on the seven most intense ions from the FT survey scan. Charge state filtering, where unassigned precursor ions were not selected for fragmentation, and dynamic exclusion (repeat count, 1; repeat duration, 30 s; exclusion list size, 500) were used. Fragmentation conditions in the LTQ were: 35% normalized collision energy, activation q of 0.25, 50-ms activation time, and minimum ion selection intensity of 500 counts.

**Database Searching**—The acquired MS/MS data, together with MS/MS data acquired in our previous LC-MS/MS cartilage proteomics study (12), were analyzed using Mascot version 2.2.06 (Matrix Science). Proteome Discoverer version 1.2 (Thermo Scientific) was used to extract tandem mass spectra from Xcalibur raw files and submit searches to an in-house Mascot server according to the following parameters: S-carboxamidomethylation of cysteine residues specified as a fixed modification and cyclization of N-terminal glutamine to pyroglutamic acid, deamidation of asparagine, hydroxylation of proline, oxidation of methionine specified as variable modifications. Parent ion tolerance of 20 ppm and fragment ion mass tolerances of 0.8 Da were used, and enzyme cleavage was set to trypsin, allowing for a maximum of two missed cleavages. The database searched consisted of 46,137 sequences, comprising the UniProt complete proteome set for Mus musculus (45,889 sequences downloaded on May 23, 2011) and sequences for common contaminants appended (downloaded from the Max Plank Institute http://maxquant.org). The automatic Mascot decoy database search was performed for all data sets.

**Criteria for Protein Identification**—The Mascot search results were loaded into Scaffold version 3.0.08 to assign probabilities to peptide and protein matches (15). Peptide-spectrum matches were accepted if the peptide was assigned a probability greater than 0.95 as specified by the Peptide Prophet algorithm (16). The probability threshold of 0.95 showed good discrimination between the predicted correct and incorrect peptide-spectrum assignments, and only peptides with charge states of +1, +2, and +3 were retained as confident identifications because the Peptide Prophet models were not a good fit to the data for charge states ≥4. Protein identifications were accepted if the protein contained at least two unique peptides (in terms of amino acid sequence), and the protein was assigned a probability >0.99 by the Protein Prophet algorithm (17). This threshold will constrain the protein false discovery rate (FDR) to <1%. The minimal list of proteins that satisfy the principal of parsimony is reported.

**RESULTS**

**Femoral Head Cartilage as a Model for Mouse Cartilage Development in Vivo**—Endochondral ossification, the process...
by which cartilaginous precursors are remodeled into bone, begins in different skeletal elements at different developmental stages. Therefore, our first aim was to establish a developmental model suitable for proteomic analysis. In the mouse femur, secondary ossification of the distal epiphyseal cartilage (corresponding to the lower boxed region I in Fig. 1) begins early in post-natal development (23). This limits the available cartilage to the growth plate, which can only be collected by tissue sectioning and microdissection (9). In contrast, the proximal femoral head at the hip (corresponding to the upper boxed region II in Fig. 1) remains devoid of trabecular bone until the onset of skeletal maturity (24) and can be dissected as an intact explant of articular cartilage and underlying primary growth plate (25).

Developmental changes in the femoral head were characterized by hematoxylin and eosin staining of sagittal sections cut through P3 and P21 cartilage explants (Fig. 2A). In P3 cartilage, with the exception of the proliferative chondrocytes in the growth plate, most of the cells were round and immature, with little morphological distinction between the articular cartilage surface and the presumptive ossification center. In contrast, the P21 ossification center, also described in the femoral head as the articular cartilage deep zone (26), was predominantly replaced by hypertrophic cartilage, and the articular cartilage showed evidence of differentiation into superficial and middle chondrocyte zones.

To quantify this cellular maturation process, cells released from P3 and P21 cartilage were analyzed by FACS (Fig. 2B). We used validated cell surface markers of defined chondrocyte differentiation states, previously identified by microarray profiling of mouse growth plate chondrocytes (27). CD24a is expressed on both immature and mature chondrocytes, and CD200 expression is restricted to the prehypertrophic and hypertrophic cells. The increased population of CD24a⁺/CD200⁺ chondrocytes in P21 cartilage (66 ± 1.2%) compared with P3 cartilage (37.3 ± 2.3%) provided further evidence of chondrocyte differentiation. On this basis, we used P3 and P21 femoral head cartilage to investigate protein expression changes during chondrocyte maturation and cartilage ECM development.
Sequential Extraction and Analysis of Femoral Head Cartilage Protein Fractions—A significant fraction of the cartilage proteome consists of extracellular matrix that must be dissociated using a strong denaturant such as GdnHCl. We used sequential extraction based on differential solubility in 1 M NaCl and 4 M GdnHCl followed by centrifugal ultrafiltration to further separate the guanidine-soluble components (13). This method, shown schematically in Fig. 3A, partitions the cartilage proteome into three distinct fractions, effectively reducing sample complexity and increasing proteome coverage (12). Protein yields from P3 and P21 cartilage extracts were consistent with previous studies (10, 13).

Analysis of the P3 and P21 cartilage protein fractions by SDS-PAGE revealed consistent band profiles between replicate extractions, but marked differences were observed in the ratios of readily soluble (E0) and poorly soluble (E1 and E2) proteins between the two maturation states (Fig. 3B). In P3 cartilage, the articular chondrocytes (AC) are homogeneous, whereas in P21 cartilage, superficial (arrows) and mid-zone (filled arrows) articular chondrocytes are more clearly distinguishable. At both stages, flattened columns of proliferative (PR) chondrocytes are evident in the growth plate. B, fluorescence-activated cell sorting of P3 and P21 chondrocytes to differentiate between chondrocyte differentiation states. Chondrocytes were prepared and sorted in three independent experiments, and representative plots are shown. All chondrocytes are labeled with antibodies to CD24a (y axis), whereas CD200 (x axis) is a marker for prehypertrophic and hypertrophic chondrocytes. The increased proportion of CD24a+/CD200- cells (Q2) relative to CD24a+/CD200+ cells (Q1) is consistent with an increased number of mature chondrocytes.

Fig. 2. Characterization of femoral head model for cartilage development at the tissue and cellular level. A, hematoxylin and eosin staining to show changes in cartilage histology between 3 days (P3) and 21 days (P21) of post-natal development. The presumptive ossification center (OC) of P3 cartilage, populated by immature chondrocytes, is largely replaced at P21 by hypertrophic cartilage. In P3 cartilage, the articular chondrocytes (AC) are homogeneous, whereas in P21 cartilage, superficial (arrows) and mid-zone (filled arrows) articular chondrocytes are more clearly distinguishable. At both stages, flattened columns of proliferative (PR) chondrocytes are evident in the growth plate.
cartilage, a higher proportion of the material was soluble in 1 m NaCl, whereas in P21 cartilage, more protein was extracted in 4 m GdnHCl. This increase in the relative abundance of poorly soluble proteins is consistent with the biosynthesis and integration of increasingly insoluble networks during maturation of the cartilage extracellular matrix.

![Sequential extraction of femoral head cartilage](image)

**Fig. 3. Sequential extraction of femoral head cartilage.** A, protein extraction from cartilage using a combination of physical (pulverization), chemical (4 m GdnHCl), and enzymatic (deglycosylation using chondroitinase ABC) methods to disrupt the extracellular matrix. The readily soluble 1 m NaCl extract (E0) is not fractionated further, whereas the 4 m GdnHCl extract is further separated by centrifugal ultrafiltration into nominal low (E1) and high (E2) molecular mass fractions. B, SDS-PAGE profiles of femoral head cartilage protein extracts to show reproducibility between biological replicates and the effective partitioning of cartilage proteins into three distinct fractions. Aliquots of replicate sequential extracts from 3-day (P3) and 21-day (P21) cartilage equivalent to 2% of the protein yield were resolved by Bis-Tris 4–12% NuPAGE, and the proteins were visualized by silver staining.
probability of identified with two or more unique peptides and a protein abundance. Using Scaffold resulted in the identification of 70,318 peptide-spectrum matches with a probability greater than 0.95 (charge states ≤ 3). The FDR at the peptide-spectrum level was 0.56%, based on the ratio of 392 decoy matches to 69,926 target matches. The complete list of peptides identified in this study is included in supplemental Table 1. After the peptides were assembled into proteins, and the principle of parsimony was applied there were 703 proteins (and one decoy protein) identified with two or more unique peptides and a protein probability of >0.99.

The number of peptide-spectrum matches assigned to each protein was used as a measure of relative protein abundance, and global normalization was used to correct for any systematic variations. The complete set of normalized spectral counts is presented in supplemental Table 2A. The beta-binomial test, which estimates the likelihood that a difference of the same or greater magnitude would be observed by chance (18), was used to compare normalized counts for P3 and P21 samples. To correct for multiple comparisons, q values were assigned to each protein in the data set, where the q value is the minimum FDR at which an observation can be called significant (28). To select an appropriate significance threshold, normalized counts for P3 and P21 were represented using MA plots (29). Significant proteins are highlighted at q value thresholds of 0.001, 0.01, and 0.05, and the null distribution for two biological replicates are presented (Fig. 4). We chose a threshold of q < 0.01, at which 146 proteins were identified as differentially abundant, and less than two of these are expected false positives.

Protein Functional Categories Associated with Cartilage Development—We used the web-based resource DAVID (20) to extract biological meaning from the set of 146 differentially abundant proteins. Using a functional annotation tool set to high stringency, 20 clusters were significant with an enrichment score of >2 (shown in supplemental Table 3A). Annotation clusters with the greatest number of entries were the cellular components “extracellular matrix” (cluster 1; n = 28) and “intracellular organelle lumen” (cluster 19; n = 22). Of the significant ECM components, 11 were more abundant in P3 cartilage, and 17 were more abundant in P21 cartilage, signifying extensive remodeling and changes in matrix structure during cartilage maturation. The modular nature of ECM constituents allows multiple interactions and modified functionality in different tissue matrices. Accordingly, three enriched terms were protein structural domains that occur frequently in ECM components: thrombospondin, type III repeat (cluster 11; n = 3), EGF-like region, conserved site (cluster 9; n = 10), and leucine-rich repeat (cluster 18; n = 7) that comprised the small leucine-rich repeat proteoglycans. The critical role of cartilage proteoglycans was also highlighted by the term polysaccharide binding (cluster 2; n = 12), which included the major cartilage proteoglycan aggrecan (Acan), one aggrecan-binding protein (cartilage oligomeric matrix protein, COMP), and the superficial zone proteoglycan lubricrin (Prg4). A further group of components involved in cell-matrix interactions, described by the term positive regulation of cell adhesion (cluster 15; n = 5), were all significantly increased in P21 cartilage. Thrombospondin-1 is a known chondrocyte-binding protein (30), whereas the other four cell adhesion proteins were either entirely novel cartilage constituents (tenascin X and emilin-1) or have never been described in post-natal cartilage (coiled-coil domain-containing protein 80/Urb and vitrin). The ECM and cell-matrix adhesion proteins described by these six enriched functional terms are presented in Table I.

Several annotation clusters were related to the rough ER and protein secretion, and the trends for these intracellular protein groups are represented in Fig. 5 (A and B). The enriched terms “oxoglutarate and iron-dependent oxygenase” and “procollagen-proline dioxygenase activity” (clusters 6 and 10, respectively) included prolyl 4-hydroxylase, lysyl hydroxylase, and prolyl 3-hydroxylase enzymes (n = 5), which are involved in the post-translational modification of collagen α chains (31). The prolyl 4-hydroxylase β subunit (P4hb) is an ER protein disulfide isomerase and among the proteins in the enriched annotation term “protein disulfide isomerase activity” (cluster 8; n = 4). One further cellular protein subset related to protein biosynthesis was classified as “ribonucleoprotein” (cluster 4; n = 12). The final group of ER proteins were the calcium-binding lumenal proteins classified by the PIR superfAMILY “reticulocalbin” (cluster 14; n = 3). With the notable exception of the reticulocalbins, the proteins associated with these enriched annotation clusters were more abundant in P3 cartilage.

The sequential extraction method used in this study partitions cartilage proteins according to solubility, for example separating macromolecular complexes from readily soluble cytosolic enzymes (12). The ratios of E0, E1, and E2 fractions between P3 and P21 developmental stages changed significantly (Fig. 3). Therefore, we examined three differentially soluble protein subsets to verify that the described differences in protein abundance did not result from a global shift in protein extractability. Accordingly, the 14-3-3 proteins (1 M NaCl-soluble), tubulin chains, and T-complex chaperonin subunits (4 M GdnHCl-soluble) showed no evidence for differential abundance in P3 and P21 cartilage (Fig. 5C).

Meta-analysis of Two Cartilage Developmental Models—We recently identified a cohort of ECM components and markers for chondrocyte differentiation by comparison of mouse juvenile epiphyseal cartilage (P3 knee cartilage) and 3-week high density chondrocyte cultures (culture day 21 neocartilage) (12). However, cartilage development in vivo involves environmental signals and factors such as mechanical loading that are absent in vitro. Meta-analysis of the in vivo and in vitro
proteomics data sets was therefore used to: (i) identify potential components with specific roles in vivo and (ii) identify components that are involved in both processes and are therefore suitable candidates for further investigation in vitro.

To generate an equivalent data set for cartilage maturation in vitro, MS/MS data from our previous study (12) were searched against the *M. musculus* complete proteome set, resulting in 44,960 confident peptide-spectrum matches (FDR = 0.43%) and 839 protein identifications (supplemental Table 2B). Excluding eight contaminants, 102 proteins were significantly different between P3 knee cartilage and in vitro chondrocyte culture day 21 neocartilage extracts (q < 0.01), listed in supplemental Table 2B. Almost half of these proteins (n = 45) were also significant in femoral head cartilage maturation (highlighted in Fig. 6A and details provided in supplemental Table 2C). As an initial evaluation of the in vitro and in vivo proteomics data sets, protein subsets classified by the functional terms “extracellular matrix,” “endoplasmic reticulum,” and “ribonucleoprotein” were represented as MA plots (Fig. 6B). Consistent with the in vivo data set, most of the significant ER components and ribonucleoproteins in vitro were enriched in P3 cartilage. In addition, significant ECM components were evenly distributed above and below y = 0 both in vitro and in vivo.

To further investigate the similarities and differences between neocartilage formation and femoral head cartilage...
Development, the list of significant proteins in vitro were submitted to DAVID for functional annotation (supplementary Table 3B). Several GO annotations enriched in the in vitro data set were similar to those enriched in the in vivo data set. In particular, the cellular component with the most entries was “extracellular matrix” (n = 22). Additional ECM-related terms associated with cartilage development both in vivo and in vitro included “polysaccharide binding” (cluster 4; n = 8), “thrombospondin, C-terminal” (cluster 7; n = 3), and “EGF-like, type 3” (cluster 9; n = 7). However, the ECM-related terms “lamin G, thrombospondin-type, N-terminal” (cluster 5; n = 4), “fibrillar collagen” (cluster 6; n = 3), and “von Willebrand factor, type C” (cluster 8; n = 4) were specifically enriched in vitro.

These results indicate fundamental roles for ECM components in cartilage development in both environments, but the development, the list of significant proteins in vitro were submitted to DAVID for functional annotation (supplementary Table 3B). Several GO annotations enriched in the in vitro data set were similar to those enriched in the in vivo data set. In particular, the cellular component with the most entries was “extracellular matrix” (n = 22). Additional ECM-related terms associated with cartilage development both in vivo and in vitro included “polysaccharide binding” (cluster 4; n = 8), “thrombospondin, C-terminal” (cluster 7; n = 3), and “EGF-like, type 3” (cluster 9; n = 7). However, the ECM-related terms “lamin G, thrombospondin-type, N-terminal” (cluster 5; n = 4), “fibrillar collagen” (cluster 6; n = 3), and “von Willebrand factor, type C” (cluster 8; n = 4) were specifically enriched in vitro.

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**Table I**

**Proteomics Analysis of Cartilage Development in Vivo**

Of the 146 proteins that were differentially expressed in P3 and P21 cartilage, a subset of 34 significant (q < 0.01) ECM and related proteins were annotated by the functional terms extracellular matrix (ECM), thrombospondin, type III repeat (TSP), EGF-like region, conserved site (EFG), leucine-rich repeat (LRR), polysaccharide binding (PSB), and positive regulation of cell adhesion (Adhesion). Classification of each entry is indicated by ×. Five additional extracellular proteins, thrombospondin-1 (Thbs1), thrombospondin-4 (Thbs4), lactadherin (Mfge8), lubricin (Prgr4), and matrilin-4 (Matn4), were included as extracellular matrix-related components on the basis of their classification under one or more of the enriched functional terms.

| Protein name                  | Gene name | Gene number | Enriched functional terms | q value | Fold (P21/P3) | Accession number |
|-------------------------------|-----------|-------------|---------------------------|---------|---------------|------------------|
| Vitrin                        | Vit       |            | ECM PSB LRR TSP EGF Adhesion | 0.00044 | 18.0          | Q8VH15           |
| Osteomodulin                  | Omd       |            |                           | 0.00059 | 17.3          | Q35103           |
| Uncharacterized protein (cartilage intermediate matrix protein, isofrom 2) | Clip2<sup>a</sup> |            |                           | 0.00102 | 10.7          | D327H8           |
| Thrombospondin 4              | Thbs4     |            |                           | 0.00384 | 10.7          | P61750           |
| Coiled-coil domain-containing protein 80 (Urb) | Ccdc80 |            |                           | 0.00030 | 10.0          | Q8R2G6           |
| Uncharacterized protein (lubricin) | Prgr4    |            |                           | 0.00273 | 9.3           | E0CZ58           |
| SPARC                         | Sparc     |            |                           | 0.00053 | 8.6           | P07214           |
| Tenascin X                    | Tnxb      |            |                           | 0.00500 | 7.0           | Q35452           |
| Collagen α-1(X) chain         | Col10a1   |            |                           | 0.00032 | 6.5           | Q05306           |
| Collagen α-2-HS-glycoprotein  | Ahsq      |            |                           | 0.00195 | 4.9           | P29699           |
| Fibromodulin                  | Fmod      |            |                           | 0.00048 | 4.1           | P50608           |
| Decorin                       | Dcn       |            |                           | 0.00044 | 3.9           | P28654           |
| Galectin-3                    | Lgals3    |            |                           | 0.00554 | 3.8           | P16110           |
| Uncharacterized protein (lactadherin) | Mfge8 |            |                           | 0.00123 | 3.8           | E9OKW0           |
| Collagen α-1(III) chain       | Col3a1    |            |                           | 0.00711 | 3.5           | P08121           |
| Cartilage oligomeric matrix protein | Comp     |            |                           | 0.00210 | 3.4           | Q9R0G6           |
| Chondroadherin                | Chad      |            |                           | 6.29E-05 | 2.8         | Q53226           |
| Fibronecin                    | Fn1       |            |                           | 0.00053 | 2.8           | P12726           |
| Matrilin-3                    | Martn3    |            |                           | 0.00032 | 2.4           | Q54302           |
| EMLIN-1                       | Emilin1   |            |                           | 0.00375 | 2.4           | Q99K41           |
| Thrombospondin 1              | Thbs1     |            |                           | 0.00264 | 1.8           | Q80YQ1           |
| Prolin arginine-rich end leucine-rich repeat | Prelp |            |                           | 0.00234 | 1.5           | Q54350           |
| Aggrecan                      | Acan      |            |                           | 0.00555 | -1.3          | E9QLS9           |
| Matrilin-4                    | Matn4     |            |                           | 0.00048 | -1.9          | Q99029           |
| Collagen α-3(IX) chain        | Col9a3<sup>a</sup> |            |                           | 0.00095 | -2.7          | A2ACT7           |
| Epiphycan                     | Epyc      |            |                           | 0.00131 | -2.7          | P70186           |
| Transforming growth factor, beta induced | Tgfb1 |            |                           | 0.00209 | -2.7          | A1L353           |
| Leprecan 1                    | Lrepl     |            |                           | 0.00321 | -2.8          | A6PW84           |
| Collagen α-2(IX) chain        | Col9a2    |            |                           | 0.00045 | -3.6          | Q07643           |
| Collagen α-2(V) chain         | Col9a2    |            |                           | 0.00901 | -3.8          | Q3U962           |
| Tenascin                      | Tnc       |            |                           | 0.00018 | -4            | Q80YX1           |
| Cartilage-associated protein  | Crtap     |            |                           | 0.00500 | -5            | QBC8C5           |
| Asporin                       | Aspn      |            |                           | 0.00304 | -5.7          | A6H6K1           |
| Collagen α-1(IX) chain        | Col9a1    |            |                           | 0.00018 | -7.1          | Q05722           |

<sup>a</sup> The 29 differentially abundant proteins classified as ECM components (GO:0031012) included Clip2 and Col9a3, which are annotated as ECM based on the human homologues.
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two processes are likely to involve distinct protein subsets. The 34 significant ECM and related proteins identified in vivo (Table I) and the 27 significant in vitro components belonging to the ECM and related GO terms described above were compared using Venn diagrams. Fig. 7A shows the protein significantly increased in abundance in vitro and/or in vivo, and Fig. 7B shows the proteins significantly decreased in abundance in vitro and/or in vivo.

Twenty-two ECM components were increased during cartilage development in vivo (group II/III). Of these, eight were also increased during cartilage maturation in vitro (group II). The remaining 14 proteins enriched in P21 femoral head cartilage (group III) could be subclassified into proteins that were also detected but not significantly increased during cartilage maturation in vitro (n = 6) and proteins that were detected exclusively in vivo (n = 8). For example, of the small leucine-rich repeat proteoglycans in group III, chondroadherin (Chad), fibromodulin (Fmod), proline arginine-rich end leucine-rich repeat protein (Prep), and decorin (Dcn) were also detected in neoarticular cultures, whereas osteomodulin was detected only in P21 femoral head cartilage.

Twelve ECM and related proteins were significantly decreased in P21 cartilage relative to P3 cartilage (group V/VI), five of which were also down-regulated during cartilage ECM maturation in vitro (group V). This comparison highlights four key protein subsets: components that are increased during ECM development both in vitro and in vivo (group II), components that are associated in general with a less "mature" cartilage ECM (group V), and proteins involved in specific aspects of ECM development, either in vitro (group I) or in vivo (group III).

Immunohistochemical Analysis of Novel Proteins Associated with Maturation of the Extracellular Matrix—Chondrocyte hypertrophy and development of articular cartilage are the major histologic features of femoral head cartilage development (Fig. 2). The prototypic markers for these processes (collagen X and lubricin, respectively) were among the significant proteins identified by our proteomic analysis. In addition, ECM components with no known role in these processes were identified. Therefore, we used a complementary approach to validate our proteomics data and further investigate the region-specific expression patterns in femoral head cartilage. Three proteins that were only identified in P21 cartilage

![Fig. 5. Functional groups associated with femoral head cartilage development. Each data point in the plot represents the relative abundance of a protein between P3 and P21 cartilage, where proteins above the horizontal axis are more abundant at P21. Both axes are log2 transformed, where the horizontal axis represents the product of the spectral counts and the vertical axis represents the ratio of the spectral counts. In A and B, colored data points represent differentially abundant proteins (q < 0.01), and in C, none of the proteins have significant differences in abundance. A, three distinct protein groups associated with the endoplasmic reticulum: oxidoreductase activity proteins (Lepre1, Plod2, Plod1, P4ha1, and P4ha2) are highlighted as green filled circles, protein disulfide isomerase activity proteins (P4hb, Pdia3, Pdia4, and Pdia6) are highlighted as blue filled circles, and PIRSFO36326:reticulocalbin proteins (Calu, Rcn1, and Rcn2) are highlighted as red filled circles. The fourth member of the reticulocalbin family identified (Rcn3) is highlighted as an open red circle. B, ribonucleoproteins (Hnrnpab, Hnrnpu, Rpl9, Rpl27a, Rps4x, Rps6, Rps8, Rpl4, Rpl6, Rpl32, Rps15, and Rpl122). C, housekeeping protein families that are not differentially abundant between P3 and P21 cartilage: the tubulin chains Tuba1b, Tuba1a, Tubb2a, Tubb2c, Tubb5, and Tubb6 (red filled circles); the 14-3-3 proteins Ywhaq, Ywhab, Ywhae, Ywhah, Ywhag, and Ywhaz (blue filled circles); and the 1D-complex chaperonin subunits Cct2, Cct3, Cct4, Cct5, Cct6a, Cct7, Cct8, and Tcp1 (green filled circles).]
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A  In vitro  In vivo

B  In vitro  In vivo

ECM

ER

RIBO
samples and therefore likely to be involved specifically in cartilage development in vivo (vitrin, tenascin X and CILP-2) and one protein that increased in abundance both in vitro and in vivo (Urb) were analyzed by immunohistochemistry. Anti-bodies for tenascin X did not give a reliable positive signal under the conditions tested; therefore only Urb, vitrin, and CILP-2 results are shown.

Analysis of Urb, vitrin, and CILP-2 in P3 cartilage revealed intracellular immunostaining of all three proteins, but little evidence of region-specific expression (Fig. 8A). In contrast, Urb, vitrin, and CILP-2 immunostaining in P21 cartilage was localized specifically with the ECM and chondrocyte populations of different maturation stages. Strong immunostaining for Urb was observed in the interterritorial matrix of the presumptive ossification center and the underlying clusters of maturing/prehypertrophic chondrocytes (Fig. 8B), but no signal was detected in proliferative growth plate cartilage matrix. Vitrin immunostaining overlapped with Urb expression in the

Fig. 7. Comparison of extracellular matrix and related proteins involved in cartilage maturation in vitro and in vivo. Proteins associated with cartilage maturation in vitro were identified by comparing P3 knee epiphyseal cartilage with neocartilage cultures. Of the differentially abundant proteins (n = 102), the proteins annotated by the significant functional terms extracellular matrix; polysaccharide binding; thrombospondin, C-terminal; EGF-like, type 3; lamin G, thrombospondin-type, N-terminal; fibrillar collagen; and von Willebrand factor, type C comprise the in vitro subsets. The proteins comprising the in vivo subsets are the ECM and matrix-related components associated with P3 and P21 femoral head cartilage development in vivo that are described in full in Table I. A, Venn diagram of the proteins increased during cartilage development specifically in vitro (I), specifically in vivo (III), or both in vitro and in vivo (II). The eight proteins in group III highlighted in bold were detected exclusively in P21 cartilage, whereas the remaining six proteins were also detected, but not significantly enriched, in neocartilage. B, Venn diagram of the proteins decreased during cartilage development specifically in vitro (IV), specifically in vivo (VI), or both in vitro and in vivo (V). Ucma (unique cartilage matrix associated protein) was included in group V because it was enriched in P3 cartilage in both comparisons; however, it was only statistically significant (q < 0.01) in vitro.

Fig. 6. Global evaluation of in vitro and in vivo models of cartilage development. MA plots of the in vitro (left panels) and in vivo (right panels) spectral count data. The in vitro spectral count data was based on the proteomic comparison of P3 mouse knee epiphyseal cartilage and neocartilage cultures (12), and the in vivo spectral count data is based on the P3 and P21 femoral head cartilage. The horizontal axis represents the product of the spectral counts, and the vertical axis represents the ratio of the spectral counts. Axes are log2 transformed. Proteins that are not differentially abundant are plotted as gray filled circles. A, proteins with significant differences in abundance (q < 0.01) either in vitro or in vivo are represented by open circles, and proteins that are differentially abundant in both developmental systems are represented as black filled circles. B, protein subsets in vitro and in vivo classified by the functional terms extracellular matrix (ECM), endoplasmic reticulum (ER), and ribonucleoprotein (RIBO) are highlighted as filled circles. Differentially abundant proteins are represented as black filled circles.
maturing/prehypertrophic region but was relatively much weaker in the hypertrophic cartilage of the presumptive ossification center. Strong vitrin expression was also observed in the proliferative zone from which Urb was absent. The immunopositive signal for CILP-2 was more restricted than vitrin and Urb, with a tight band of staining at the margin of the presumptive ossification center and the underlying growth plate. This pattern is consistent with the localized expression of CILP-2 in the calcified deeper zones of knee articular cartilage (22).

According to our proteomics analysis, Urb was detected both in vitro and in vivo, whereas CILP-2 and vitrin were only identified in P21 femoral head cartilage (Fig. 7). Consistent with these results, only Urb was detected in the ECM of chondrocyte neocartilage cultures (Fig. 8A). High magnification images of the chondrocyte cultures showed intracellular Urb staining and pericellular staining that was particularly strong in enlarged chondrocytes, most likely undergoing hypertrophy (Fig. 8B). These results support a role for Urb in chondrocyte hypertrophy, whereas the lack of CILP-2 and vitrin in neocartilage suggest roles in processes that are not recapitulated in culture. Collectively, the results of CILP-2, Urb, and vitrin analysis by immunohistochemistry agree...
closely with the proteomics data and provide evidence for very distinct roles in cartilage development.

**DISCUSSION**

The formation of skeletal elements by endochondral ossification involves tightly regulated chondrocyte differentiation. In the mouse, this developmental process has been investigated "spatially" using microdissection and microarray analysis of chondrocyte populations from histomorphologically defined zones of growth plate cartilage (5–7). This is a challenging target for proteomics particularly because of the very limited amount of protein available from individual cartilage zones. To overcome this, we used a "temporal" approach based on developmental stages of femoral head cartilage with a high proportion of immature chondrocytes (day 3) or prehypertrophic and hypertrophic chondrocytes (day 21). Label-free quantitative mass spectrometry identified 703 proteins, of which 67 were significantly more abundant in P21 cartilage and 79 were more abundant in P3 cartilage (q < 0.01). The prototypic markers for chondrocyte differentiation (collagen X) and development of the articular surface (lubricin) were significantly enriched in P21 cartilage, demonstrating the utility of our model for discovery of new proteins and processes involved in cartilage development. The significance of our results is discussed below.

**Overview of Significant Biological Processes and Protein Functional Categories Enriched in P3 and P21 Cartilage Extracts**—The highest ranking protein functional category in femoral head cartilage development was extracellular matrix (GO:0031012), consistent with the increased abundance Gdn-HCl-extracted proteins relative to NaCl-extracted proteins in (GO:0031012), consistent with the increased abundance Gdn-HCl-extracted proteins relative to NaCl-extracted proteins in femoral head cartilage development was extracellular matrix (GO:0031012), consistent with the increased abundance Gdn-HCl-extracted proteins relative to NaCl-extracted proteins in (GO:0031012), consistent with the increased abundance Gdn-HCl-extracted proteins relative to NaCl-extracted proteins in (GO:0031012), consistent with the increased abundance Gdn-HCl-extracted proteins relative to NaCl-extracted proteins in (GO:0031012), consistent with the increased abundance Gdn-HCl-extracted proteins relative to NaCl-extracted proteins in (GO:0031012), consistent with the increased abundance Gdn-HCl-extracted proteins relative to NaCl-extracted proteins in. Label-free quantitative mass spectrometry identified 703 proteins, of which 67 were significantly more abundant in P21 cartilage and 79 were more abundant in P3 cartilage (q < 0.01). The prototypic markers for chondrocyte differentiation (collagen X) and development of the articular surface (lubricin) were significantly enriched in P21 cartilage, demonstrating the utility of our model for discovery of new proteins and processes involved in cartilage development. The significance of our results is discussed below.

**Novel Cell Adhesion Proteins and Evidence for Sushi Domain-mediated Interactions in Cartilage**—Four of the significant proteins classified by the GO term "regulation of cell adhesion" are novel in the context of post-natal cartilage development (emilin-1, tenascin X, vitrin, and Urb). Urb and vitrin were previously identified by *in silico* screening for novel ECM components and verified as promoting cell attachment and matrix assembly *in vitro* (21). The diffuse immunostaining for Urb throughout P3 cartilage is consistent with observations in mouse embryonic ribs and vertebrae (21, 32). The restricted Urb expression within the prehypertrophic cartilage and presumptive ossification center at P21 suggests a role in the ECM prior to bone formation. In addition, the "extraction profile" (the distribution between readily soluble and guanidine-soluble P3 and P21 extracts) of Urb was consistent with the integration of Urb within the ECM during cartilage development (data not shown).

Urb is a member of the sushi repeat-containing protein superfamily. The sushi domain, also known as a complement control protein module, is a structural motif of ~60 amino acids that mediates protein-protein interactions. We identified three further sushi repeat proteins that have not previously been reported in cartilage at the protein level. Fibulin-7 (Fbln7), the only fibulin with a sushi repeat domain, has been detected in growth plate and articular cartilage at the mRNA level (33), whereas the X-linked sushi repeat containing protein (Srpx) and homolog (Srpx2) are entirely novel cartilage components. Preliminary studies in our lab showed similar localization of Srpx2 expression in P21 cartilage to that of Urb (data not shown). The identification of ligands for these sushi domain-containing secreted proteins will help elucidate their roles in cell attachment and/or matrix protein assembly in cartilage.

The dramatic increase in vitrin abundance during femoral head cartilage development was unexpected. Vitrin is a modular protein containing two von Willebrand factor A domains at the C terminus that forms a separate subfamily of noncollagenous A domain-containing proteins related to the matrilins (34). The role of matrilins in bridging the collagen and proteoglycan macromolecular networks in cartilage is well known, whereas little has been reported on vitrin function since its discovery in bovine vitreous (35). Localization of vitrin by immunohistochemistry revealed particularly strong staining in the proliferative region of the P21 growth plate cartilage, from which Urb staining was absent. Also unlike Urb, vitrin was more abundant in the matrix surrounding the prehypertrophic chondrocytes than in the adjacent hypertrophic cartilage. Consistent with this region-specific pattern, the transition from prehypertrophic to hypertrophic chondrocytes in the growth plate is marked by a 10-fold decrease in vitrin mRNA. This tightly regulated expression signifies a potential role in maintaining ECM structure in the proliferative and prehypertrophic cartilage matrix, making vitrin a novel candidate for human chondrodysplasias and an important target for further analysis *in vivo.*

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2 D. Belluoccio, and J. F. Bateman, unpublished observations.
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Similarities between Femoral Head Cartilage Development and Neocartilage Formation in Vitro—Our previous investigation of cartilage matrix formation in chondrocyte “neocartilage” cultures identified a cohort of ECM components based on differential protein solubility compared with P3 mouse knee epiphyseal cartilage (12). Meta-analysis of the two model systems revealed matrix components and cell adhesion proteins that were significant both in vitro and in vivo. These included matrilin-3 (Matn3), COMP, thrombospondin-1 (Thbs1), and fibronectin (Fn1), supporting fundamental roles for these components in ECM network formation and tissue stability in cartilage.

Chondrocyte hypertrophy and associated ECM remodeling creates the niche for vascular invasion and replacement of the cartilage remnants with bone. In addition to this developmental role, hypertrophy of articular chondrocytes in osteoarthritis leads to neovascularization and osteophyte formation (37). Our proteomics analysis provided evidence for chondrocyte hypertrophy in both femoral head cartilage development and in neocartilage. In addition to collagen X, the cardinal marker of late stage chondrocyte differentiation, lesser known components of hypertrophic cartilage such as annexin VIII (Anxa8), galectin-3 (Lgals3), and lysyl oxidase C (Loxl4) (38) were all significantly increased. The reduction in collagen IX α1, α2, and α3 chains extracted from P21 cartilage suggests an inverse relationship between collagen IX and collagen X synthesis. Epiphycan (Epyc) and unique cartilage matrix-associated protein (Ucmα), two known markers for immature chondrocytes (39, 40), were also enriched in P3 cartilage relative to P21 cartilage. The relative abundance of these differentiation markers in P3 and P21 cartilage, consistent with their expression changes in vitro, reveals key similarities in matrix development and remodeling in vitro and in vivo.

Specific and Novel Proteins Involved in Femoral Head Cartilage Development—We anticipated that cartilage development in vivo would involve specific proteins related to the unique physiological conditions in the developing joint that cannot be recapitulated in chondrocyte cultures. In support of this hypothesis, eight of the P21-enriched ECM and matrix-related proteins in Table I were specific to femoral head cartilage development and remodeling in vivo. Examples include the transforming growth factor β1 (TGFβ1)-regulated proteins, tenasin C, asporin, and TGFβ-induced Ig-h3 (46–48). These results support a role for TGFβ1 signaling during early cartilage development in vivo, consistent with the positive effect of TGFβ1 on the differentiation of mesenchymal chondrocyte precursors in vitro (49, 50). Altered TGFβ1 signaling is implicated in cartilage degeneration associated with aging and arthritis (51), and pathologic changes in cartilage homeostasis and chondrocyte phenotype include re-expression of early markers of cartilage development. Of the TGFβ-regulated proteins enriched in P3 cartilage, asporin and tenasin C are absent from normal adult cartilage but expressed in osteoarthritis, whereas TGFβ-induced Ig-h3 warrants investigation as a potential novel biomarker.

The overlap in protein expression between terminally differentiated chondrocytes and osteoblasts at the chondro-osseous junction has generated debate over chondrocyte fate and the respective roles of chondrocytes and osteoblasts in mineralization of the hypertrophic cartilage (reviewed in Ref. 52). Osteomodulin was detected exclusively in P21 femoral head cartilage and so is potentially a product of osteoblasts recruited into the hypertrophic cartilage. In contrast, three proteins involved in normal and pathologic mineralization of the cartilage matrix that were enriched in P21 cartilage (alkaline phosphatase/Alpal, osteopontin/Spp1 and osteonectin/Sparc) are also synthesized by chondrocytes in culture (53–55). Further analysis of these constituents in vitro and in vivo will determine their cellular origin and elucidate their roles in late stage chondrocyte differentiation and hypertrophic cartilage mineralization.

Regulation of Specific Endoplasmic Reticulum-resident Proteins and Elements of the Secretory Protein Trafficking and Quality Control System—Development of the cartilage ECM during endochondral ossification is essential for tissue integrity and normal chondrocyte differentiation, and we have identified new components involved in matrix development in vivo. In addition to changes in the cartilage matrix proteome, which are the net result of protein accumulation and turnover, we identified changes in ER proteins involved in matrix protein biosynthesis. In particular, cartilage maturation was accompanied by changes in the repertoire of enzymes and molecular chaperones involved in the post-translational modification, folding, and assembly cartilage collagens.
One significant group of proteins enriched in P3 cartilage belong to the iron-dependent dioxygenases, specifically five members of the prolyl 3-hydroxylase, prolyl 4-hydroxylase, and lysyl hydroxylase families, which catalyze post-translation modification of collagen α chains. Prolyl 4-hydroxylation of the Xaa-Pro-Gly collagen tripeptide repeat is essential for triple helical stability, whereas lysyl hydroxylation of Xaa-Lys-Gly generates carbohydrate attachment sites that stabilize intermolecular collagen interactions. Both prolyl 4-hydroxylases α subunit isoforms (P4ha1 and P4ha2), two lysyl hydroxylases (Plod1 and Plod2), and the procollagen galactosyl transferase-1 (Glt25d1) were significantly more abundant in P3 cartilage. A further modification essential for collagen triple helix formation is the cis-trans isomerization of peptide prolyl bonds. In our study, two ER-associated peptidyl prolyl cis-trans isomerase, Fkbp10 and Ppib, were significantly more abundant in P3 chondrocytes, whereas levels of the cytosolic enzymes Ppia and Ppibc were unchanged.

Prolyl 3-hydroxylation, a highly site-specific modification of collagen α chains, appears to regulate the transit of correctly folded procollagen from the ER, in addition to proposed structural roles (56). Prolyl 3-hydroxylase 1 (Lepre1) combines with Ppib and cartilage-associated protein (Crtap) to form a chaperone complex that is critical for the quality control of collagen secretion. Mutations in any one of these subunits cause severe bone, tendon, and skin phenotypes in mice and osteogenesis imperfecta in humans (57, 58). Strikingly, all three components were more abundant in P3 cartilage, signifying an important developmental role for this complex in the assembly and secretion of cartilage collagens. Another ER chaperone involved in collagen-specific quality control, Hsp47 (Serpinh1), has also recently been associated with osteogenesis imperfecta (59). Hsp47 was detected at high levels in P3 and P21 chondrocytes, albeit 1.3-fold reduced at P21, suggesting that Hsp47 is required throughout chondrocyte maturation. Although development of the cartilage ECM is marked by increased levels of certain collagen subtypes (e.g. collagen X) and a decrease in others (e.g. collagen IX), our results indicate that collagen biosynthesis is highest overall during early stages of cartilage maturation.

The changing secretory function of the chondrocyte was also marked by reduced levels of protein disulfide isomerase (P4hb), Pdia3, Pdia4, and Pdia6, indicating reduced capacity for folding and assembly of disulfide-linked proteins in the ER of late stage chondrocytes. The chaperone activity of Pdia4 (ERp72) is of particular interest because ERp72 forms stable complexes with disease-causing COMP and matrilin-3 mutants. Accumulation of these complexes in dilated ER cisternae is a hallmark of multiple epiphyseal dysplasia and pseudoachondrodysplasia (60–62), and the resulting ER stress affects normal chondrocyte differentiation and organization in the growth plate (63–65). ER stress induced by misfolding of collagen X in hypertrophic chondrocytes also contributes to the pathology of Schmid metaphyseal chondrodysplasia, type Schmid (66, 67), but the role of specific ER chaperones is not known. Although ERp72 was significantly reduced at P21, the master regulator of the unfolded protein response BiP (Hspa5) was detected at approximately equal levels in P3 and P21 cartilage. The changing repertoire of ER chaperones during the chondrocyte lifespan, and thus potential mechanisms of ER stress, may alter the impact of different disease-causing mutations on chondrocyte function.

Further biochemical evidence of changing ER function during chondrocyte differentiation was reflected in the significant increased in P21 cartilage of the reticulocalbins Rcn1, Rcn2, and Calu (reviewed in Ref. 68). Reticulocalbins are thought to regulate calcium-dependent functions in the ER and secretory pathway on the basis of ER retention motifs and multiple EF hand domains. However, their roles in chondrocytes are presently unknown. Collectively, our results reveal the down-regulation of secretory pathway elements related to collagen biosynthesis but a significant increase of the reticulocalbins. It has been reported that the cytosolic concentration of calcium increases with respect to chondrocyte maturation (69). Whether the reticulocalbins modify the bioavailability of ER luminal calcium during cartilage development is a target for future studies.

Benefits of Our Approach and Future Perspectives—Endochondral ossification is a complex and fundamentally important developmental process that is difficult to study in vitro because of the interdependence of chondrocyte phenotype and tissue architecture. Using femoral head cartilage to investigate this process within its physiological context is both a strength and, because of increased complexity, a limitation. However, the integrated analysis of the in vitro and in vivo proteomic data sets enabled us to discover novel components, characterize the two developmental systems in greater detail, and pinpoint targets for further analysis. The expression and observed distribution of Urb supports a role in chondrocyte hypertrophy in vitro and in vivo, whereas the immunohistochemistry and proteomics data signified more specialized roles for vitrin and CILP-2 in cartilage development in vivo. Using this approach in cartilage from knockout mice and models of human skeletal dysplasias will enable proteomics level investigation of the roles of specific cartilage components and effects of disease-causing mutations on skeletal development in vivo.

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