MECHANICAL REGULATION OF PROGENITOR CELL DIFFERENTIATION
DURING ENDOCHONDRAL OSSIFICATION

A Dissertation

Submitted to the Graduate School
of the University of Notre Dame
in Partial Fulfillment of the Requirements
for the Degree of

Doctor of Philosophy

by

Anna M. McDermott

Joel D. Boerckel, Director

Graduate Program in Bioengineering
Notre Dame, Indiana
July 2019
MECHANICAL REGULATION OF PROGENITOR CELL DIFFERENTIATION
DURING ENDOCHONDRAL OSSIFICATION

Abstract
by
Anna M. McDermott

Endochondral ossification is an indirect path of bone formation involving the condensation of progenitor cells into a cartilaginous anlage that grows in size, matures, and releases growth factors to promote blood vessel invasion and cartilage remodeling, resulting in a robust, vascularized bone organ. This is the dominant pathway of bone formation in both long bone development and fracture healing and has emerged as an attractive tissue engineering template to address the lack of angiogenesis and poor engraftment seen in current intramembranous engineering approaches. Though it is known that mechanical cues are essential to the progression of endochondral ossification in both processes, the role of mechanical loading in endochondral bone defect regeneration has not been investigated. In vivo, we applied mechanical loading to chondrogenically-primed hMSCs within a critically-sized rat femoral defect either immediately after implantation, or delayed until 4 weeks into chondrogenesis, compared to unloaded controls. Delaying loading significantly improved bone regeneration through increased bone volume, bridging across the defect, and recovery of mechanical properties.
compared to unloaded controls, and this was further potentiated by supplementation of additional growth factors. Conversely, early loading increased bone volume but did not enhance bridging or mechanical properties. There was significantly decreased vascular invasion and persistent unmineralized gaps of cartilage, resulting in lack of functional repair. This suggests that the timing of in vivo mechanical loading during progenitor cell maturation is an important factor in directing tissue formation. To this end, we investigated, in vitro, the role of endochondral priming length prior to dynamic compression in a custom-made bioreactor. Mechanical load initiated chondrogenic gene expression at all stages, but only enhanced endochondral progression when applied after 6 weeks of priming. Loading at earlier stages of priming had an inhibitory effect on chondrocyte maturation, consistent with in vivo findings. Together these results suggest that mechanical loading is necessary to induce successful endochondral progression for bone tissue engineering.
CONTENTS

Figures........................................................................................................................................ v

Tables.......................................................................................................................................... xx

Abbreviations................................................................................................................................ xxi

Acknowledgments....................................................................................................................... xxiv

Chapter 1: Introduction.................................................................................................................. 1
  1.1 Significance............................................................................................................................. 1
  1.2 Research objectives............................................................................................................... 2
    1.2.1 Utilize mechanical load for bone healing ................................................................. 2
    1.2.2 Recapitulate development......................................................................................... 2
    1.2.3 Investigate temporal mechanical and chemical cues ........................................... 3

Chapter 2: Literature Review: Mechanical forces in endochondral ossification and tissue
  engineering approaches............................................................................................................. 4
  2.1 Step 1: Mesenchymal condensation.................................................................................... 5
    2.1.1 Initiation of condensation during development ................................................... 5
    2.1.2 Mechanical forces............................................................................................... 5
    2.1.3 Mimicking condensation in tissue engineering............................................... 6
  2.2 Step 2: Chondrogenic differentiation............................................................................... 8
    2.2.1 Early chondrogenesis and immature chondrocytes (TGF-β signaling).................. 8
    2.2.2 Beginning of chondrocyte maturation (BMP signaling)....................................... 9
    2.2.3 In vitro guided differentiation.............................................................................. 9
      2.2.3.1 Cell choice and priming............................................................................... 9
      2.2.3.2 Material choices....................................................................................... 11
    2.2.4 Mechanical loading in lineage specification....................................................... 11
    2.2.5 Mechanical loading in fracture healing............................................................ 12
  2.3 Step 3: Cartilage proliferation and maturation............................................................... 13
    2.3.1 Molecular control of growth plate organization.................................................. 13
    2.3.2 Recreating the growth plate in vitro with endochondral priming.......... 15
Chapter 5: Combinatorial morphogenic and mechanical cues to mimic bone development for defect repair

5.1 Abstract ..................................................................................57
5.2 Introduction ...............................................................................58
5.3 Materials and Methods ...............................................................59

5.3.1 Study design ...........................................................................59
5.3.2 hMSC isolation and expansion ..............................................60
5.3.3 Gelatin microsphere synthesis and TGF-β1 loading ...............60
5.3.4 Microsphere-incorporated hMSC sheet preparation .............61
5.3.5 Nanofiber mesh production ..................................................62
5.3.6 Nanofiber mesh production ..................................................62
5.3.7 Preparation of microparticle ..................................................63
5.3.8 Surgical procedure ................................................................63
5.3.9 Fixation plate mechanical characterization .............................63
5.3.10 Fibrin gel preparation and dynamic compression ...............64
5.3.11 Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis .........................................................64
5.3.12 MicroCT angiography ...........................................................65
5.3.13 Statistical analysis .................................................................66

4.4 Results ......................................................................................66

4.4.1 Engineered mesenchymal condensations .................................66
4.4.2 Mechanical regulation of bone regeneration in vivo ...............67
4.4.3 Endochondral matrix formation .............................................67
4.4.4 Recovery of mechanical properties .......................................67
4.4.5 Dual contrast-enhanced microCT imaging of vasculature and cartilage .......................................................................68
4.4.6 Temporal effects of load on progenitor lineage specification ....68

4.5 Discussion ..................................................................................69

4.5.1 Limitations .............................................................................70

4.3 Endochondral matrix formation ....................................................70

4.3.1 Study design ...........................................................................70
4.3.2 Experiments and study groups ..............................................70
4.3.3 hMSC isolation and expansion ..............................................70
4.3.4 Microsphere synthesis and TGF-β1 loading .........................71
4.3.5 Nanofiber mesh production ..................................................71
4.3.6 Nanofiber mesh production ..................................................71
4.3.7 Preparation of microparticle ..................................................72
4.3.8 Fibrin gel preparation .............................................................72
4.3.9 Biomechanical testing ............................................................72
4.3.10 Ex vivo microCT .................................................................72
4.3.11 Biomechanical testing ............................................................72
4.3.12 MicroCT angiography ...........................................................72
4.3.13 Statistical analysis .................................................................73

4.3.14 qRT-PCR analysis .................................................................73

4.3.15 Dynamic compression ............................................................73

4.3.16 Temporal effects of load on progenitor lineage specification ....73

4.3.17 MicroCT angiography ...........................................................74

4.3.18 Statistical analysis .................................................................74

4.3.19 Dynamic compression ............................................................74

4.3.20 Temporal effects of load on progenitor lineage specification ....74

4.3.21 MicroCT angiography ...........................................................74

4.3.22 Statistical analysis .................................................................74

Chapter 5: Combinatorial morphogenic and mechanical cues to mimic bone development for defect repair .........................................................106

5.1 Abstract ..................................................................................106
5.2 Introduction ...............................................................................107
5.3 Materials and Methods ...............................................................107

5.3.1 Study design ...........................................................................107
5.3.2 Experiments and study groups ..............................................108
5.3.3 hMSC isolation and expansion ..............................................108
5.3.4 Hydroxyapatite microparticle mineral coating and BMP-2

loading ............................................................................................108
5.3.5 Gelatin microsphere synthesis and TGF-β1 loading ...............108
5.3.6 Nanofiber mesh production ..................................................109
5.3.7 Preparation of microparticle-incorporated mesenchymal

condensations ..................................................................................109
5.3.8 Surgical procedure .................................................................110
5.3.9 In vivo X-ray and microCT .....................................................110
5.3.10 Ex vivo microCT .................................................................110
5.3.11 Biomechanical testing ............................................................110
5.3.12 Histological analysis .............................................................111
Chapter 6: The timing of mechanical loading modulates endochondral ossification of chondrogenically primed MSCs

6.1 Introduction ................................................................. 154
6.2 Methods and Materials .................................................. 156
  6.2.1 Study design ......................................................... 156
  6.2.2 Cell culture ............................................................ 157
  6.2.3 Fibrin gel preparation and chondrogenic culture ................. 157
  6.2.4 Dynamic compression .............................................. 158
  6.2.5 Biochemical analysis ............................................. 158
  6.2.6 RNA isolation and qPCR .......................................... 159
  6.2.7 Histology and immunohistochemistry .......................... 160
  6.2.8 Mechanical testing ................................................ 161
  6.2.9 Statistics ............................................................. 161
6.3 Results part 1: ............................................................... 161
  6.3.1 Biochemical content ............................................. 163
  6.3.2 Chondrogenic gene expression .................................. 165
  6.3.3 Hypertrophic maturation ......................................... 166
  6.3.4 Osteogenic expression in response to load .................... 167
  6.3.5 Mechanical properties ........................................... 170
  6.3.6 Pericellular matrix formation ................................... 170
6.4 Results part 2: ............................................................... 171
  6.4.1 Biochemical content ............................................. 171
  6.4.2 Gene expression .................................................. 173
6.5 Discussion and conclusion ............................................. 175

Chapter 7: Conclusions and future directions .............................................. 178
Appendix A: Hematoxylin & eosin and Safranin-o panels ..................................................180
A.1 Full histological analysis of endochondral bone formation at week 4........180
A.2 Full histological analysis of endochondral bone formation at week 12.......182
A.3 Effects of morphogen priming of engineered mesenchymal condensations and
   in vivo mechanical loading on tissue-level bone regeneration.................184
A.4 Effects of morphogen priming of engineered mesenchymal condensations and
   in vivo mechanical loading on tissue-level bone regeneration..............185

Appendix B: Picro-sirius red panels ..................................................................................186
B.1 Collagen organization .................................................................................................186

References...........................................................................................................................188
FIGURES

Figure 2.1: Paralysis of the fetal chick *in utero* (A) decreases mineralization of the tibia, and (B) rigid fracture fixation inhibits a fracture callus from forming.

Figure 2.2: Endochondral ossification in development of long bones starts with condensation of MSCs (A), followed by chondrogenic differentiation and maturation (B-C), and ending with vessel infiltration and cartilage remodeling to bone (D-F).

Figure 3.1: Tissue engineering constructs and stiff and compliant fixation plate configurations. A) Structural PLDL/ALG scaffolds featured both random and axially-oriented porosity and 1.5 mm central core filled with RGD-functionalized alginate hydrogel containing rhBMP-2, while non-structural ALG constructs featured RGD-alginate and rhBMP-2, contained within a flexible perforated nanofiber mesh tube. Magnified images at left show porous scaffold architecture. Scale bars: 0.5 mm. In the ALG image at right, the alginate hydrogel is indicated in gray, while the perforated nanofiber mesh tube is shown in white. B) For *in vivo* mechanical loading, control limbs received stiff plates (left), while experimental limbs received compliant plates initially implanted in the locked configuration for 4 weeks (center) and were unlocked at week 4 through week 12 (right).

Figure 3.2: Structural and mechanical properties of PLDL scaffolds prior to and after eight weeks dynamic culture for degradation *in vitro*. Degradation had no effect on structural morphology, as measured by porosity (A), strut thickness (B), strut spacing (C), or connectivity (D) and did not alter the effective compressive modulus (E), but significantly reduced 0.2% offset yield strain (F) and stress (G) and ultimate stress (H). Representative stress-strain curves for scaffolds pre- and post-degradation (I) illustrate mechanical property changes. All data points plotted with mean ± s.d. * p < 0.05, unpaired Student’s t-test. N = 6 per group.

Figure 3.3: Effects of mechanical loading on bone regeneration in structural PLDL/ALG scaffolds. Delayed *in vivo* loading had no effect on defect bridging (A,B), bone formation (C,D), or torsional mechanical properties at week 12 (E,F). Binary bridging scores (bridged - B or not bridged - NB) are indicated on representative radiographs. Box plots show 25th, and 75th percentiles, with whiskers at 5th and...
95th percentiles, respectively. Mean values indicated by +. NS: p > 0.05, Student’s t-test. N = 9-10 per group........................38

Figure 3.4: Comparison of bone formation in PLDL/ALG and ALG constructs independent of mechanical loading. ALG constructs exhibited a significantly increasing trend in bridging rate over time (Chi Square test for trend, p<0.01), while PLDL/ALG did not (p > 0.73) (A). Comparison of bridging rates between groups at week 12 was statistically significant (p < 0.05). Bridging rates were evaluated from 2D radiographs, shown at weeks 4, 8, and 12 with bridging scores (bridged - B or not bridged - NB) as indicated (B). ALG constructs had significantly greater bone volume (C), illustrated by high-resolution post-mortem microCT reconstructions (D). Box plots show 25th, and 75th percentiles, with whiskers at 5th and 95th percentiles, respectively. Mean values indicated by +. **** p < 0.0001, unpaired Student’s t-test. N = 6-19 per group.........................41

Figure 3.5: Region of interest analysis of bone distribution. Bone formation was evaluated in three regions of interest defined by the inner core region, the cortex region, which was coincident with the annular PLDL scaffold, and the ectopic region, respectively (A). Bone formation in the PLDL/ALG and ALG groups was evaluated in each region (B) and quantified for total bone volume (C) and bone volume fraction, BV/TV (D). Comparison of BV and BV/TV among and between groups was performed by two-way ANOVA with Tukey’s multiple comparisons test. Box plots show 25th, and 75th percentiles, with whiskers at 5th and 95th percentiles, respectively. Mean values indicated by +. Letters shared in common between or among groups indicate no significant difference. N = 6-19 per group. ..................................43

Figure 3.6: Functional regeneration assessed by mechanical testing in torsion to failure. Torsional stiffness (A) and maximum torque at failure (B) were significantly greater in the ALG group (* p < 0.05, unpaired Student’s t-test), though both were lower than the properties of intact limbs (0.030 ± 0.001 N-m/deg and 0.31 ± 0.02 N-m, respectively) 192. Box plots show 25th, and 75th percentiles, with whiskers at 5th and 95th percentiles, respectively. Mean values indicated by +. N = 8-19 per group. ..........................................................44

Figure 3.7: Histological comparison PLDL/ALG and ALG-mediated regeneration at week 12. Haematoxylin and Eosin (H&E) (A) and Safranin-O/Fast Green (B) staining showing bone formation (b), cartilage formation (c), alginate hydrogel (a), PLDL scaffold (p), nanofiber mesh (m). Scale bars: top row - 250 μm, all others - 50 μm. ...........................................................................................................45

Figure 3.8: BMP-2 release in vitro. Cumulative release data (A) were fit to a one-phase exponential curve, and the half-life of protein release was calculated: 6.8 ± 2.8 and 3.9 ± 2.1 days for ALG and PLDL/ALG groups, respectively. The remaining BMP-2 retained in the constructs was evaluated after alginate dissolution in sodium citrate at day 21. (B) There were no differences in release kinetics, total
release, or protein retention between groups (Student’s t-test, p > 0.05). Error bars in (A) indicate standard deviations. All data points shown with nonlinear regression to exponential decay function (A) or mean ± s.d. (B). N = 3 per group.

Figure 4.1: Engineered mesenchymal condensations. (A) Photograph of hMSC sheets containing TGF-β1-loaded gelatin microspheres self-assembled for two days on Transwell inserts (left) prior to combination into engineered mesenchymal condensations for implantation (right). Each condensation was assembled from three sheets and enclosed within a perforated electrospun nanofiber mesh tube of polycaprolactone. (B) Photograph of a critically-sized (8 mm) segmental bone defect created in the femora of an RNU nude rat. Engineered mesenchymal condensations were implanted into bone defects. (C, D) Timelines of in vitro and in vivo analyses. (C) hMSC sheets were evaluated in vitro at 2 days (the time point of transplantation) or at 23 days of culture in chondrogenesis-supportive medium. (D) Bone regeneration, neovascularization, and endochondral ossification was evaluated over 3-12 weeks after transplantation. (E) Safranin-O/fast green staining of hMSC sheets with empty or 600 ng TGF-β1-containing gelatin microspheres, cultured for two or 23 days in vitro. Red indicates sGAGs; fast green counterstain shows cells and other matrix. (F) Messenger RNA expression of chondrogenic, osteogenic, and YAP pathway genes at day 2 and day 23 in vitro; qRT-PCR results normalized to GAPDH and expressed as fold-change over empty microsphere control sheets (n = 3 sheets/group). (G) Immunoblot of phosphorylated-SMAD3 activity at day 2 in vitro with β-actin control and (H) band intensity of p-SMAD3/SMAD3 ratio expressed as fold change over sheets without growth factor. (I) Immunostaining for YAP and CYR61 at days 2 and 23 in vitro. DAB peroxidase was used to produce a brown reaction product at locations of immunolabeled antigens. Right: negative control isotype IgG (rabbit, top; mouse, bottom). *P < 0.05, **P < 0.01, ***P < 0.001 TGF-β1-treated vs. empty, unpaired two-tailed Student’s t-test for each independent gene. Data shown are mean ± s.d. Scale bars, 100 μm.

Figure 4.2: Mechanical loading enhanced endochondral bone regeneration. (A) Schematic of fixation plate configurations for dynamic control of ambulatory load transfer. (B) Schematic of loading timeline. Early loading features compliant plate actuation at implantation; delayed loading features unlocking at week 4. (C) Representative in vivo microcomputed tomography (microCT) reconstructions at week 4. (D) Safranin-O/fast green staining of sagittal histological sections at week 4 (left) in comparison to the native rat distal femur growth plate (right). Bottom row: magnification of boxed areas. (E) Longitudinal microCT quantification of bone volume at week 4 [n = 11, 11, 9, and 8 for stiff, early, delayed, and BMP-2/collagen (stiff), respectively], week 8 (n = 10, 9, 8, 8) and 12 (n = 10, 8, 8, 8). Repeated significance indicator letters (a,b,c) signify P > 0.05 (not significant); groups with distinct indicators (a vs b) signify P < 0.05 at each time point. (F) Representative 3D microCT reconstructions at week 12. (G) Local trabecular
thickness mapping on transverse sections, indicated by boxed arrows in (F), in comparison to the native bone of the ipsilateral femoral head (H). (I) MicroCT quantification of trabecular thickness (Tb.Th), number (Tb.N), and spacing (Tb.Sp) in reference to that of the ipsilateral femoral head (femoral head mean ± s.d. shown as dotted line and shaded pink region). (J) H&E-stained histological sections at week 4 (representative sample from n = 1 per group). (K) Representative 3D microCT reconstruction of BMP-1/collagen group at week 12. (L) Trabecular thickness mapping on the section indicated in (K) illustrating heterotopic bone. All scale bars, 100 µm. Data shown with mean ± s.e.m. *p<0.05, ** p<0.01, **** p<0.0001, NS = not significant, one or two-way ANOVA with Tukey’s post-hoc analysis.

Figure 4.3: Fixation plate characterization. (A, B) Axial compression testing of fixation plates prior to implantation with fixed-fixed or fixed-free boundary conditions, as illustrated. (C, E) Four-point bend testing of fixation plates prior to implantation was performed in three directions, as illustrated, with supports and load applied to maintain constant bending moment on the plate. Statistical comparisons were evaluated by one-way ANOVA on log-transformed data to ensure normality of residuals followed by Tukey’s post hoc test, where groups sharing a letter are not significantly different. Summary data shown as individual data points with mean ± s.d.

Figure 4.4: Bone accumulation and bridging rates. (A) Bone volume fraction (BV/TV) over time where bone volume is normalized to total volume of the defect area (8mm length x 5mm diameter). Individual data points shown with mean ± s.d. Samples with bony bridging are shown in shaded data points, while open data points indicate non-bridged. (B) Bone volume accumulation rate, defined as bone volume accrual over each 4-week interval where BV(t) = bone volume over time, ti = time at t ≥ 4 weeks, ti−4 = time 4 weeks prior to ti. Box plots display median as horizontal line, inter-quartile range as boxes, and min/max range as whiskers. Mean values are indicated by +. *p<0.05, **p<0.01, ***p<0.001, two-way ANOVA with Tukey’s post-hoc analysis. (C) High resolution (20 µm voxel size) microCT reconstructions of excised femurs at week 12 showing best- and worst-case regeneration for each group. (D) Representative x-ray images for each group at 4, 8, and 12 weeks illustrating bridged (filled circles) and non-bridged (open circles) samples. (E) Longitudinal analysis of bone bridging in vivo at 4 (n = 11, 11, 9, for stiff, early, and delayed, respectively), 8 (n =10, 9, 8) and 12 weeks (n = 10, 8, 8) determined via x-ray as mineral fully traversing the defect. Significance of trend was analyzed by chi-square test for trend (**p < 0.001) while differences between groups were determined by chi-square test at each time point with Bonferroni correction (*p < 0.05).

Figure 4.5: MicroCT region of-interest analysis demonstrating formation of cortical and trabecular bone compartments. (A) Region of interest (ROI) analyses of bone volume in total, defect, and periphery ROIs, defined by regions either inside a
5mm diameter cylinder (defect) or annulus with 7 mm outer diameter and 5 mm inner diameter (periphery, $n = 10, 8, 8$ for stiff, early, and delayed respectively) at 12 weeks. (B) ROI analysis of bone volume fraction, BV/TV. (C) Mean mineral density, (D) Proximal vs. distal region of interest analysis, expressed as ratio of bone volume in proximal to distal halves of the defect. (E) The location of minimum pMOI for each sample. In panel E, filled data points indicate bridged samples and open data points indicate non-bridged samples. Box plots show interquartile range with whiskers at minimum and maximum values, center lines at median, and + symbols at the mean. Bar graphs show data with mean ± s.d., where groups with shared indicator letters have no significant differences and *$P < 0.05$, **$P < 0.01$, ****$P < 0.0001$, one or two-way ANOVA with Tukey’s post-hoc analysis.

Figure 4.6: Transplanted cell function. (A) Timeline depicting devitalization of mesenchymal condensations performed day 2, prior to transplantation. (B-C) MicroCT analysis of bone formed at week 12 in live and devitalized groups ($n = 10, 5$ for live and devitalized, respectively). Dotted lines illustrate the location of the native bone ends. (D) Representative Safranin-O/fast green staining at the center of the defects at week 12 ($n = 1-3$ per group). Representative samples selected based on mean bone volume. Scale bar, 100 $\mu$m. (E) Human nuclear antigen (HuNu) staining of live and devitalized samples (stiff plates) at week 12. DAB peroxidase produced a brown reaction product at locations of immunolabeled antigens. Dotted lines indicate the edges of the native cortical bone at the distal end of the defect. Devitalized samples exhibited some matrix-associated non-specific staining, as shown in IgG controls. (F) Immunostaining of HuNU, YAP, and CYR61 in defects of the delayed loading group (live cells). In each case, DAB peroxidase was used to produce a brown reaction product at locations of immunolabeled antigens. Isotype-matched IgG controls were used to demonstrate specificity. Bottom row shows magnification of boxed areas. Scale bars, 100 $\mu$m.

Figure 4.7: Endochondral matrix formation. Tile-scan images of Safranin-O/fast green-stained histological sections of representative samples from stiff, early, and delayed loading groups at (A) week 4 and (B) week 12. Scale bar: 3mm. All samples oriented distal (left) to proximal (right). Dotted lines in top-left indicate the native cortical bone ends. Labels “dh” indicate location of fixation plate drill holes. Scale bar, 3 mm. (C) Magnified images of dotted boxed regions in (B) showing endochondral cartilage remnants at week 12. Scale bar, 100 $\mu$m. Bottom row: magnification of boxed regions in upper row. (D) Polarized light microscopy of picrosirius red-stained histological sections at week 12. Increased birefringent intensity indicates increased collagen fibril organization associated with bone matrix remodelling to lamellar bone; reduced birefringence indicates greater amounts of woven bone. Scale bar, 100 $\mu$m. Bottom row: magnification of boxed regions in upper row. All images were taken from a representative sample that most closely matched the average in vivo microCT morphometry of that group.
Figure 4.8: Restoration of mechanical function. Structural mechanical properties were measured by torsion to failure at week 12. Age-matched intact bone properties are shown as dotted line/gray shading indicating mean ± s.d. Samples with full defect bridging are shown in filled data points; open data points indicate non-bridged samples. (A) Analysis of torsional stiffness, (B) maximum torque at failure, (C) minimum polar moment of inertia (pMOI), and (D) average pMOI (n = 8, 7, 7 for stiff, early, and delayed, respectively). Best subsets regression analysis with lowest AIC value for measured and predicted torsional stiffness (E) and maximum torque at failure (F) indicating significant contributions of minimum pMOI (Jmin) and binary bridging score. Error bars show mean ± s.d. with individual data points. Statistical comparisons between groups for each measure were performed by one-way ANOVA with Tukey’s post-hoc analyses, * P < 0.05; † P < 0.05 vs. intact bone.

Figure 4.9: Mechanical control of neovascularization. (A) Schematic of stiff versus early loading featuring compliant plate actuation at implantation and contrast agent perfused at week 3. (B-C) Representative microCT reconstructions of bone (B) and blood vessels with local vessel diameter mapping (C) under stiff and early loading conditions at week 3. (D) Quantification of bone volume. (E-H) 3D vascular network morphometry quantifying vascular volume (E), connectivity (F), and vessel orientation and distribution, as measured by degree of anisotropy (G) and the angle with respect to the bone-axis of the maximum principal eigenvector (H2) of the mean intercept length (MIL) tensor (H), indicating the dominant direction of vessel orientation. Degree of anisotropy represents the ratio of the longest and shortest MIL eigenvalues; DA = 1 indicates isotropy. (I) Schematic of stiff versus delayed loading featuring compliant plate unlocking at week 4 and contrast agent perfused at week 7. (J-L) Representative microCT reconstructions of bone (J) and blood vessels with local vessel diameter mapping (K) under stiff and early loading conditions at week 7. (L) Quantification of bone volume. (M-P) Vascular network morphometry measured by vascular volume (M), connectivity (N), degree of anisotropy (O), and maximum principal vector angle (P). Quantification is in a 5-mm ROI, paired data shown either as mean ± s.e.m. or superimposed on box plots displaying median as horizontal line, inter-quartile range as boxes, and min/max range as whiskers. Mean values are indicated by +. Comparisons between groups evaluated by paired two-tailed Student’s t-tests (* P < 0.05). .

Figure 4.10: Vasculature in defect periphery. Vessel volume, connectivity, and anisotropy of (A) early loaded limbs compared to contralateral stiff controls in a 7mm region of interest that included vasculature from peripheral muscle (*P < 0.05, ** P < 0.01, two-way paired student’s t-test). Paired individual data points are superimposed on box plots displaying median as horizontal line, inter-quartile range as boxes, and min/max range as whiskers. Mean values are indicated by +. (B) Region of interest analysis in the 5mm diameter defect region compared to the 7mm-5mm peripheral region that included surrounding muscle (inset; two-way
ANOVA with Tukey’s post-hoc comparisons). Box plots display median as horizontal line, inter-quartile range as boxes, and min/max range as whiskers. Mean values are indicated by +. (C) Histograms of vessel diameter bins indicating similar vessel thickness distribution between groups, and (D) representative microCT angiography vessel thickness mapping. Vessel volume, connectivity, and anisotropy of (E) delayed loaded limbs \((n = 8)\) compared to contralateral stiff controls in the same 7mm with (F) region of interest analysis. (G) Histograms of vessel diameter bins and (H) representative microCT angiography vessel thickness mapping.

Figure 4.11: (A) Axial view of 3D neovessel diameter mapping under stiff and early loading conditions at week 3, \(n = 10\). (B) Region of interest analysis to quantify vascular volume fraction in a 1.5 mm diameter core region compared to a 5mm-1.5mm annular region (inset). (C) Cationic (CA4+) cartilage contrast agent-enhanced microCT quantification of cartilage in annulus and core regions. \(n = 5-6\). (D) Axial view of 3D neovessel diameter mapping under stiff and delayed loading conditions at week 7, \(n = 8\). (E) ROI analysis of vascular volume fraction. (F) Cartilage contrast agent-enhanced microCT quantification of cartilage in annulus and core regions at week 7. (G) Representative image of co-registered contrast agent-enhanced cartilage with microCT angiography of neovascularure. Cartilage is shaded blue and vessels are red. (H) Safranin-O/fast green-stained histological sections of vascular contrast agent-perfused tissues (3 weeks). Residual contrast agent exhibits dark dots in vessel lumens. Scale bar, 100 \(\mu\)m. Data shown either as mean \(\pm\) s.e.m. with individual data points or with box plots displaying median as horizontal line, inter-quartile range as boxes, and min/max range as whiskers. Mean values on box plots are indicated by +. (* \(P < 0.05\), two-way ANOVA with Tukey’s post-hoc comparisons)..........................................................94

Figure 4.12: In vitro analysis of mechanical load on chondrogenic lineage progression. (A) Photograph of hMSC-laden hydrogel and schematic of custom-made bioreactor applying dynamic compression. (B) Timeline of the four loading groups evaluated: free-swelling (FS) controls, early loading (continuous for 5 weeks), delayed loading (free-swelling for 3 weeks followed by 2 weeks of loading), and reversed loading (loading for 2 weeks followed by 3 weeks free-swelling). (C) Quantification of DNA, sGAG, and total collagen content \((n = 5)\). (D) Alcian blue and (E) pericellular COL6a1 immunostaining. DAB peroxidase was used to produce a brown reaction product at locations of immunolabeled antigen. Images shown at 20x with 10x insets, scale bars 100 \(\mu\)m. (F-G) Quantitative PCR at week 5 \((n = 4-5\) per group) of (F) COL6a1 and (G) SOX9, COL10a1, OPN, and VEGF. Relative expression was calculated as fold change over free swelling controls. Data are shown as mean \(\pm\) s.d. with individual data points. Statistical comparisons between groups for each measure were performed by one-way ANOVA with Tukey’s post-hoc analyses, where groups sharing a letter (a,b,c) are not statistically different. ..........................................................100
Figure 4.13: mRNA expression of hMSCs in dynamically compressed hydrogels. A, COL2a1 deposition in samples after 5 weeks in vitro with hematoxylin counterstain (10x images, scale bars 100µm, n=2). B, Samples analyzed at 5 weeks for mRNA expression of ACAN, COL2a1, RUNX2, Col1a1, YAP, and CYR61 via qPCR (n=4-5) calculated as fold change over free swelling controls. Data are shown as mean ± s.d. with individual data points. One-way ANOVA and multi comparison by tukey’s post hoc was used to determine significance (*P < 0.05) where groups sharing a letter are not statistically different.

Figure 5.1: Effects of morselized autografts and in vivo mechanical loading on longitudinal bone formation and bone accumulation rate. (A) Stiff and compliant fixation plate configurations for dynamic control of ambulatory load transfer, and loading timeline with compliant plate unlocking at week 4. (B) Longitudinal quantification of bone volume at weeks 4, 8, and 12 by in vivo microCT (N = 6-8 per group). (C) Bone volume accumulation rate, defined as bone volume accrual over each 4-week interval. Data shown with mean ± SD. Box plots display median as horizontal line, mean as +, inter-quartile range as boxes, and min/max range as whiskers. Comparisons between groups were evaluated by two-way ANOVA with Tukey’s post-hoc tests. Repeated significance indicator letters (a,b,c) signify p > 0.05, while groups with distinct indicators signify p < 0.05 at each time point.

Figure 5.2: Effects of BMP-2-primed engineered mesenchymal condensations and routine clinical therapies on new bone quantity and architecture in absence of mechanical cues. (A) Representative 3-D microCT defect reconstructions of mid-shaft transverse (top) and sagittal (bottom) sections at week 12, selected based on mean bone volume. Dashed circles show 5 mm defect region. Rectangular boxes illustrate transverse cutting planes. Scale bar, 1 mm. (B) Morphometry analysis of bone volume fraction, (C) trabecular number, (D) trabecular thickness, (E) trabecular separation, shown with native femoral head properties (N = 3; dotted lines with gray shading: mean ± SD; †p<0.05 vs. femoral head), and (F) ectopic bone formation (i.e., bone extending beyond the 5-mm defect diameter) at week 12 (N = 7-10 per group). Individual data points shown with mean ± SD. Comparisons between groups were evaluated by two-way ANOVA with Tukey’s post-hoc tests. Repeated significance indicator letters (a,b,c) ........................................130

Figure 5.3: Effects of morphogen priming of engineered mesenchymal condensations and in vivo mechanical loading on longitudinal bone formation and bone accumulation rate. (A) Longitudinal quantification of bone volume at weeks 4, 8, and 12 by in vivo microCT (N = 4-11 per group). Data shown with mean ± SD. (B) Bone volume accumulation rate, defined as bone volume accrual over each 4-week interval. Box plots display median as horizontal line, mean as +, inter-quartile range as boxes, and min/max range as whiskers. (C) Representative 3-D microCT reconstructions showing bone formation per group over time. Representative samples were selected based on mean bone volume at week 12. Scale bar, 3 mm.
Comparisons between groups were evaluated by two-way repeated measures ANOVA with Tukey’s post-hoc tests. Repeated significance indicator letters (a,b,c) signify $p > 0.05$, while groups with distinct indicators signify $p < 0.05$ at each time point. Comparisons between time points were not assessed.

Figure 5.4: Effects of morphogen priming of engineered mesenchymal condensations and in vivo mechanical loading on defect bridging. (A) Longitudinal determination of defect bridging by in vivo radiography, defined as mineral fully traversing the defect ($N = 4-11$ per group). (B) Representative radiography images at 4, 8 and 12 weeks showing defect bridging per group over time, selected based on mean bone volume at week 12 (Figure 3). Significance of trend was analyzed by chi-square test ($**p<0.01$, ***$p<0.001$, ****$p<0.0001$). Differences between groups were determined by chi-square test at each time point with Bonferroni correction ($p<0.01$, correction factor of 5. Repeated significance indicator letters (a,b,c) signify $p > 0.05$, while groups with distinct indicators signify $p < 0.05$.

Figure 5.5: Effects of morphogen priming of engineered mesenchymal condensations and in vivo mechanical loading on new bone quantity and architecture. (A) Representative 3-D microCT reconstructions, with bone formation illustrated at mid-shaft transverse (top) and sagittal (bottom) sections at week 12, selected based on mean bone volume. Dashed circles show 5 mm defect region. Rectangular boxes illustrate transverse cutting planes. Note, due to minimal bone regeneration, additional transverse sections for stiff and compliant no growth factor controls were derived from the proximal end of the defect (small dashed circles and arrows). Scale bar, 1 mm. (B) Morphometric analysis of bone volume fraction, (C) trabecular number, (D) trabecular thickness, and (E) trabecular separation at week 12 ($N = 4-11$ per group), shown with corresponding measured parameters of femoral head trabecular bone ($N = 3$; dotted lines with gray shading: mean ± SD; †$p<0.05$ vs. femoral head). Individual data points shown with mean ± SD. Comparisons between groups were evaluated by two-way ANOVA with Tukey’s post-hoc tests. Repeated significance indicator letters (a,b,c) signify $p > 0.05$, while groups with distinct indicators signify $p < 0.05$.

Figure 5.6: Effects of morphogen priming of engineered mesenchymal condensations and in vivo mechanical loading on new bone distribution and architecture. (A) Morphometry analysis of proximal vs. distal bone volume distribution ($N = 4-11$ per group; $p<0.05$), and (B) ectopic bone formation (i.e., bone extending beyond the 5-mm defect diameter) shown with BMP-2 soaked on collagen data ($N = 9$; dotted line with green shading: mean ± SD). (C) Representative 3-D microCT reconstructions of native femoral head transverse (top) and sagittal (bottom) sections, selected based on mean bone volume. Dashed circles show 5 mm defect region. Rectangular box illustrates transverse cutting plane. Scale bar, 1 mm. (D) Morphometry analysis of connectivity density, (E) degree of anisotropy, and (F) structure model index within the defect region ($N = 4-11$ per group), shown with native femoral head properties ($N = 3$; dotted lines with gray shading: mean ± SD;
Individual data points shown with mean ± SD. Comparisons between groups were evaluated by two-way ANOVA with Tukey’s post-hoc tests. Repeated significance indicator letters (a,b,c) signify p > 0.05, while groups with distinct indicators signify p < 0.05.

Figure 5.7: Effects of morphogen priming of engineered mesenchymal condensations and *in vivo* mechanical loading on functional defect regeneration. (A) Torsional stiffness, (B) maximum torque at failure, (C) mean polar moment of inertia (pMOI), and (D) minimum pMOI. Best subsets regression analysis ($R^2$) with lowest Akaike’s information criterion (AIC) value for measured and predicted (E) torsional stiffness and (F) maximum torque at failure indicating significant contributions of bone volume fraction (BV/TV), trabecular separation (Tb.Sp), and minimum pMOI ($J_{\text{min}}$). Individual data points shown with mean ± SD (N = 3-10 per group). Comparisons between groups were evaluated by two-way ANOVA with Tukey’s post-hoc tests. Repeated significance indicator letters (a,b,c) signify p > 0.05, while groups with distinct indicators signify p < 0.05. Biomechanical and structural parameters are shown with age-matched intact bone properties, with pMOI obtained from the same midshaft ROI as used for the defects (N = 3; dotted lines with gray shading: mean ± SD; †,#p<0.05 vs. intact bone).

Figure 5.8: Effects of morphogen priming of engineered mesenchymal condensations on *in vitro* chondrogenic lineage specification at the time of implantation. Histological Safranin-O/Fast green staining of representative microparticle-containing hMSC sheets at the time of implantation (2 days; N = 3 per group). Scale bars, 100 μm (10x: top; 40x: bottom, magnification of dotted squares). (B) Normalized mRNA fold-change over control of key chondrogenic or osteogenic markers by qRT-PCR (N = 3 per group; *p<0.05, **p<0.01, ***p<0.001 vs. empty/control; ‡p<0.05 vs. BMP-2-containing hMSC sheets). (C) Representative immunoblots and (D) relative quantification of p-SMAD5/SMAD5 and (E) p-SMAD3/SMAD3 in lysates of day 2 hMSC sheets (N = 3 per group). β-Actin served as loading control. Individual data points shown with mean ± SD. Comparisons between groups were evaluated by one-way ANOVA with Tukey’s post-hoc tests. Repeated significance indicator letters (a,b,c) signify p > 0.05, while groups with distinct indicators signify p < 0.05.

Figure 5.9: Effects of morphogen priming of engineered mesenchymal condensations and *in vivo* mechanical loading on tissue-level bone regeneration. Representative histological (A) H&E and (B) Safranin-O/Fast green staining of defect tissue at week 4 (left) and week 12 (right), with stiff (top) and compliant fixation (bottom), selected based on mean bone volume. Scale bars, 100 μm (40x).

Figure 6.1: Bioreactor setup and loading timeline. (A) hMSC-laden hydrogels (P3, 600,000 cells, 50mg/mL fibrinogen) were subjected to different degrees of chondrogenic priming (0wks, 2wks, 4wks, 6wks) followed by 2 weeks of (B) dynamic compression in a custom-made bioreactor. (C) All samples were
collected after their loading cycle and compared to free-swelling controls at the same time. .................................................................162

Figure 6.2: Biochemical content of mechanically stimulated hydrogels. (A) DNA, sulfated glycosaminoglycans (sGAG), and collagen content calculated after each loading cycle in comparison to free swelling controls. Data are displayed as mean ± s.d. with individual data points. Two-tailed student t-test was used to determine significance (*p<0.05) (B) Alcian blue staining for sGAG with nuclear fast red counter stain (scale bar =100µm). ............................................................164

Figure 6.3: Normalized biochemical content. (A) sGAG content and (B) Collagen content normalized to hydrogel DNA quantity after their loading cycle in comparison to free-swelling controls. Data are displayed as mean ± s.d. with individual data points. Two-tailed student t-test was used to determine significance (*p<0.05). 165

Figure 6.4: Chondrogenic differentiation of hydrogels. (A) Samples were analyzed at the end of their loading cycle for message level expression of SOX9, ACAN, and Col2a1 calculated as fold change over free swelling controls. Data are shown as mean ± s.d. with individual data points. Two-tailed student t-test was used to determine significance (*p<0.05) (B) Immunohistochemistry for Col2a1 with hematoxylin counterstain (scale bar= 100µm). .........................................................................................166

Figure 6.5: Hypertrophic maturation of hydrogels in response to mechanical load. (A) Samples were analyzed at the end of their loading cycle for message level expression of hypertrophic genes Col10a1 and VEGF as fold change over free swelling controls. Data are displayed as mean ± s.d. with individual data points. Two-tailed student t-test was used to determine significance (*p<0.05) (B) Immunohistochemistry for Col10a1 with hematoxylin counterstain (scale bar= 100µm). .........................................................................................167

Figure 6.6: Osteogenic suppression in response to load. (A) Samples were analyzed at the end of their loading cycle for message level expression of osteogenic genes RUNX2 and OPN calculated as fold change over free swelling controls. Data are shown as mean ± s.d. with individual data points. Two-tailed student t-test was used to determine significance (*p<0.05) (B) Alizarin Red stain for hydroxyapatite (scale bar= 100µm). .........................................................................................169

Figure 6.7: Mechanical properties of hydrogels over time. Samples were tested in unconfined compression for (A) equilibrium modulus and (B) dynamic modulus at the end of the loading cycle in comparison to free swelling controls. Data are shown as displayed as mean ± s.d. with individual data points. Two-tailed students t-test was used to determine significance (*p<0.05). ..................................................170

Figure 6.8: Pericellular matrix formation. (A) Immunohistochemistry for Col6a1 with hematoxylin counterstain (scale bar= 100µm).........................................................................................171
Figure 6.9: Matrix production in response to load without the presence of growth factor. Hydrogels were cultured with or without 1ng/mL TGF-β3 (+/-GF) and were subjected to dynamic compression for 2 weeks, or allowed to free swell. (A) Gross quantity of DNA, sGAG, or sGAG normalized to DNA. Data are shown as mean ± s.d. with individual data points. Significance between groups was determined by one-way ANOVA and multi comparison by tukey’s post hoc where groups sharing letters (a,b,c) are not statistically different. (B) Alcian blue with nuclear fast red counter staining for sGAG, (scale bar = 100µm)..........................172

Figure 6.10: Chondrogenic gene expression in response to loading without the presence of growth factor. Hydrogels were cultured with or without 1ng/mL TGF-β3 (+/-GF) and were subjected to dynamic compression for 2 weeks, or allowed to free swell (+/-DC). (A) Message level expression of Sox9, ACAN, and Col2a1 calculated as fold change over free swelling samples without growth factor (GF-, DC-). Data are shown as mean ± s.d. with individual data points. Significance between groups was determined by one-way ANOVA multi comparison by tukey’s post hoc where groups sharing letters (a,b,c) are not statistically different. (B) Col2a1 immunohistochemistry (IHC) with nuclear fast red counter stain. (scale bar = 100µm)........................................................................174

Figure A.1: (A-F) Tile-scan images of H&E- (top) and Safranin-O/Fast green-stained (bottom) histological sections of representative samples from Stiff, Early, and Delayed loading groups at week 4. All samples oriented distal (left) to proximal (right). Dotted lines in panel A indicate the native cortical bone ends. Labels “dh” indicate location of fixation plate drill holes. Dotted boxes (1-3) indicate location of magnifications. Scale bar, 3mm. (A1-3-F1-3) Magnified images of sections in (A-F). Dotted boxes indicate location of magnifications. Scale bars, 100 µm. All images were taken from the sample that most closely matched the average in vivo microCT morphometry of that group.................................................................180

Figure A.2: (A-F) Tile-scan images of H&E- (top) and Safranin-O/Fast green-stained (bottom) histological sections of representative samples from Stiff, Early, and Delayed loading groups at week 4. All samples oriented distal (left) to proximal (right). Dotted lines in panel A indicate the native cortical bone ends. Labels “dh” indicate location of fixation plate drill holes. Dotted boxes (1-3) indicate location of magnifications. Scale bar, 3mm. (A1-3-F1-3) Magnified images of sections in (A-F). Dotted boxes indicate location of magnifications. Scale bars, 100 µm. All images were taken from the sample that most closely matched the average in vivo microCT morphometry of that group.................................................................182

Figure A.3: Overview histology of bone formation at 4 weeks. (A-F) Representative histological H&E and (G-L) Safranin-O/Fast green staining of defect tissue at week 4, selected based on mean bone volume. Scale bar, 100 µm..............................184
Figure A.4: Overview histology of bone formation at 12 weeks. (A-F) Representative histological H&E and (G-L) Safranin-O/Fast green staining of defect tissue at week 12, selected based on mean bone volume.

Figure B.1: Photomicrographs of picrosirius red staining obtained using polarized light microscopy on sections at week 4 (A-C) and 12 (D-F) (n = 1 per group at each time point, chosen by proximity to mean bone volume in vivo at 4 and 12 weeks). Under polarized light, large collagen fibers birefringe yellow and orange, while thinner fibers are green. Images are shown at 10x (top) and 40x (bottom) magnification of dotted boxes. Scale bars, 100 μm.
TABLES

Table 4.1 Oligonucleotide primer sequences for qRT-PCR. ..................................................64
Table 5.1 Oligonucleotide primer sequences for qRT-PCR. ..................................................124
Table 5.2 Experimental Design.............................................................................................132
Table 6.1 Study design...........................................................................................................157
Table 6.2 qPCR target genes.................................................................................................160
ACAN: Aggrecan
AIC: Akaike’s information criterion
ALG: Non-structural alginate scaffold
ALP: Alkaline Phosphatase,
ANOVA: Analysis of variance
BMP-2: Bone Morphogenic Protein
BV/TV: Bone volume fraction
COL1a1: Collagen 1a1
COL2A1: Collagen 2a1
COL6a1: Collagen 6a1
Conn.D: Connectivity density
CYR61: cysteine-rich angiogenic inducer 61
DA: Degree of anisotropy
DC: Dynamic Compression
DMEM: Dulbecco’s modified Eagle’s medium
EDTA: ethylenediaminetetraacetic acid
ELISA: enzyme-linked immunosorbent assay
FBS: Fetal bovine serum
FGF-2: Fibroblast growth factor
FS: Free swelling
GF: Growth factor
GM: Gelatin microspheres
H&E: Haematoxylin and Eosin,
hMSC: Human mesenchymal stem cell
HuNu: Human nuclear antigen
IACUC: Institutional Animal Care and Use Committees
IgG: Immunoglobulin G
MCM: Mineral coated microparticle
microCT: Micro computed tomography
MSC: Mesenchymal stem cell
OPN: Osteopontin
OSX: Osterix,
PBS: Phosphate buffered saline
PCL: Polycaprolactone
PCM: Pericellular matrix
PLDL: poly(DL-lactide)
pMOI: Polar moment of inertia
qRT-PCR: Quantitative real time polymerase chain reaction
RGD: Arginine-Glycine-Aspartic acid
rhBMP-2: recombinant human bone morphogenetic protein-2
RNA: Ribonucleic acid
RNU: Rowett nude (rats)
ROI: Region of interest,
RUNX2: Runt related transcription factor-2
Saf-O: Safranin-O/Fast Green
sGAG: Sulfated glycosaminoglycan
SOX9: Sry-box 9
Tb.N: Trabecular number
Tb.Sp: Trabecular spacing
Tb.Th: Trabecular thickness
TCP: Tricalcium Phosphate
TGF-β1: Transforming growth factor-beta1
VEGF: Vascular endothelial growth factor
YAP: Yes-associated protein
ACKNOWLEDGMENTS

There are a lot of people that I need to thank for making all of this possible, but first and foremost is my advisor, Joel. You chose me to be one of your first students, and put amazing faith in me to complete this work. Your mentorship, guidance, and unrelenting kindness over the years have made all of this possible. I can never thank you enough for this amazing opportunity. I’d also like to thank my committee, Dr. Daniel Kelly, and Dr. Glen Niebur, for their insightful and probing questions that have vastly improved the quality of my work and my own inquisitiveness as a researcher.

Next I want to thank my lab for all of their support over the years. Being a new lab, we had to lean on each other for help and friendship more often than not, so I thank Devon, Chris, Joe, and Jay for being my lab brothers and family. Your willingness to lend a helping hand or act as a soundboard for all of my questions greatly influenced my journey as a researcher. In that respect, I would also like to thank the Notre Dame bioengineering department, for asking insightful questions during my seminars, and for creating a family-like atmosphere where everyone is welcome.

For two years of my PhD I had the opportunity to travel to Ireland and collaborate with Dr. Daniel Kelly. I’d like to acknowledge the Naughton family for the generosity
and support necessary to make this happen, as well as being excellent hosts during my time abroad. The ability to collaborate with a second lab in another country has immeasurably improved my research, and forever changed me as a person.

Finally I’d like to give my biggest thanks to my family for all of their love and support over the years. We’re a very close crew and it wasn’t easy for me to move around the world over the past couple of years; so thank you to my parents, Tom and Susan, for your unconditional love, and pushing me to be better at the times when I didn’t think I could do it. Thanks to my siblings Bonnie, Chris, and Alyssa; you guys are my best friends. And thanks to Ron, for being my rock.
CHAPTER 1:
INTRODUCTION

1.1 Significance

Bone fractures are the most common traumatic injury\(^1\), but despite their frequency and their functional impairment they heal remarkably well through formation of a stabilizing callus that remodels to bone with a success rate of 90-95\(^%\)\(^1\). Conversely, critically-sized bone defects (exceeding 3cm), such as those that accompany bone tumor resections, gunshot wounds, non-union fractures, and other traumatic injuries, cannot heal without medical intervention and often result in life-long disability for the patient.

The clinical gold standard for repairing large bone defects is currently autograft bone from the iliac crest\(^2\), but it is limited by amount of source material available as well as adverse side effects including donor site morbidity\(^3\). Another popular clinical procedure is the application of recombinant human bone morphogenetic protein-2 (BMP-2) administered on a collagen sponge\(^4\). BMP is a powerful growth factor that stimulates mineralization at the defect site without need for donor tissue. However, large doses of this growth factor can cause harmful ectopic bone formation and soft tissue inflammation\(^5\)\(^-\)\(^7\), and it’s continued clinical use is currently under review. Additionally, long-term outcomes of these procedures suffer high rates of failure and complications, and many patients report that their quality of life after such interventions are no better
than amputation. As such, there remains a clinical need to engineer alternative approaches and graft materials for large bone defect repair that requires little or easily obtainable donor material, remains localized to the defect region, and successfully vascularizes and integrates into the defect for functional regeneration of the limb.

1.2 Research objectives

1.2.1 Utilize mechanical load for bone healing

Mechanical stimuli have long been implicated as critical regulators of bone structure and behavior. Mechanical loads control nearly all aspects of bone development, homeostasis, and disease, including load-induced bone modeling and remodeling, disuse-associated osteopenia, and peri-implant resorption caused by stress shielding, but utilizing mechanical load as a tool to enhance large defect repair has not been fully investigated. Therefore, our first objective is to investigate the effects of mechanical load on clinically relevant BMP-2 scaffolds for large defect regeneration in vivo.

1.2.2 Recapitulate development

Skeletal development occurs through a combination of intramembranous and endochondral ossification. In intramembranous ossification, progenitor cells progress directly to osteogenesis and bone formation, contributing the flat bones of the face and skull, while long bone development is dominated by the indirect process of endochondral ossification, in which a cartilage intermediate is later remodelled to bone. Much like development, the majority of fracture healing occurs indirectly with the formation of a
stabilizing cartilage callus that remodels to bone\textsuperscript{14}. Because this endochondral paradigm is recapitulated so successfully in response to injury, it is as an attractive tissue engineering approach for repair of large defects.

In addition, in both skeletal development and fracture healing mechanical loading is important to induce endochondral ossification. Paralysis \textit{in utero} generates neonatal abnormalities\textsuperscript{15}, reduces chondrocyte proliferation and delays ossification\textsuperscript{16–20}. Similarly, some interfragmentary motion is required for callus formation, while rigidly fixed fractures only heal intramembranously\textsuperscript{21–25}. Therefore, our second objective is to combine the principles of cartilage template maturation and mechanical loading to investigate endochondral lineage progression \textit{in vivo} for regeneration of large bone defects.

1.2.3 Investigate temporal mechanical and chemical cues

The induction of a cartilage callus in response to interfragmentary motion suggests that there is a significant relationship between mechanical loading and lineage specification of progenitor cells during endochondral ossification that has not been thoroughly investigated, and it is unclear the role that load timing and cartilage template maturation state play in this progression. Our third objective is to investigate, \textit{in vitro}, how progenitor cells respond to mechanical loading after different degrees of chondrogenic priming.
CHAPTER 2:
LITERATURE REVIEW: MECHANICAL FORCES IN ENDOCHONDRAL OSSIFICATION AND TISSUE ENGINEERING APPROACHES

Bone is the second most commonly transplanted tissue worldwide after blood products\(^2\). The gold standard for repairing large bone defects is still autograft bone\(^{26}\), but limited source material and adverse reactions such as donor site morbidity\(^3\) have prompted research into tissue engineering methods that can provide alternative graft materials. Early approaches concentrated on facilitating direct bone formation through intramembranous ossification, however these constructs often suffer from poor vascular infiltration, resulting in necrosis at the core from limited diffusion of nutrients, increasing fracture non-unions\(^{27}\). Endochondral ossification, in which bone formation occurs indirectly by first laying down a cartilage template that is later remodeled into bone, is the mechanism by which long bones form during development and fractures heal through callus formation, and has emerged as an attractive tissue engineering pathway due to its ability to form robust, vascularized bone\(^{28}\). However, current endochondral engineering approaches have achieved limited success recapitulating this developmental process, in part due to poor understanding of the mechanical and morphogenic cues necessary to stimulate the pathway.
2.1 Step 1: Mesenchymal condensation

2.1.1 Initiation of condensation during development

Development of the appendicular and axial skeleton begins with condensation of mesoderm-derived cells into a precartilaginous mesenchyme. Interactions between the mesoderm and epithelium induce migrating progenitor cells to begin condensing through transforming growth factor (TGF-β) signaling\textsuperscript{29,30} to bone morphogenic protein (BMP) receptors within the mesenchyme. TGF-β in turn regulates the molecules tenascin and N-cadherin necessary for cell adhesion, and N-CAM for stabilization and maintenance\textsuperscript{31,32}. Adhesions in the condensing mesenchyme are dominated by cell-cell interactions (mediated by n-cadherin) as opposed to the more mature cell-extracellular matrix (ECM) interactions mediated by fibronectin focal adhesions later in development. Cell adhesion in the condensation is particularly important for chondrogenic differentiation as inhibition of N-cadherin in the limb bud limits chondrogenesis while conversely overexpression promotes it\textsuperscript{33}. Indeed, cell-cell contact has been shown to inhibit osteogenesis due to a decrease in cell-ECM contact area\textsuperscript{34}. Condensations must reach a critical size before subsequent differentiation can occur, as small and delayed condensations have been shown to cause hypoplasia of long bones\textsuperscript{35}.

2.1.2 Mechanical forces

Mechanical forces within the condensing mesenchyme are also important to condensation maintenance and the progression of endochondral ossification. Within the condensation there are opposing stimulatory and repulsive migratory factors, in the form of fibroblast growth factor (FGF)-8 and Sema3f, that cause mesenchymal stem cells
(MSCs) to pack tightly together during development\textsuperscript{36}. In low density cell cultures, without compaction there is no Pax-9 expression and subsequent ECM synthesis; where Pax-1 and Pax-9 are strongly expressed at condensation stage and important regulators of epithelial-mesenchymal interactions\textsuperscript{37}. Lack of condensation in these cultures cannot be rescued by exogenous supplementation of FGF-8 and Sema3f, nor do these factors enhance condensation in high density cultures, suggesting that they play a role in maintenance, but that physical compaction and cell to cell contact is necessary for induction of condensation. Once a condensation of sufficient size is reached, growth is halted and differentiation of the progenitor cells can begin.

2.1.3 Mimicking condensation in tissue engineering

In general, methods to culture articular chondrocytes or MSC-derived chondrocytes in 2D \textit{in vitro} is difficult as the cells often fail to maintain their phenotype and dedifferentiate to a more fibrocartilage lineage\textsuperscript{38}. Pellet, micromass, and high density 3D cell cultures that mimic embryonic condensations better maintain the cartilage phenotype\textsuperscript{39} by recapitulating the spherical morphology and cell-cell contact needed for embryonic condensation\textsuperscript{40}. Micromass assays, in which progenitor cells in high density cell suspension are placed on culture plastic in a small drop and allowed to attach have long been used as a tool for study (reviewed in Klumpers et al\textsuperscript{41}). The small micromass becomes a 3D culture after abundant proliferation and fibronectin deposition\textsuperscript{42}, and hypertrophy is observed in the core within 14 days\textsuperscript{43}. Different factors can be added to the mass to observe their effects on chondrogenesis and endochondral ossification.
Similar studies have been performed in culture with cellulose\textsuperscript{44}, alginate beads\textsuperscript{45} or cell pellets\textsuperscript{46}.

Maintenance of a condensation in culture is also dependent on soluble factors added to the growth media. Addition of FGF-2 and other factors involved in condensation formation and growth can maintain MSC phenotype and redifferentiation ability, and increases their chondrogenic ability\textsuperscript{39,47}. FGF-2 works to inhibit TGF-\beta2 and sox9 signaling to suppress cellular senescence\textsuperscript{14}, allowing cells to form larger pellets and more matrix. Increased basal Sox9 levels follow cells expanded in FGF-2 as well as a decrease in OCT3/4 and Nanog suggesting a reduction in multipotentiality and priming for chondrogenesis\textsuperscript{48}. Priming of cells with a mixture FGF-2 and Wnt3a also stimulated MSC proliferation in 3D culture\textsuperscript{49}, as well as lowered oxygen tension to mimic the prevascularized environment in the mesenchyme\textsuperscript{50}.

Culture with TGF-\beta is also used for inducing aggregation of MSCs. It can induce condensation of cellular bodies\textsuperscript{51} with best cohesion when introduced early, and progenitor cells at a high density with factors such as TGF-\beta1 can produce condensation-like masses for cartilage or bone tissue engineering\textsuperscript{28,52,53}. Through recapitulation of the condensing mesenchyme, in many cases a synthetic 3D matrix is not needed to regenerate large defects. Among other studies using cell aggregates, MSC spheroids alone have been shown to heal calvarial defects\textsuperscript{54}. And in fact, hydrogels and other material-cell interactions can reduce cell to cell contact and inhibit embryonic condensation\textsuperscript{55}. This can be overcome by addition of newly developed HAVDI peptides to hydrogels that mimic cell-cell interactions\textsuperscript{56}. As the first essential building block to long bone formation, future
studies will attempt to recapitulate mesenchymal condensations to potentially regenerate full bone organs.

2.2 Step 2: Chondrogenic differentiation

2.2.1 Early chondrogenesis and immature chondrocytes (TGF-β signaling)

TGF-β signaling plays a critical role in induction and maintenance of chondrogenesis. TGF-β activated Smad 2/3 signaling promotes early chondrogenesis of the mesenchymal condensation by inducing expression of Sox9, Sox8, and Sox10 before any phenotypical changes are detectable \cite{30,57,58}. At the same time, high endogenous expression of Twist1 suppresses Runx2 and prevents a transition to osteogenesis \cite{59}. Downstream expression of L-sox5 and sox6 occurs shortly afterward (their expression is absent in sox9 deficient limbs \cite{58}), and they cooperate in the control of type II Collagen and Aggrecan gene expression \cite{60-64}, the main ECM components of cartilage. Sox signaling occurs upstream and is induced independently of BMP signaling to maintain the chondrogenic phenotype \cite{30}. Indeed, without Sox signaling severe bone defects occur. Misexpression of sox9 in chick limbs results in the formation of ectopic cartilages \cite{65}, and haploinsufficiency in mice causes campomelic dysplasia syndrome, defective cartilage primordia, and premature skeletal mineralization \cite{35}. In addition, L-sox5 and sox6 double null mice die in utero with severe cartilage defects \cite{64}, and sox8 deficient mice exhibit reduced skeletal size \cite{66}.

Early TGF-β signaling works in concert with Wnt/beta-catenin signaling to together stabilize chondrogenesis (inhibit maturation) \cite{50}; overexpression of sox9 or
inactivation beta-catenin results in dwarfism and reduced chondrocyte proliferation while the converse causes chondrodysplasia\textsuperscript{67}, and it is thought that interactions between these two pathways are crucial for chondrogenesis.

2.2.2 Beginning of chondrocyte maturation (BMP signaling)

There are low endogenous levels of runx2 in early chondrogenesis to maintain the cells in an immature state\textsuperscript{68}. Subsequent BMP signaling in the limb bud promotes the beginning of chondrocyte maturation through phosphorylation of Smad 1/5/8, raising runx2 levels and initiating ALP production, an early marker of osteogenesis. Knockouts of BMP receptors, BMPR1A and BMPR1B, in mice have missing or severely affected skeletal elements and compromised growth plate formation\textsuperscript{69}. Similarly, knockouts of Smad 1/5/8, have severe chondrodysplasia\textsuperscript{70}, indicating that canonical BMP signaling is necessary for chondrocyte differentiation, proliferation, and maturation.

2.2.3 In vitro guided differentiation

2.2.3.1 Cell choice and priming

When engineering a cartilage graft for either cartilage engineering or bone tissue engineering through endochondral ossification, many cell types have been considered. Articular chondrocytes are an advantageous choice due to their pre-differentiated state, however, these cells have limited proliferative potential\textsuperscript{71}, high variability in the quality of donor cells\textsuperscript{72}, and donor site morbidity is a problem due to the tissue’s limited innate ability to repair itself. In addition, in 2D culture these cells tend to dedifferentiate into a fibrocartilage-like phenotype\textsuperscript{38} and lose their ability to redifferentiate. For these reasons,
stem cells are a more attractive option with their high proliferation rates and multipotency.

Embryonic stem cells isolated from the inner cell mass of the blastocyst have been used in bone tissue engineering approaches\textsuperscript{73}, but the process to maintain their pluripotency and induce differentiation is extensive, and there remain ethical concerns with their use. A more obvious choice is the MSC, readily available and more easily obtained from bone marrow, adipose tissue, infrapatellar fat pad, and synovium\textsuperscript{74–79}, and they can undergo numerous passages \textit{in vitro} while still maintaining their potential to differentiate osteogenically, chondrogenically, or adipogenically. MSCs also tend to progress through hypertrophy and ossification naturally, making them challenging for cartilage engineering, but advantageous for bone engineering\textsuperscript{80}.

Chondrogenic differentiation is \textit{in vitro} is usually induced by TGF-\(\beta\), Smad3 phosphorylation and expression of sox9 with production of collagen II and aggrecan using a variety of morphogens. TGF-\(\beta1\) and TGF-\(\beta3\) are the most common factors used in chondrogenic differentiation, and their downstream synthesis of ECM occurs in a dose dependent manner, with different sources of MSC requiring more or less morphogen to obtain a chondrogenic gene expression profile. The effects of TGF-\(\beta\) can be enhanced by inclusion of other chondrogenic factors. Addition of dexamethasone, BMP-6, BMP-2, or IGF-1 with TGF-\(\beta\) all promote better chondrogenesis together than any factor alone\textsuperscript{81,82}. Temporal administration of morphogens can have differing effects on differentiation as well. The addition of BMP-6 after TGF-\(\beta3\) decreases the chondrogenic effect and begins increasing collagen-I transcription\textsuperscript{81}, but if it is applied earlier it upregulates collagen-II\textsuperscript{83}. In addition, environmental factors such as oxygen tension can induce chondrogenic
differentiation of MSCs as well as steer them away from hypertrophy and towards hyaline cartilage through metabolic programming. Consensus on the synergism or temporal administration of multiple morphogens for ideal chondrogenic culture has yet to be determined.

2.2.3.2 Material choices

Delivery of morphogens in 2D and 3D culture can be tailored by the use of materials. They can be bound through electrostatic association to many naturally occurring materials and hydrogels; fibrin and collagen, heparin, and micro/nano-particles, or covalently bound with the use of RGD motifs. Delivery of TGF-β in gelatin microspheres or hydroxyapatite microspheres can induce uniform differentiation of MSCs in vitro without exogenous supplementation, or promote healing of calvarial defects when implanted in vivo. Release kinetics of morphogens can be partially controlled by altering binding affinity, cross-linking density, or degradation of these materials (reviewed in detail by Caliari et al).

2.2.4 Mechanical loading in lineage specification

Another, often overlooked, necessity in differentiation and endochondral ossification is the application of mechanical load. The absence of mechanical stimulation via muscle contraction results in failure of progenitor cells to commit to a chondrogenic fate. Application of muscle relaxants to rats during gestation generates neonatal abnormalities, and reduces chondrocyte proliferation and delays ossification in chick embryos. Similarly, in fracture healing, some interfragmentary motion is required
for endochondral bone healing to occur, while rigidly fixed fractures only heal intramembranously\textsuperscript{93} (Fig. 2.1). Flexibly fixed osteotomies show increase in chondrogenic markers such as col2a1, aggregan, and sox9, as well as Wnt signaling inhibitors\textsuperscript{94}. However the effects of loading depend on the mode\textsuperscript{95}, rate\textsuperscript{22,96}, magnitude as well as gap size\textsuperscript{25} and time of applied load\textsuperscript{97}.

![Figure 2.1](image)

**Figure 2.1**: Paralysis of the fetal chick *in utero* (A) decreases mineralization of the tibia\textsuperscript{16}, and (B) rigid fracture fixation inhibits a fracture callus from forming\textsuperscript{21}.

### 2.2.5 Mechanical loading in fracture healing

In many cases, early loading of high strain to a fracture callus tends to produce a more robust cartilage response and possible psuedoarthrosis\textsuperscript{98,99} and rarely results in full bone healing\textsuperscript{100}. In the case of cartilage tissue engineering, this is beneficial; high strain compressive loads *in vitro* have been shown to increase cartilage matrix production\textsuperscript{101} while suppressing expression of hypertrophic markers\textsuperscript{102,103}, a possible tool to prevent ossification of MSC derived cartilage *in vitro*. Other types of loading, namely hydrostatic pressure, are physiologically relevant for cartilage engineering and increase
glycosaminoglycan (GAG) synthesis\textsuperscript{104–106}, but high magnitudes can have an inhibitory effect\textsuperscript{107,108}.

Typically, delayed loading in fracture or critically sized defect models is more beneficial to ossification. Claes et al, observed greater fracture healing in osteotomies dynamized late than those loaded early, and reported it due to the formation of a stiffer callus decreasing interfragmentary strains\textsuperscript{109}. In fracture healing, as the callus matures it becomes stiffer, reducing interfragmentary movement enough for bridging by bony callus to occur\textsuperscript{110}. Reversed dynamization (low stiffness to high stiffness) in critically sized defects saw enhanced healing, but again saw that persistent loading resulted in connective tissue and non-union\textsuperscript{111}. \textit{In vitro}, delayed loading (21 days) promotes more matrix synthesis in chondrogenically primed MSCs\textsuperscript{112}, indicating that some chondrogenic differentiation may be necessary before loading is beneficial.

2.3 Step 3: Cartilage proliferation and maturation

2.3.1 Molecular control of growth plate organization

The growth plate forms as a result of chondrocytes undergoing rapid proliferation and maturation in an orderly columnar fashion to form the endochondral skeleton\textsuperscript{113}. This maturation begins through Smad 1/5/8 signaling\textsuperscript{114} and is reliant on an increase in runx2 accompanied by a decrease in Twist1\textsuperscript{59}. During chondrocyte maturation, the columns are divided into horizontal zones of chondrocytes appearing at different stages of differentiation based on distinct morphological differences with an axial orientation; these are in progressing pools of proliferating, prehypertrophic, and hypertrophic
chondrocytes. The regulation and maintenance of the growth plate organization is subject to several important signaling pathways and related transcription factors. The transition from proliferating chondroblasts to maturing prehypertrophic chondrocytes is regulated by Indian Hedgehog (Ihh), in turn Ihh stimulates the expression of Parathyroid Hormone-Related Protein (PTHrP)\textsuperscript{115} which works to keep chondrocytes in the proliferative pool. PTHrP delays the production of Ihh by binding to a PTHrP receptor on proliferating cells, creating a negative feedback loop to control the size of the developing chondrocytes\textsuperscript{116}. Prehypertrophic chondrocytes express sox9 (as mentioned previously) and Ihh activation of PTHrP actively represses the osteogenic differentiation factor, runx2, to maintain chondrogenesis and delay ossification. For example, knockout mice for PTHrP show accelerated chondrocyte differentiation resulting in dwarfism while knockout mice for the analogous PTH/PTHrP receptor showed a similar, yet far more extreme phenotype\textsuperscript{117}. In addition, Ihh null mutant embryos having a severe reduction in proliferation and premature hypertrophy in chondrocytes\textsuperscript{118}.

Though it is not fully understood, it is believed that hypertrophy of the chondrocyte proceeds when chondrocytes extend far enough from the source of PTHrP, the pool of proliferating chondroblasts\textsuperscript{113}. The loss of PTHrP slows proliferation and allows for Ihh regulated chondrocyte maturation characterized by a loss of sox signaling and up to a 20-fold increase in size\textsuperscript{119}. No longer suppressed by PTHrP, hypertrophic chondrocytes also begin to express runx2, followed by the expression of important factors such as collagen-X\textsuperscript{120}, bone morphogenetic protein-2 (BMP-2), matrix metalloproteinase-13 (MMP-13)\textsuperscript{121}, alkaline phosphatase (ALP), and vascular endothelial growth factor (VEGF)\textsuperscript{122,123}. Mice globally lacking runx2 fail to develop chondrocytes.
and severely lack vascularization in the growth plate area\textsuperscript{124}, while its overexpression accelerates endochondral ossification\textsuperscript{68}. Hypertrophic chondrocytes are sometimes considered the primary engines of bone growth; this change in gene expression and matrix synthesis recruits blood vessels and osteoprogenitors to the condensation and begins the process of ossification.

2.3.2 Recreating the growth plate in vitro with endochondral priming

One of the main goals of endochondral tissue engineering is creating a cartilage template \textit{in vitro} that will grow and ossify \textit{in vivo}; a pseudo growth plate to mimic long bone maturation\textsuperscript{28}. Typically this is achieved through chondrogenic priming of cells, generally MSCs with previously discussed morphogens such as TGF-\(\beta\)\textsubscript{1}, TGF-\(\beta\)\textsubscript{2}, and TGF-\(\beta\)\textsubscript{3}, known to induce chondrogenesis in MSCs and stimulate aggrecan and collagen-II synthesis\textsuperscript{125}. There are no standards in place that indicate how the stage of chondrogenesis achieved in \textit{in vitro} will affect bone growth \textit{in vivo}, but generally the goal is to reach some level of hypertrophy in order to obtain inductive factors released by the mature chondrocytes, such as VEGF, for vascularization and matrix remodelling. For example, chondrogenically primed constructs implanted subcutaneously\textsuperscript{126–128} and orthotopically\textsuperscript{129} show progression toward endochondral bone formation with an upregulation of key signaling pathways involved in endochondral ossification. However, recent work has shown MSCs at different stages of chondrogenesis were all able to release VEGF regardless of priming time\textsuperscript{130}, suggesting that a lengthy \textit{in vitro} period may not be necessary. In some cases priming may not be necessary at all; incorporation of microparticles that control the release of growth factor into cultures can allow immediate
implantation for differentiation and maturation of cellular constructs in vivo\textsuperscript{131}. Because there is no consensus of the most appropriate priming time, shorter in vitro periods may be more economical without being any less effective.

2.3.3 Final fate of hypertrophic chondrocytes

There is some debate about the fate of hypertrophic chondrocytes during development and within the growth plate. It was traditionally believed that they are the terminal stage of chondrogenesis, become apoptotic, and are resorbed and replaced by invading osteoprogenitor cells while leaving behind a collagen scaffold\textsuperscript{113,132,133}. However, the loss of sox9 during hypertrophy followed by the expression of bone markers such as runx2, osterix, osteocalcin, and osteopontin suggest a direct conversion to osteoblasts\textsuperscript{134}. Recent lineage tracing studies using Cre-recombination under control of a Coll-X promotor show that hypertrophic chondrocytes can become collagen-I expressing osteoblasts and sclerostin expressing osteocytes\textsuperscript{135,136}, and these bone cells are present in both neonatal and adult bone. In addition, a tracing of donor cells from a fracture callus implanted into a segmental defect show that the regenerated bone was graft-derived\textsuperscript{137}. Similarly, Scotti et al. showed that in defect regeneration, donor cells contributed to and were detected in the inner trabecular bone, while cortices were derived from host cells\textsuperscript{127}, suggesting that in addition to invading osteoprogenitor cells, hypertrophic chondrocytes can also contribute directly to new bone formation. Therefore, in addition to creating a template for host tissue invasion, hypertrophic scaffolds may also contribute directly to tissue regeneration.
2.3.4 Mimicking the ECM with biomaterials

When recapitulating endochondral ossification with scaffolds, aggregates that mimic embryonic mesenchymal condensations are the clearest approach, but a challenge of cell-based scaffolds is achieving adequately sized constructs for large defects, so biomaterials are often used to scale-up. Hydrogels are popular and promising because they mimic the native extracellular matrix, are mechanically similar to soft tissues, and can support cell adhesion and protein sequestration\textsuperscript{91}. Naturally derived materials such as collagen\textsuperscript{138}, gelatin, and fibrin\textsuperscript{139} are frequently used due to their biocompatibility, site for cell attachment, and ability to be remodeled. However they have a lower stiffness than many other hydrogels, and because they are easily remodeled, they are quickly contracted and resorbed by cells, and may not be a good long term option for \textit{in vitro} culture. Other hydrogels such as alginate and hyaluronic acid have more flexibility in tailoring properties, but must be modified with functional groups, i.e. RGD peptides\textsuperscript{140} to ensure cell attachment. Cells do not readily degrade these hydrogels, so though better for long term \textit{in vitro} culture, they may not sufficiently be remodeled \textit{in vivo}\textsuperscript{141}.

Other material approaches employ the use of structural scaffolds from a range of polymers and metals to mimic the mechanical properties of native bone and bear load upon implantation. They may also bring out the osteogenic potential of MSCs by incorporating materials such as hydroxyapatite (HA) and β-tri calcium phosphate (β-TCP)\textsuperscript{142,143}. However there is some evidence suggesting that structural scaffolds may inhibit the stimulatory mechanical loads, invasion of progenitor cells, and matrix remodeling necessary to the endochondral pathway\textsuperscript{144}. Construct design that mimics the initial, non-structural stages of growth that later fully matures and integrates with bone \textit{in}
vivo, may be better suited to obtain unobstructed endochondral ossification and observe the effects of mechanical stimuli.

2.3.5 Spatial control of cell organization

Part of what makes the growth plate unique is how critical its organization is to the progression of bone morphogenesis. The polarity of the growth plate is unaffected by surrounding tissues; excision, inversion, and reimplantation results in bone growth in the opposite direction\textsuperscript{145}. In both bone and cartilage tissue engineering, current approaches generally lack the native structural complexity and organization, and fail to maintain their boundaries when diffusible morphogens are used\textsuperscript{146}. In response, many have attempted to artificially organize cells in order to direct the desired tissue growth. The first was Thompson et al, who showed that embryonic avian heart tubes cut into rings and placed on supporting tubular framework would fuse and morph overnight into a single beating heart tube. It was hypothesized that components of tissue can inherently fuse together, and the concept of bioprinting; printing cells, hydrogels, and other materials layer by layer until a 3D form is achieved, was developed.

Commonly, multicellular spherical aggregates are utilized as bio-ink particles\textsuperscript{147}, or hydrogels and polymers are printed in precise patterns/layers for cell seeding and implantation \textit{in vivo}\textsuperscript{148}. Different cell populations and densities can be incorporated into separate inks, and switched on demand to create cell gradients. This has recently been beneficial in zonal cartilage engineering\textsuperscript{149}, and could be potentially useful in recreating the organization if the growth plate. Another method, without the need for computer-assisted printing devices, is the use of multilayer constructs. Sheehy et al recreated a bone
organ by layering a hydrogel with articular chondrocytes on top of a larger hydrogel with MSCs\textsuperscript{150}. To date, regulation of engineered growth plate organization by manipulating a morphogen gradient such as seen in development has not yet been attempted.

2.4 Step 4: Blood vessel invasion and remodelling

2.4.1 Morphologic steps

The progression to hypertrophy in chondrocytes is a major turning point in the endochondral pathway after which ossification begins. The release of VEGF by hypertrophic chondrocytes stimulates angiogenesis\textsuperscript{151}, and the release of MMP-13 begins degrading the cartilage matrix, making it possible for invading vessels to deliver osteogenic and hematopoietic precursors that will lay down the initial woven bone and form a primary ossification center (POC)\textsuperscript{152}. It was recently discovered that oxygen rich blood enters the growing bone preferentially through phenotypically unique capillaries (termed type H by the authors) near the growth plate, and that osteoprogenitor cells are found selectively near these vessels, indicating the importance of hypertrophic chondrocyte morphogen signaling\textsuperscript{153}. Without the release of VEGF there is little blood vessel formation, and trabecular bone formation is seriously impeded\textsuperscript{122}. After vessel invasion and formation of the POC, the diaphysis is remodeled to form a medullary cavity and a secondary ossification center (SOC) forms in the epiphysis (Fig. 2.2). Between the POC and SOC remains an epiphyseal growth plate full of hypertrophic chondrocytes responsible for continued long bone formation.
Figure 2.2: Endochondral ossification in development of long bones starts with condensation of MSCs (A), followed by chondrogenic differentiation and maturation (B-C), and ending with vessel infiltration and cartilage remodeling to bone (D-F)\textsuperscript{154}.

2.4.2 Engineering approaches to vascularize tissue

Vascular invasion of implanted cellular constructs is essential to combat necrosis at the core and maintain viability. Similarly in fracture repair, a process that recapitulates endochondral ossification, lack of an active blood supply is detrimental to repair and can cause non-union\textsuperscript{155}. Past approaches to bone tissue engineering focusing on intramembranous ossification have struggled with adequate vascularization due to excessive mineralization pre-implantation and inefficient remodeling\textsuperscript{156}. Conversely, a hypertrophic scaffold \textit{in vitro}, achieved through chondrogenic priming, hypothetically contains all the developmentally inherent signals necessary for blood vessel invasion, growth, and remodeling when implanted \textit{in vivo}. For example, Scotti et al showed that engineered hypertrophic cartilage could recreate features of a full ‘bone organ’ with mature vasculature when implanted ectopically \textit{in vivo}\textsuperscript{127}. Similarly, Bahney et al showed that a mature fracture callus could regenerate an orthotopic defect when transplanted\textsuperscript{157}. 

20
Depending on material, cell source, and priming time, hypertrophic grafts can still suffer necrosis at the core during in vitro culture due to poor nutrient diffusion. Altering scaffold architecture with nutrient channels is one simple way to overcome this\textsuperscript{158}. Other methods attempt to prevascularize the grafts using a coculture of MSCs with endothelial cells or HUVECS. The interactions between the two cell types have shown to promote the release of proangiogenic factors\textsuperscript{149,159}, and a mixture of chondrogenic and angiogenic priming increases the osteogenic potential of MSCs when implanted in vivo\textsuperscript{159}. In a few recent large animal experiments, prevascularization outperformed unvascularized controls in bone graft operations\textsuperscript{160}. Conversely, it may be beneficial to impede vascularization in cases such as articular cartilage tissue engineering. As stated previously, bone marrow MSCs tend mature to hypertrophy and ossify naturally, and keeping them as stable chondrocytes is difficult. Recently it was shown that MSCs transduced to express decoy VEGF receptors that sequester the morphogen were able to induce chondrogenic differentiation in MSCs, prevent hypertrophic lineage progression, and inhibit vascular invasion in subcutaneous pouches in vivo\textsuperscript{161}.

2.4.3 Mechanical load in vascularization

The effect of mechanical stimulus on vessel growth and invasion is still poorly understood. Loading of in vivo critically sized rat femoral defects too early has a detrimental effect on angiogenesis, but late loading is less sensitive\textsuperscript{162}. As mentioned previously, early loading in vitro of primed MSCs produced a more stable cartilaginous phenotype\textsuperscript{101}, suggesting that results seen in vivo maybe be due to a lack of inductive angiogenic factors released by hypertrophic chondrocytes rather than a direct effect of
loading. However, shear fluid forces in blood vessels may play an active role in bone formation and maintenance. It was recently found that blood shear rate is higher in the type H capillaries preferentially stationed near the growth plate and metaphysis, and that shear rate decreases in each branch as it reaches the diaphysis. Defective flow prevents bud formation in the type H vessels that give rise to further vascularization, subsequently decreasing bone volume\textsuperscript{163}. Similarly reduced blood flow in elderly women is linked to loss in bone mass and osteoporosis\textsuperscript{164}, indicating that adequate blood velocity is needed for bone homeostasis. Attempts at prevascularization \textit{in vitro} need to consider ability to withstand high shear forces once implanted \textit{in vivo} in order to promote further neovascularization and incorporation into the native tissue.

2.5 Conclusion

After the stage of vascular invasion, osteogenesis and bone growth takes over. There is still much to learn regarding the stages of endochondral ossification and how tissue engineers can utilize morphogenic and mechanicals cues to create better graft material. The work described herein utilizes mechanical loading in bone repair to recapitulate endochondral ossification by combining mesenchymal condensations and mechanical loading, and to understand how these cues affect cell lineage progression.
CHAPTER 3:
INFLUENCE OF STRUCTURAL LOAD-BEARING SCAFFOLDS ON
MECHANICAL LOAD- AND BMP-2- MEDIATED BONE REGENERATION

3.1 Abstract

A common design constraint in functional tissue engineering is that scaffolds intended for use in load-bearing sites possess similar mechanical properties to the replaced tissue. Here, we tested the hypothesis that in vivo loading would enhance bone morphogenetic protein-2 (BMP-2)-mediated bone regeneration in the presence of a load-bearing PLDL scaffold, whose pores and central core were filled with BMP-2-releasing alginate hydrogel. First, we evaluated the effects of in vivo mechanical loading on bone regeneration in the structural scaffolds. Second, we compared scaffold-mediated bone regeneration, independent of mechanical loading, with alginate hydrogel constructs, without the structural scaffold, that have been shown previously to facilitate in vivo mechanical stimulation of bone formation.

Contrary to the hypothesis, mechanical loading had no effect on bone formation, distribution, or biomechanical properties in structural scaffolds. Independent of loading,

---

1 This chapter is published in: J Mech Behav Biomed Mater. PMID: 27208510. Authors: McDermott AM, Mason DE, Lin ASP, Guldberg RE, Boerckel JD.
the structural scaffolds reduced bone formation compared to non-structural alginate, particularly in regions in which the scaffold was concentrated, resulting in impaired functional regeneration.

This is attributable to a combination of stress shielding by the scaffold and physical or chemical inhibition of bone formation into the defect. Collectively, these data question the necessity of scaffold similarity to mature tissue at the time of implantation and emphasize regeneration by the end of the healing time course.

3.2 Introduction

Mechanical stimuli have long been implicated as critical regulators of bone structure and behavior \(^9\). Mechanical loads control nearly all aspects of bone development, homeostasis, and disease, including load-induced bone modeling and remodeling \(^10\), disuse-associated osteopenia \(^11\), and peri-implant resorption caused by stress shielding \(^12\). The process of fracture healing is also acutely sensitive to mechanical stimuli, with both the magnitude and mode of interfragmentary motion influencing tissue differentiation, speed of recovery, and ultimate clinical outcome \(^23,165,166\).

The consensus of many experimental \(^25,93,167\) and theoretical \(^110,168–171\) studies on bone fracture healing demonstrates that cellular lineage specification and tissue differentiation in the fracture callus is controlled by local mechanical conditions, with thresholds and modes of interfragmentary stress and strain regulating callus formation and remodeling. However, the conditions necessary for functional regeneration of critical sized bone defects, which cannot heal without intervention, remain poorly understood and cannot be predicted by the classical theory alone \(^172–174\).
In recent years, attempts to re-engineer diseased and damaged tissues have demonstrated the importance of both intrinsic and extrinsic mechanical cues for functional regeneration. Intrinsic mechanical cues include inherent properties of the extracellular matrix such as elastic rigidity and viscoelasticity \(^{175-177}\), while extrinsic cues include both static and dynamic forces applied via boundary conditions \(^{162,178}\). Each of these is of particular importance in bone tissue engineering, where tissue function is fundamentally mechanical. For example, intrinsic matrix mechanical properties are sufficient *per se* to control lineage specification of stem and progenitor cells \(^{175}\), an observation that has inspired many to pursue controllable, defined matrices for use in tissue engineering, frequently through hydrogels \(^{176,179,180}\). Similarly, the importance of extrinsic mechanical conditions in tissue formation and adaptation is apparent in the prevalence of dynamic bioreactors for *in vitro* tissue conditioning and culture \(^{181-183}\).

Efforts to control the biomechanical environment *in vivo* have also revealed profound effects of both intrinsic \(^{176,184,185}\) and extrinsic \(^{111,162,186,187}\) mechanical stimuli on tissue regeneration.

It has long been posited that the ideal biomaterial for tissue engineering would have identical properties to the tissue being replaced \(^{188,189}\), balanced by other factors including microstructure, degradation, cell adhesion, and inflammation \(^{190}\). This principle, termed “mechanical similarity,” has been particularly influential in bone tissue engineering, where a common design criterion is to match the properties of native bone, or at least enable physiologic loading without additional stabilization \(^{189,191}\).

Recently, we developed a model system to evaluate the role of *in vivo* mechanical loading on large bone defect regeneration \(^{192}\). In this model, a critically sized (8 mm)
bone defect is created in the rat femur, requiring treatment to induce healing. To stimulate bone formation, we evaluated delivery of rhBMP-2 using a non-structural alginate hydrogel which released the protein over a time-course of 21 days in vivo. We then tested the effects of in vivo loading and load timing on bone formation, tissue differentiation, and neovascularization by modifying the fixation plates to allow elective actuation of ambulatory load transfer through compliant fixation plates designed to constrain loading to axial compression. These studies found that limb fixation with the compliant plates implanted in the unlocked configuration at day 0 (i.e. early loading) prevented vascular ingrowth and inhibited bone formation while increasing cartilage formation; however, delaying load initiation to week 4, after onset of bone formation and initiation of defect bridging, significantly enhanced bone formation, biomechanical properties, and local tissue adaptation and remodeling.

The purpose of the present study was two-fold: first, to evaluate the influence of in vivo mechanical loading on large bone defect regeneration in the presence of a structural load-bearing scaffold capable of supporting and transmitting ambulatory loads to the defect, and second, to compare bone regeneration in the structural and non-structural constructs independent of mechanical stimulation. In Part 1, we tested the hypothesis that mechanical loading would enhance large bone defect regeneration in the presence of a tissue engineering scaffold featuring structural properties and microarchitectural features (i.e. porosity & anisotropy) similar to those of trabecular bone. In Part 2, we compared bone regeneration in the structural scaffolds, independent of mechanical loading, with non-structural hydrogel constructs composed of the same alginate hydrogel but without the structural scaffold support.
3.3 Methods

3.3.1 Scaffold production

We evaluated two bone tissue-engineering scaffolds selected for their microstructural, mechanical, and functional properties (Figure 3.1B). First, mechanically competent, structural scaffolds (PLDL) were fabricated through amorphous co-polymerization of poly(L-lactide) and poly(DL-lactide) with an L:DL ratio of 70:30 (PLDL; Purac America). PLDL pellets were mixed with 10% tricalcium phosphate (TCP) particles (<200nm diameter; Sigma Aldrich) and 30% azodicarbonamide (azo; Sigma Aldrich) as a heat-activated porogen. Porous scaffolds with combined longitudinal and random interconnected porosity were created, as described previously \(^{196,197}\), through layered deposition of PLDL-azo on removable longitudinal fibers followed by rapid porogen decomposition at 260°C \(^ {196}\). Cylindrical PLDL scaffolds were fabricated with 4 mm outer diameter and cut to 8 mm length, followed by punch removal of a 1.5 mm diameter core (Figure 3.1A, left). TCP particles were included to enhance cell adhesion and osteoconductivity of the PLDL scaffolds, and to buffer potential acidic degradation products of PLDL \(^ {196-198}\). The pores and inner core of each scaffold was infused with alginate hydrogel, described below, to create composite PLDL/ALG constructs.
Figure 3.1: Tissue engineering constructs and stiff and compliant fixation plate configurations. A) Structural PLDL/ALG scaffolds featured both random and axially-oriented porosity and 1.5 mm central core filled with RGD-functionalized alginate hydrogel containing rhBMP-2, while non-structural ALG constructs featured RGD-alginate and rhBMP-2, contained within a flexible perforated nanofiber mesh tube. Magnified images at left show porous scaffold architecture. Scale bars: 0.5 mm. In the ALG image at right, the alginate hydrogel is indicated in gray, while the perforated nanofiber mesh tube is shown in white. B) For in vivo mechanical loading, control limbs received stiff plates (left), while experimental limbs received compliant plates initially implanted in the locked configuration for 4 weeks (center) and were unlocked at week 4 through week 12 (right).

Second, non-structural hydrogel constructs (ALG) were fabricated using alginate hydrogel contained in electrospun nanofiber mesh tubes (Fig.3.1A, right). Briefly, 5M rad-irradiated, Arg-Gly-Asp (RGD)-functionalized alginate polysaccharide chains were reconstituted to final concentration of 2% w/v and cross-linked with 0.21 g/ml calcium sulfate (Sigma Aldrich) at a ratio of 25:1 by volume. Alginate hydrogels were injected in situ into electrospun poly(ε-caprolactone) (PCL) nanofiber mesh tubes, which
contained the hydrogel in the defect but did not provide axial structural support, as described previously 193,194,200. Briefly, PCL pellets (Sigma Aldrich) were dissolved in a 90:10 v/v solution of hexafluoropropanol:dimethyl formamide, and electrospun onto a static collector plate at a distance of 20 cm with a voltage potential of ~20 kV, resulting in a ~300 μm-thick mesh of randomly-oriented nanofibers.

In both scaffold systems, sustained delivery of recombinant human BMP-2 (R&D Systems) was achieved through release from alginate hydrogel 194. In the structural PLDL scaffolds, the pores and core regions were infused with alginate gel, while in the non-structural ALG constructs, crosslinked alginate was injected into non-structural nanofiber mesh tubes to contain the hydrogel in the defect 193,194. These alginate hydrogels, formed though ionic crosslinking of alginate polysaccharides, exhibit shear-reversible gelation, enabling injection 201. Samples in the alginate hydrogel group have been previously described in the dose-response study 194 and analysis was expanded to enable comparison.

3.3.2 Scaffold characterization

To evaluate the structural characteristics and mechanical behavior of the PLDL scaffolds, scaffolds were evaluated by microCT imaging (microCT40, Scanco Medical) prior to and following 8 week dynamic incubation in basal cell culture medium at 37°C in vitro to simulate effects of hydrolytic degradation in vivo (N = 6 per group). Samples were scanned at 21 μm resolution at a voltage of 55 kVp and 109 μA. A global threshold was applied to segment scaffold material, and applied consistently across samples. Porosity was defined as 1-SV/TV, where SV is the scaffold volume and TV is the total
volume enclosed by the scaffold, exclusive of the central cored region. Separate samples were tested in unconfined axial compression between flat plates to failure at 0.1 mm/s without and after in vitro degradation (N = 6 per group). The effective modulus for each sample was computed as the slope from a linear regression of the engineering stress (defined as current force divided by microCT-derived average cross-sectional referential area) vs. linearized strain curve within the linear range (Figure 3.2). The yield stress and strain were defined using the 0.2% offset approach. The ultimate stress was the maximum stress reached in the course of the test.

3.3.3 Surgical procedure and experimental design

Bilateral, critically-sized (8 mm) segmental defects were surgically created in femora of 13 week-old SASCO Sprague Dawley rats, as previously described 192,194,197. Prior to creation of the defect, limbs were stabilized by two types of custom fixation plates, described below, and implanted with two types of scaffolds containing rhBMP-2. The experimental design featured two studies, the first evaluating the influence of in vivo mechanical loading on large bone defect regeneration in the presence of a structural load-bearing scaffold, and the second comparing regeneration in the structural scaffolds with regeneration in non-structural hydrogel constructs, without mechanical loading (N = 9-10 per group). Post-surgery, animals were given subcutaneous injections of buprenorphine every 8 h for three days. All procedures were approved by the Institutional Animal Care and Use Committees (IACUC) of the University of Notre Dame (protocol #14-05-1778) and the Georgia Institute of Technology (protocol #A08032).
3.3.4 Fixation plates

To modulate the \textit{in vivo} mechanical loading conditions, limbs were stabilized using custom internal fixation plates capable of modulating ambulatory load transfer, enabling two levels of \textit{in vivo} loading, as characterized previously \cite{192}. Control limbs were stabilized by stiff plates (Fig. 3.1B, left) that allowed minimal transfer of axial, bending, or torsional loads \cite{192}. Experimental limbs received implantation of compliant fixation plates, which were initially implanted in the locked configuration (Fig. 3.1B, middle), preventing loading, but were unlocked at week 4 (Fig. 3.1B, right), allowing delayed axial load transfer, but maintaining a high stiffness to bending and torsional loads. \( N = 9-10 \) per group. Fixation plate mechanical behavior is controlled by plate geometry as well as composition, with compliance conferred by deformability against integrated elastomers (shown in Fig. 3.1B in blue). The stiff fixation plates featured an axial stiffness of 214 N/mm, while the stiffness of the unlocked compliant plates was \( \sim 8.4 \) N/mm \cite{192}.

3.3.5 Radiographs and microCT

Two-dimensional radiographs (Faxitron MX-20 Digital, Faxitron X-ray Corp.) were taken at 2, 4, 8, and 12 weeks post-surgery. Three blinded observers evaluated the bridging rate as a binary score, determined by the number of samples at each time point featuring continuous bone connectivity across the defect in standardized orientation X-ray images. Results were verified using post-mortem quantification of bone formation & morphology was evaluated by microCT (microCT40, Scanco Medical) at 21.0 \( \mu \)m voxel size at a voltage of 55 kVp and a current of 109 \( \mu \)A. Bone tissue was segmented by
application of a global threshold corresponding to 386 mg hydroxyapatite/cm$^3$, and a low-pass Gaussian filter (sigma: 0.8, support: 1) was used to suppress noise.

To quantitatively assess bone distribution, bone volumes were re-evaluated using a region of interest (ROI) analysis, featuring three ROIs (Fig. 3.5A). The 1.5 mm diameter core and cortex ROIs coincided with the hollow core and porous structure regions of the PLDL scaffolds, respectively (cf. Fig. 3.1B). The cortex region was contained by a 4 mm diameter circle, centered on the defect region, with the 1.5 mm core removed. The radiodensity of the TCP particles within the PLDL scaffolds was indistinguishable from that of physiologic mineral, and was not removed from these groups since the degree of degradation and release was unknown in vivo; however, the total mineral volume represented by the TCP particles prior to any degradation was no greater than 2.3 mm$^3$, contributed exclusively in the cortex region. The ectopic region included any bone formation outside the outer diameter of the scaffold/defect. In the second study, post-mortem scans of ALG samples from a prior study$^{194}$, which had previously been quantified only in vivo, precluding comparative analysis, were evaluated simultaneously with limbs receiving PLDL/ALG scaffolds, N = 8-9 per group.

3.3.6 Mechanical testing

Mechanical function was evaluated in torsion to failure as described previously$^{197}$. Briefly, limbs were potted in low-melting temperature ($T_m = 70^\circ$C) Wood’s metal, and tested in torsion to failure at a rate of 3.0$^\circ$/min. Torsional stiffness and ultimate torque were quantified. N = 8-9 per group.
3.3.7 Histology

One representative sample per group was selected based on average microCT-quantified bone volume for qualitative histology at week 12. Limbs were decalcified for 14 days in a formic acid-based decalciﬁer containing 10% neutral buffered formalin (Cal-ExII, Fisher Scientiﬁc), or 0.25M ethylenediaminetetraacetic acid (EDTA, Fisher Scientiﬁc) at pH 7.4, embedded in paraffin, sectioned to 4 μm in the sagittal plane, and stained with Haematoxylin and Eosin (H&E) and Safranin-O/Fast Green (Saf-O).

3.3.8 ELISA

BMP-2 release kinetics from ALG and PLDL/ALG scaffolds were evaluated by enzyme-linked immunosorbent assay (ELISA) in vitro. Scaffolds from each group (N = 3 per group) were loaded with 500ng rhBMP-2 per scaffold, and incubated in 37°C PBS for 21 days. Supernatant (1 ml) was collected and replaced at 1, 2, 4, 7, 10, 14, and 21 days, and frozen for simultaneous analysis. The BMP-2 amount was selected to facilitate detection within the linear range of the ELISA assay for the given supernatant volume. At day 21, the alginate in each group was dissolved by incubation in 2% (w/v) sodium citrate. BMP-2 concentration in the collected buffer at each time point and in the sodium citrate solutions were assessed using an ELISA kit (R&D Systems) according to manufacturer instructions.

3.3.9 Statistical analysis

Summary statistics are presented as mean ± standard deviation or median with boxes indicating interquartile range and whiskers at 5th and 95th percentiles, as indicated. Multiple group comparisons were analyzed by analysis of variance (ANOVA), with
pairwise post-hoc comparisons by Tukey’s multiple comparisons test. Comparisons between two groups were assessed using unpaired, two-tailed Student’s t-tests. Normality and homoscedasticity were verified by D’Agostino-Pearson omnibus normality test and Brown-Forsythe tests, respectively. Bridging trends within groups were assessed using the Chi square test for trend, with comparisons between groups by Chi square test, with Bonferroni corrections for multiple comparisons.

3.4 Results Part 1: Effects of mechanical loading on BMP-2-mediated bone regeneration in structural PLDL/ALG scaffolds

First, we evaluated the effects of in vivo mechanical loading on large bone defect regeneration in structural, load-bearing scaffolds. Structural PLDL scaffolds were infused, including the cored center region, with alginate hydrogel containing a total of three micrograms rhBMP-2, and implanted in bone defects stabilized by either stiff fixation plates that allowed minimal load sharing, or axially compliant fixation plates, initially implanted in a locked configuration to prevent load transfer, but unlocked at week 4 to initiate ambulatory load transfer. We assessed bone regeneration over 12 weeks, allowing 8 weeks of loading in the compliant plate group.

3.4.1 Structural PLDL scaffold properties

MicroCT scanning and mechanical testing were performed to characterize the architectural and mechanical properties of the PLDL scaffolds, prior to and after in vitro hydrolytic degradation for 8 weeks. Scaffolds had initial porosity of 73.3 ± 2.3% and effective modulus of 20.3 ± 4.1 MPa. Following 8 weeks in vitro degradation, there were
no gross morphometric changes in either scaffold architecture (Fig. 3.2A-D) or elastic behavior (Fig. 3.2E); however, *in vitro* degradation significantly altered the failure properties, reducing yield strain by 57% (Fig. 3.2F), yield stress by 46% (Fig. 3.2G) and ultimate stress by 41% (Fig. 3.2H). These changes in material properties are illustrated in representative stress-strain curves for PLDL samples pre- and post-degradation (Figure 3.2I).
Figure 3.2: Structural and mechanical properties of PLDL scaffolds prior to and after eight weeks dynamic culture for degradation *in vitro*. Degradation had no effect on structural morphology, as measured by porosity (A), strut thickness (B), strut spacing (C), or connectivity (D) and did not alter the effective compressive modulus (E), but significantly reduced 0.2% offset yield strain (F) and stress (G) and ultimate stress (H). Representative stress-strain curves for scaffolds pre- and post-degradation (I) illustrate mechanical property changes. All data points plotted with mean ± s.d. * p < 0.05, unpaired Student’s t-test. N = 6 per group.
3.4.2 Radiographic bridging

Radiographic assessment of bony bridging at weeks 4, 8, and 12 found no significant differences in rate or incidence of bridging between loading conditions at any time point (Fig. 3.3A,B). At week 4, the time point of compliant plate actuation, the stiff and compliant plate groups achieved bridging incidence of 30 and 10%, respectively, but were not statistically distinguishable. Both groups plateaued at a bridging incidence of 40% by weeks 8-12.

Figure 3.3: Effects of mechanical loading on bone regeneration in structural PLDL/ALG scaffolds. Delayed in vivo loading had no effect on defect bridging (A,B), bone formation (C,D), or torsional mechanical properties at week 12 (E,F). Binary bridging scores (bridged - B or not bridged - NB) are indicated on representative radiographs. Box plots show 25th, and 75th percentiles, with whiskers at 5th and 95th percentiles, respectively. Mean values indicated by +. NS: p > 0.05, Student’s t-test. N = 9-10 per group.
3.4.3 Bone formation

Post mortem microCT evaluation of bone formation revealed no differences between stiff or delayed loading groups for total bone volume within the defect ($p = 0.88$, $\beta = 0.94$; Figure 3.3C,D). In addition, a similar pattern of bone deposition was observed for the two groups, with bone formation concentrated in the core region and around the periphery of the scaffold (Fig. 3.3D).

3.4.4 Biomechanics

Torsion testing of excised limbs to failure revealed no significant differences in torsional stiffness or maximum torque to failure between stiff and compliant plate groups, indicating no differences in functional regeneration ($p = 0.34$, $\beta = 0.83$ and $p = 0.54$, $\beta = 0.75$, respectively; Fig. 3.3E,F).

3.5 Results Part 2: Influence of structural scaffold on bone regeneration induced by alginate-mediated BMP-2 delivery.

To evaluate the effect of the structural scaffold on bone regeneration without mechanical loading, regeneration in the structural scaffolds was compared to non-structural alginate constructs (ALG) in which the alginate hydrogel was contained in the defect by a perforated, non-structural nanofiber mesh, described previously \(^{193,194}\). The samples from the 2.5 $\mu$g rhBMP-2 group in a prior dose-response study for the ALG group \(^{194}\) were re-evaluated for comparison with the PLDL/ALG samples from Part 1.
3.5.1 Radiographic bridging

PLDL/ALG constructs had a significantly lower bridging rate compared to ALG at weeks 8 and 12 (p < 0.05, Chi square test), with the ALG group exhibiting a significantly positive bridging rate over time (p < 0.001, Chi square test for trend) to a maximum of 80% at week 12 (Fig. 3.4A,B).
Figure 3.4: Comparison of bone formation in PLDL/ALG and ALG constructs independent of mechanical loading. ALG constructs exhibited a significantly increasing trend in bridging rate over time (Chi Square test for trend, p<0.01), while PLDL/ALG did not (p > 0.73) (A). Comparison of bridging rates between groups at week 12 was statistically significant (p < 0.05). Bridging rates were evaluated from 2D radiographs, shown at weeks 4, 8, and 12 with bridging scores (bridged - B or not bridged - NB) as indicated (B). ALG constructs had significantly greater bone volume (C), illustrated by high-resolution post-mortem microCT reconstructions (D). Box plots show 25\textsuperscript{th}, and 75\textsuperscript{th} percentiles, with whiskers at 5\textsuperscript{th} and 95\textsuperscript{th} percentiles, respectively. Mean values indicated by +. **** p < 0.0001, unpaired Student’s t-test. N = 6-19 per group.
3.5.2 Bone formation

Post-mortem microCT evaluation revealed a significant 77% lower bone volume in the PLDL/ALG group compared to ALG (Fig. 3.4C,D). When observing representative images, bone formation in ALG samples appeared more evenly distributed throughout the defect compared to the PLDL/ALG group (Fig. 3.4D).

3.5.3 Bone distribution

Volumetric region of interest analysis (Fig. 3.5A) indicated the ALG group exhibited a significantly higher bone volume in both the ectopic and cortex regions, but not in the core region compared to the PLDL/ALG group (Fig. 3.5B,C). To enable direct comparison of bone distribution within each scaffold type, the bone volume fraction (BV/TV) was computed in each region (Fig. 3.5D). There were no significant differences in BV/TV between regions for ALG scaffolds, while PLDL/ALG scaffolds had significantly higher BV/TV in the ectopic and core regions compared to the cortex, in which the scaffold itself was located. In addition, bone volume fraction was lower in the PLDL/ALG group in all regions except the ectopic region.
Figure 3.5: Region of interest analysis of bone distribution. Bone formation was evaluated in three regions of interest defined by the inner core region, the cortex region, which was coincident with the annular PLDL scaffold, and the ectopic region, respectively (A). Bone formation in the PLDL/ALG and ALG groups was evaluated in each region (B) and quantified for total bone volume (C) and bone volume fraction, BV/TV (D). Comparison of BV and BV/TV among and between groups was performed by two-way ANOVA with Tukey’s multiple comparisons test. Box plots show 25th, and 75th percentiles, with whiskers at 5th and 95th percentiles, respectively. Mean values indicated by +. Letters shared in common between or among groups indicate no significant difference. N = 6-19 per group.
3.5.4 Biomechanics

Post-mortem mechanical testing revealed a significantly greater torsional stiffness and maximum torque at failure in the ALG group compared to PLDL/ALG (Fig. 3.6).

![Graph showing biomechanical testing results](image)

**Figure 3.6:** Functional regeneration assessed by mechanical testing in torsion to failure. Torsional stiffness (A) and maximum torque at failure (B) were significantly greater in the ALG group (* p < 0.05, unpaired Student’s t-test), though both were lower than the properties of intact limbs (0.030 ± 0.001 N-m/deg and 0.31 ± 002 N-m, respectively) \(^{192}\). Box plots show 25\(^{th}\) and 75\(^{th}\) percentiles, with whiskers at 5\(^{th}\) and 95\(^{th}\) percentiles, respectively. Mean values indicated by +. N = 8-19 per group.

3.5.5 Histology

Histological evaluation of tissue formation was evaluated by Haematoxylin and Eosin (H&E) and Safranin-O/Fast Green staining at week 12 post surgery. Bone formation was present throughout the defect in the ALG group, forming appositionally on and around particles of alginate hydrogel (Fig. 3.7). In the PLDL/ALG group, bone formation was predominantly localized to the core and ectopic regions, with significant remaining PLDL scaffold evident in the cortex region (Fig. 3.7). Interestingly, substantial
amounts of cartilage formation (c) were present in the PLDL/ALG group, frequently adjacent to the scaffold struts, indicated by presence of chondrocytes and staining for negatively charged glycosaminoglycans by Safranin-O. Both groups exhibited residual alginate in the defect at week 12 post-surgery.

Figure 3.7: Histological comparison PLDL/ALG and ALG-mediated regeneration at week 12. Haematoxylin and Eosin (H&E) (A) and Safranin-O/Fast Green (B) staining showing bone formation (b), cartilage formation (c), alginate hydrogel (a), PLDL scaffold (p), nanofiber mesh (m). Scale bars: top row - 250 μm, all others - 50 μm.

3.5.6 BMP-2 release kinetics

*In vitro* BMP-2 release kinetics were similar for both groups, exhibiting a total release of ~40ng over 28 days with a half-life of release of 6.8 ± 2.8 and 3.9 ± 2.1 days (mean ± std. dev.) for ALG and PLDL/ALG groups, respectively (Fig. 3.8). Neither release rate nor total release was significantly different (p > 0.05) between groups. The amount of BMP-2 left in each scaffold at day 28 was 41.4 ± 21.9 and 71.1 ± 20.0 ng
(mean ± std. dev.) in ALG and PLDL/ALG groups, respectively. Differences in remaining protein were not significant (unpaired Student’s t-test, p > 0.05).

Figure 3.8: BMP-2 release in vitro. Cumulative release data (A) were fit to a one-phase exponential curve, and the half-life of protein release was calculated: 6.8 ± 2.8 and 3.9 ± 2.1 days for ALG and PLDL/ALG groups, respectively. The remaining BMP-2 retained in the constructs was evaluated after alginate dissolution in sodium citrate at day 21. (B) There were no differences in release kinetics, total release, or protein retention between groups (Student’s t-test, p > 0.05). Error bars in (A) indicate standard deviations. All data points shown with nonlinear regression to exponential decay function (A) or mean ± s.d. (B). N = 3 per group.

3.6 Discussion

Tissue engineering scaffolds for bone regeneration are frequently designed to match, as closely as possible, the mechanical properties of the native tissue. Particularly in bone, structural scaffolds are attractive for load-bearing bone defects to enable load sharing between the construct and fixation hardware to promote functional use as soon as possible. However, if the fixation system is sufficiently durable to withstand in vivo loading, this constraint may not be necessary, allowing use of either
structural or non-structural materials as graft substitutes. This presents the question of which are preferable for mechanical load-induced bone regeneration: structural scaffolds that can bear physiologic loads or non-structural scaffolds that enable rapid replacement with newly-formed tissue.

This study tested the hypothesis that mechanical loading would enhance bone regeneration in the presence of a structural, load-bearing scaffold, similar to our prior observations on mechanical regulation of bone regeneration in non-load-bearing hydrogel constructs. In this experiment, we replicated the loading time-course that had a positive effect on bone regeneration in the hydrogel system: delayed loading with compliant plates unlocked at week 4 post-implantation. Contrary to the hypothesis, delayed in vivo mechanical loading had no effect on bone formation, distribution, or functional regeneration at week 12 in the presence of a structural scaffold. Slight differences in bridging incidence were apparent at week 4 but these were not statistically significant and fell within the range of experimental variability typical of this model.

One rationale for using load-bearing scaffolds in combination with mechanical loading is that the scaffolds may promote transfer of stimulatory loads to in-growing bone prior to complete defect bridging. However, as loading was not effective under these conditions, the high stiffness of the scaffold may have contributed a stress-shielding effect, sheltering the ingrowing tissue from stimulation. This is supported by the observation that the elastic properties of the scaffolds within the range of expected loads in vivo did not change by in vitro degradation, suggesting the scaffolds maintained mechanical integrity and load-bearing, and were not functionally replaced with new bone. The axial load amplitude in vivo in the compliant plate group was previously calculated at
approximately 3N\textsuperscript{195}, which corresponds to a maximum effective interfragmentary strain amplitude of 0.6\% in the scaffolds, assuming no tissue ingrowth, indicating the scaffolds remained within the elastic range \textit{in vivo}.

A potential limitation of the bilateral defect model for studies of the influence of mechanics is the possibility of differential weight-bearing between limbs since this is essential for ambulatory mechanical stimulation in this model. While we did not directly measure differential weight bearing in this experiment, several lines of evidence from our prior and ongoing studies suggest that bilateral weight bearing is uniform and sufficient for mechanical stimulation\textsuperscript{195,203,204}. First, we previously performed a quantitative dynamic gait analysis on animals receiving unilateral defects and found no significant difference in either duty cycle or paw print area compared to contralateral unoperated limbs, suggesting that the bone defect surgery and fixation do not induce preferential limb unloading\textsuperscript{203,204}. Second, we have observed consistent enhancement of bone regeneration by delayed mechanical loading in multiple studies\textsuperscript{162,195}. Finally, recent data on paired stiff-compliant or compliant-compliant limbs do not exhibit differential responses (data not shown).

In the second part of the study, we compared bone regeneration in the structural scaffolds with the non-structural hydrogel constructs\textsuperscript{162,194,195}, independent of mechanical loading. In this analysis, the presence of the structural scaffold significantly reduced bone formation and functional mechanical properties and altered the distribution of new bone in the defect. Specifically, bone formation was reduced in the core and cortex regions, but was not significantly different in the ectopic region. Bone formed least in the cortex region, which was coincident with the annular scaffold, and the difference in bone
volume fraction compared to the ectopic region (0.28) was identical to the average post-degradation volume fraction of that region which contained the scaffold (i.e. 72% scaffold porosity). Consistently, while the core region exhibited lower bone volume than the cortex region, the core also had a significantly higher volume fraction than the cortex region. Compared to the ALG group, however, the bone volume fraction in both the core and cortex regions were significantly lower than just the amount of space occupied by the scaffold. Taken together, these data indicate that the scaffold itself reduced bone formation in the defect by occupying potential space and impeding bone growth into the central core from the periphery. A limitation of this analysis is that the BMP-2 dose delivered in the PLDL/ALG group was 3.0 μg, while it was 2.5 μg in the ALG group. However, the bone formation response was significantly greater in the ALG group, which received the lower dose. Further, these doses are in the middle of the range of doses we have evaluated, exhibiting a clear positive dose response between 0 and 5 μg BMP-2 194, and similar spatial distribution patterns at both 2.5 μg (Fig. 3.5) and 5 μg BMP-2 200. Therefore, since we would have expected an even greater response due to dose in the PLDL/ALG group, this further supports the stated conclusions. The sub-saturation dose of BMP-2 was selected to induce bone regeneration, but enable evaluation of potential regeneration enhancement by mechanical loading, as described previously 162.

In addition to stress shielding and structural interference, it is possible that PLDL scaffold degradation influenced bone formation. The poly(lactic acid) polymers are known to produce acidic byproducts during hydrolytic degradation, which may adversely affect osteogenesis through an enhanced inflammatory response 205. However, the slow degradation rate of the PLDL co-polymer formulation selected has been shown to limit
local acidic concentration\textsuperscript{205-207}, and low w/v tricalcium phosphate microparticles were included to buffer the degradation products\textsuperscript{198}. The poly(lactic acid) polymers are used extensively as delivery vehicles for growth factors, including BMP-2, and have been shown maintain BMP-2 activity and osteoinductivity\textsuperscript{208,209} and promote new bone growth\textsuperscript{209,210}. In the present study, histological evaluation revealed cartilage formation adjacent to PLDL scaffold struts, which were not present in the ALG group, suggesting a potential effect of the scaffold on local tissue hypoxia.

Structural scaffold inclusion did not affect protein delivery kinetics \textit{in vitro}. Though \textit{in vitro} and \textit{in vivo} release rates are likely to differ, and we did not evaluate the effects of mechanical loading on protein release\textsuperscript{211}, this data suggests that the effect of the scaffold on load-mediated bone formation was primarily through scaffold structural properties and not through interference of inductive protein presentation. As is frequently observed with the supernatant ELISA assay, the total protein release measured did not add up to the amount initially loaded as some is left in the scaffold (measured at 10-15\% in both groups), some is degraded and lost prior to detection, and some may not be detected by the assay. Together, with differences between \textit{in vitro} conditions and those \textit{in vivo}, these data suggest the \textit{in vivo} release kinetics may be underestimated by the \textit{in vitro} assay. Regardless, the differences between groups can be compared \textit{in vitro}.

The observations that intrinsic matrix mechanical properties can alter progenitor cell recruitment and fate independent of biochemical cues\textsuperscript{175} have heightened attention on scaffold mechanical properties, not only for mechanical similarity\textsuperscript{188}, but also of the microenvironmental mechanical signals presented to delivered or endogenous cells\textsuperscript{176,177}. For example, the concept of durotaxis, or cellular motility driven by gradients in matrix
rigidity, has been proposed as an explanation for the ability of MSCs to home to sites of injury \(^{212}\). Similarly, MSC lineage specification may be controlled by matrix rigidity, with preferential differentiation toward adipogenic and osteogenic lineages on soft and stiff substrates, respectively \(^{175}\). Recently, Huebsch et al. demonstrated that alginate matrix rigidity controlled bone regeneration \textit{in vivo}, with an optimal elasticity of approximately 60 kPa (bulk modulus) \(^{176}\). Thus, seeding progenitor cells onto the scaffold prior to implantation may enable local transmission of mechanical signals throughout the defect and enhance mechanosensitivity; however, the length scales governing cellular mechanotransduction differ from those of bulk properties, and elucidating the direct influence of heterogeneous scaffold properties and architecture on endogenous and exogenously-supplied cell behavior will require continued research.

Given the longitudinal orientation of the porosity and high stiffness of the PLDL scaffolds, we initially hypothesized that these would enhance cellular recruitment and osteogenic differentiation compared to the relatively soft hydrogel constructs. However, migration through the interstitial alginate, functionalized with RGD peptides, appeared to be the primary means of cellular infiltration, which may have minimized differences in mechanical signals between groups. Further, the primarily longitudinal orientation of the scaffold porosity may also have reduced the ability of cells to migrate in from the surrounding tissues. This is particularly critical for neovascularization, which we found previously is controlled by mechanical loading, and invades primarily radially from the surrounding muscle \(^{162}\).

The optimal extrinsic mechanical conditions for large bone defect regeneration remain unknown, and both the delayed loading approach described here \(^{162,192,195}\), and a
reverse dynamization approach in which moderate stiffness fixators were replaced by stiff fixators after two weeks \(^{111}\) have been shown to enhance regeneration. Consistent with the present observations, in each of these systems in which loading enhanced bone formation, BMP-2 was delivered in non-structural constructs (alginate or collagen sponge). In our previous study \(^{162}\), we found that large deformations associated with early loading inhibited neovascular ingrowth and bone formation; however, future studies will be required to test whether early loading of structural scaffolds would exhibit different behavior, as the scaffold could limit interfragmentary strains in the early stages of healing. Similarly, while excessively stiff scaffolds may inhibit load-induced regeneration, scaffolds with moderate elastic moduli may instead facilitate mechanical stimulation through both intrinsic and extrinsic cues.

3.7 Conclusions

Collectively, these observations suggest that structural scaffolds can impede the beneficial effect of mechanical loading by stress shielding and inhibition of cellular infiltration and tissue ingrowth. This suggests that the common design constraint that tissue engineering scaffolds possess similar mechanical properties to the replaced tissue is not requisite for load-bearing tissues, given appropriate fixation conditions. These data instead emphasize the importance of recreating an adequate environment to enable cells to activate matrix production and achieve ultimate functional regeneration. Indeed, like early fracture healing, optimal matrix properties at the onset of tissue regeneration may be much lower than those of the fully regenerated tissue; however, further studies will be
required to evaluate whether the stress-shielding effects of load-bearing scaffolds inhibit mechanical stimulation of bone regeneration regardless of scaffold composition.
CHAPTER 4:
RECAPITULATING BONE DEVELOPMENT THROUGH ENGINEERED MESENCHYMAL CONDENSATIONS AND MECHANICAL CUES FOR TISSUE REGENERATION

4.1 Abstract

Large bone defects cannot form a callus and exhibit high complication rates even with the best treatment strategies available. Tissue engineering approaches often use scaffolds designed to match the properties of mature bone. However, natural fracture healing is most efficient when it recapitulates development, forming bone via a cartilage intermediate (endochondral ossification). Because mechanical forces are critical for proper endochondral bone development and fracture repair, we hypothesized that mechanical forces would also be essential to recapitulate this process for large bone defect regeneration in rats. Here, we engineered mesenchymal condensations that mimic the cellular organization and lineage progression of the early limb bud in response to local transforming growth factor (TGF-β1) presentation from incorporated gelatin microspheres. We then controlled mechanical loading \textit{in vivo} by dynamically tuning

\footnote{This chapter is currently in press for publication: Science Translational Medicine. Authors: Anna M. McDermott, Samuel Herberg, Devon E. Mason, Joseph M. Collins, Hope B. Pearson, James H. Dawahare, Rui Tang, Amit N. Patwa, Mark W. Grinstaff, Daniel J. Kelly, Eben Alsberg, Joel D. Boerckel}
fixator compliance. Mechanical loading enhanced mesenchymal condensation-induced endochondral bone formation in vivo, restoring functional bone properties when load initiation was delayed to week four after defect formation. Live cell transplantation produced zonal human cartilage and primary spongiosa mimetic of the native growth plate, whereas condensation devitalization prior to transplantation abrogated bone formation. Mechanical loading induced regeneration comparable to high-dose BMP-2 delivery, but without heterotopic bone formation and with order-of-magnitude greater mechanosensitivity. In vitro, mechanical loading promoted chondrogenesis and upregulated pericellular matrix deposition and angiogenic gene expression. In vivo, mechanical loading regulated cartilage formation and neovascular invasion, dependent on load timing. This study establishes mechanical cues as key regulators of endochondral bone defect regeneration and provides a paradigm for recapitulating developmental programs for tissue engineering.

4.2 Introduction

Bone fractures are among the most common traumatic injuries, but regularly heal efficiently (90-95% success rate) by forming a stabilizing cartilage callus that remodels to bone, a process called endochondral ossification 133. Conversely, critically-sized bone defects (exceeding 3 cm) cannot form a callus, do not heal without surgical intervention, and often result in life-long disability for the patient. The clinical gold standard for repairing large bone defects is currently autograft bone transplantation from the iliac crest, but it is limited by the amount of source material available as well as donor site morbidity 213. To overcome these limitations, recombinant human bone morphogenetic
protein-2 (BMP-2) is administered via collagen sponge, but large doses of this growth factor can cause harmful heterotopic bone formation and soft tissue inflammation, and its continued clinical use is under re-evaluation\textsuperscript{194}. Despite these treatment options, long-term outcomes exhibit high rates of failure and complications, and many patients report that their quality of life after such interventions is no better than amputation\textsuperscript{8}. Thus, there remains a need for alternative graft materials that require little or easily obtainable donor material, remain localized to the defect region, and successfully vascularize and integrate for functional regeneration of large bone defects.

A common tissue engineering approach to this problem features osteoprogenitor cell and/or osteoinductive agent delivery using scaffolds designed to mimic the structure and properties of mature bone tissue\textsuperscript{188}. However, natural bone healing achieves highly efficient functional repair by re-activating developmental programs, producing new bone through endochondral ossification\textsuperscript{16,20}. Here, we sought to recapitulate the cellular, morphogenic, and mechanical cues present during bone development for regeneration of large bone defects.

Long bone morphogenesis is initiated by condensation of mesenchymal cells in the early limb bud, which differentiate and mature into the cartilaginous anlage that gives rise to endochondral bone formation. This process is dependent on both local morphogen gradients and mechanical forces \textit{in utero}\textsuperscript{20,214}. Natural bone fracture healing recapitulates this developmental program, but only under mechanical conditions of axial interfragmentary motion (i.e., cyclic compression at the fracture site)\textsuperscript{23,25,171}. In the absence of interfragmentary strain, fractures will heal through direct intramembranous bone formation; conversely, excessive motion or instability can induce non-union\textsuperscript{215}. 

56
Thus, mechanical cues are critical regulators of endochondral ossification. The emerging paradigm of biomimetic bone tissue engineering aims to replicate the endochondral process\textsuperscript{28,216}, but functional endochondral bone regeneration using transplanted human progenitor cells remains elusive, potentially due to insufficient recapitulation of the essential cellular, biochemical, and mechanical stimuli.

Here we evaluated the effects of in vivo mechanical loading on endochondral bone regeneration mediated by engineered mesenchymal condensations with local morphogen presentation. Self-assembled, scaffold-free human bone marrow-derived mesenchymal condensations containing transforming growth factor-\(\beta\)1 (TGF-\(\beta\)1)-releasing gelatin microspheres\textsuperscript{53,217} were transplanted into critical-sized bone defects in rat femora, and in vivo mechanical loading was controlled by dynamic modulation of fixation plate compliance\textsuperscript{162,192}. Mechanical loading enhanced mesenchymal condensation-induced endochondral bone formation. Mechanistically, loading regulated chondrogenesis and pericellular matrix formation, and controlled cartilage persistence and neovascularization in vivo, dependent on load timing. Together, these data demonstrate the importance of mechanical cues for mimicking development and natural repair for tissue engineering.

4.3 Materials and Methods

4.3.1 Study design

The main experimental design featured an 8-mm critically-size rat femoral defect, each defect receiving three high-density hMSC sheets (total of \(6 \times 10^6\) cells and 1.8 \(\mu\)g
TGF-β1) contained within an electrospun, perforated poly-(ε-caprolactone) (PCL) nanofiber mesh tube, under 3 loading conditions. Control limbs (stiff) were stabilized with fixation plates that limited load transfer. Early loading limbs were stabilized by axially compliant fixation plates that allowed load transfer immediately upon implantation (early). Delayed loading limbs were stabilized by the same compliant plates implanted initially in a locked configuration to prevent loading, until surgical unlocking after 4 weeks to enable load transfer (delayed). Sample sizes were determined by power analyses ($\alpha = 0.05$, $\beta = 0.2$), computed using effect sizes and variability described in prior studies. Samples were assigned to animals to balance pairing of stiff, early, and delayed loading groups between limbs.

In additional animals, hMSC sheets were devitalized by 3 freeze/thaw cycles, combined in a PCL mesh tube, and placed into segmental defects under stiff fixation. As a clinical comparison, another set of animals were treated with recombinant human BMP-2 (5 µg, rhBMP-2, R&D Systems) delivered in an absorbable type I collagen sponge (DSM) under stiff fixation. These were performed at a separate time from the main study and were therefore not included in quantitative statistical comparisons.

4.3.2 hMSC isolation and expansion

Human mesenchymal stem cells (hMSCs) were derived from the posterior iliac crest of a healthy male donor (41 years of age) using a protocol approved by the University Hospitals of Cleveland Institutional Review Board. Cells were isolated using a Percoll gradient (Sigma-Aldrich, St. Louis, MO) and cultured in low-glucose Dulbecco’s modified Eagle’s medium (DMEM-LG; Sigma-Aldrich, St. Louis, MO) containing 10%
fetal bovine serum (FBS; Sigma-Aldrich), 1% penicillin/streptomycin (P/S; Fisher Scientific), and 10 ng/ml fibroblast growth factor-2 (FGF-2, R&D Systems, Minneapolis, MN) \(^{53,217,218}\). Cells were verified to be negative for mycoplasma contamination during expansion, prior to \textit{in vivo} implantation.

4.3.3 Gelatin microsphere synthesis and TGF-β1 loading

Gelatin microspheres (GM) \(^{52,218,219}\) were synthesized from 11.1% (w/v) gelatin type A (Sigma-Aldrich) using a water-in-oil single emulsion technique and crosslinked for 4 h with 1% (w/v) genipin (Wako USA, Richmond, VA) \(^ {220}\). Hydrated GM were light blue in color and spherical in shape with an average diameter of 52.9±40.2 μm and a crosslinking density of 25.5 ± 7.0%. Growth factor-loaded microspheres were prepared by soaking crosslinked, UV-sterilized GM in 80 μg/ml solution of rhTGF-β1 (Peprotech, Rocky Hill, NJ) in phosphate buffered saline (PBS) for 2 h at 37°C. Unloaded microspheres without growth factor were hydrated similarly using only PBS. Sheet thickness was quantified by FIJI (Image J) on transverse sections.

4.3.4 Microsphere-incorporated hMSC sheet preparation

Expanded hMSCs (2 × 10^6 cells/sheet; passage 4) were mixed with TGF-β1-loaded GM (400 ng/mg; 1.5 mg/sheet) in chemically defined medium [DMEM-HG (Sigma-Aldrich), 1% ITS⁺ Premix (Corning), 1 mM sodium pyruvate (HyClone), 100 μM non-essential amino acids (Lonza), 100 nM dexamethasone (MP Biomedicals, Solon, OH), 0.05 mM L-ascorbic acid-2-phosphate (Wako), and 1% P/S (Fisher Scientific)]. Five hundred microliter of the suspension were seeded onto the pre-wetted membrane of Transwell inserts (3-μm pore size, 12-mm diameter; Corning) and allowed to self-
assemble for 2 days. After 24 h, medium in the lower compartment was replaced. Control sheets containing unloaded GM were prepared and cultured in a similar fashion. After 48 h, hMSC sheets were harvested for implantation.

4.3.5 Nanofiber mesh production

Nanofiber meshes were formed by dissolving 12% (w/v) poly-(ε-caprolactone) (PCL; Sigma-Aldrich) in 90/10 (v/v) hexafluoro-2-propanol/dimethylformamide (Sigma-Aldrich). The solution was electrospun at a rate of 0.75 ml/h onto a static aluminum collector. 9 × 20 mm sheets were cut from the product, perforated with a 1 mm biopsy punch (VWR, Radnor, PA), and glued into tubes around a 4.5 mm mandrel with UV glue (Dymax, Torrington, CT). Meshes were sterilized by 100% ethanol evaporation over ~15 hours under UV light overnight and washed 3x with sterile PBS before implantation.

4.3.6 Surgical procedure

Critical-sized (8-mm) bilateral segmental defects were created in the femora of 14 week-old male Rowett nude (RNU) rats (Charles River Labs, Wilmington, MA) under isoflurane anesthesia. Limbs were stabilized by custom internal fixation plates that allow controlled transfer of ambulatory loads in vivo and secured to the femur by four bi-cortical miniature screws (J.I. Morris Co, Southbridge, MA). Animals were given subcutaneous injections of 0.04 mg/kg buprenorphine every 8 h for the first 48 h postoperative (post-op) and 0.013 mg/kg every 8 h for the following 24 h. All procedures were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Notre Dame (Protocol No. 14-05-1778).
4.3.7 Fixation plate mechanical characterization

Characterization of the axial, torsional, and flexural plate stiffness was performed by screwing the plate onto two stainless steel 3.9-mm diameter rods through the tapped holes in the plate. Axial tests were conducted in both the fixed-fixed and fixed-free configurations on all three plate configurations with a control rate of 0.02 mm/s to a displacement of 1.2 mm for the unlocked compliant and 0.005 mm/s to a displacement of 0.2 mm for stiff and locked compliant. Torsional tests were conducted with a control rate of 0.1 deg/s to a displacement of 5 deg. Four-point bending tests were conducted using 3.9-mm square rods in the convex, concave, and side orientations on the three plate configurations with a control rate of 0.05 mm/s to a displacement of 2 mm for the unlocked compliant and 0.05 mm/s to a displacement of 1.5 mm for stiff and locked compliant. The stiffness under each loading condition was calculated as the slope of the linear region of the load-displacement curves.

4.3.8 In vivo X-ray and microCT

In vivo X-rays and microCT scans were performed at 4, 8, and 12 weeks to determine bridging and assess bone volume of the defect respectively. In vivo CT scans were performed on an Albira Si imaging system (Bruker, Billerica, MA) at 45 kVp, 0.4 mA, with best resolution (125 μm voxel size). 45 slices were analyzed in the center of each defect with a global threshold of 400 to determine bone volume. For the group treated with rhBMP-2/absorbable collagen scaffold, microCT analysis was performed using a Scanco µCT 80 system (Scanco Medical, Bassersdorf, Switzerland) at 70 kVp, 114 μA, at a resolution of 39 μm/voxel. For this group, 144 slices (144 slices × 39
\( \mu m/\text{voxel} = 5.616 \text{ mm} \) were analyzed in the center of each defect with a global threshold of 270. X-rays were taken using an Xtreme scanner (Bruker) at 45 kVp, 0.4 mA, with 2 second exposure time. A binary bridging was score was assigned by two independent, blinded observers, and determined as mineralized tissue fully traversing the defect.

4.3.9 Biomechanical testing

Femora excised at 12 weeks were biomechanically tested in torsion to failure. Limbs were cleaned of soft tissue and the fixation plate was carefully removed. Bone ends were potted in Wood’s metal (Alfa Aesar), mounted on a Bose ElectroForce biaxial load frame system (ELF 330, Bose EnduraTEC) and tested to failure at a rate of 3 degrees per second. For each sample maximum torque at failure was recorded and torsional stiffness was determined as the slope of a 5 degree linear region in the torque-rotation curve. Samples were compared to 7 age matched, un-operated femurs.

4.3.10 Fibrin gel preparation and dynamic compression

Expanded hMSCs (passage 4) were resuspended in a final hydrogel solution of 50 mg/mL fibrinogen, 2.5 U/mL thrombin. Gel was pipetted into 5mm diameter x 2mm thickness cylindrical agarose molds to create uniform constructs with a total cell volume of approximately 600,000 each. Culture was maintained in chondrogenic media where fresh media was supplied every 3 days. Dynamic unconfined compressive loading was applied to the constructs using a custom-made bioreactor. Load was applied 2 hours per day, 5 days a week at 1Hz and 10% strain after a .01N preload was applied. Load was applied either continuously for 5 weeks (early), for 2 weeks following a 3 week free
swelling period (delayed), or for 2 weeks prior to a 3 week free swelling period (reversed), in comparison to 5 week free swelling controls.

4.3.11 Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis

hMSC sheet halves and fibrin hydrogels were homogenized in TRI Reagent (Sigma-Aldrich) for subsequent total RNA extraction and cDNA synthesis (PrimeScript 1st strand cDNA Synthesis Kit; Takara Bio Inc., Kusatsu, Shiga, Japan). One hundred nanograms of cDNA were amplified in duplicates in each 40-cycle reaction using a Mastercycler (Eppendorf, Hauppauge, NY) with annealing temperature set at 60°C, SYBR® Premix Ex Taq™ II (Takara), and custom-designed qRT-PCR primers (Table 4.1; Life Technologies, Grand Island, NY). Transcript expression was normalized to GAPDH and gene expression was calculated as fold change using the comparative Ct method \(^{221}\).
### TABLE 4.1

OLIGONUCLEOTIDE PRIMER SEQUENCES FOR QRT-PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence (5’-3’)</th>
<th>Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOX9</td>
<td>Fwd CACACAGCTCACTCGACCTTG</td>
<td>NM_000346.3</td>
</tr>
<tr>
<td></td>
<td>Rev TTCGGTTATTTTTAGGATCATCTCG</td>
<td></td>
</tr>
<tr>
<td>ACAN</td>
<td>Fwd TCGGGGTCACACAGTGCCTATC</td>
<td>NM_001135.3</td>
</tr>
<tr>
<td></td>
<td>Rev CACGATGCCTTTCACCACGAC</td>
<td></td>
</tr>
<tr>
<td>COL2A1</td>
<td>Fwd GGAAACTTTTGCTGCCAGATG</td>
<td>NM_001844.4</td>
</tr>
<tr>
<td></td>
<td>Rev TCACCAGGTCCACCAGGATTGC</td>
<td></td>
</tr>
<tr>
<td>OSX</td>
<td>Fwd TGGCTAGGTGTTTGCCAGGG</td>
<td>NM_001173467.2</td>
</tr>
<tr>
<td></td>
<td>Rev TGGGCAGCTGGGGGTTCAGT</td>
<td></td>
</tr>
<tr>
<td>RUNX2</td>
<td>Fwd ACAGAAACCACAAGTGCCGTCGAA</td>
<td>NM_001015051.3</td>
</tr>
<tr>
<td></td>
<td>Rev TGGCTGCTAGTGACCTGCGGA</td>
<td></td>
</tr>
<tr>
<td>ALP</td>
<td>Fwd CCACGTCTTTCACATTTGGTG</td>
<td>NM_000478.4</td>
</tr>
<tr>
<td></td>
<td>Rev GCAGTGAGGGCTTTCTGTTC</td>
<td></td>
</tr>
<tr>
<td>COL1A1</td>
<td>Fwd GATGGAATTCAGTGCTGATG</td>
<td>NM_000088.3</td>
</tr>
<tr>
<td></td>
<td>Rev GTTTGGGTCTTTGTCTGTTCG</td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>Fwd GGGCTGGCATCTGCCCTCAA</td>
<td>NM_002046.5</td>
</tr>
<tr>
<td></td>
<td>Rev GCAGCCTCTCTCTCTCCATCTG</td>
<td></td>
</tr>
<tr>
<td>YAP</td>
<td>Fwd CAACCTCAACACAGCAGCAAAC</td>
<td>NM_001130145</td>
</tr>
<tr>
<td></td>
<td>Rev GCAGCCCTCTCTCTCTCCATCTG</td>
<td></td>
</tr>
<tr>
<td>TAZ</td>
<td>Fwd ACCCACCCACAGTGAACCCA</td>
<td>NM_015472</td>
</tr>
<tr>
<td></td>
<td>Rev GCACCCTAACCCACGAGCCAC</td>
<td></td>
</tr>
<tr>
<td>CTGF</td>
<td>Fwd AGGAGTGCTGAGTGCTGACGA</td>
<td>NM_001901</td>
</tr>
<tr>
<td></td>
<td>Rev CCAGGCAGTGGCTCTCAATC</td>
<td></td>
</tr>
<tr>
<td>CYR61</td>
<td>Fwd GAGTGGGTCTGAGCAGGGAT</td>
<td>NM_001554</td>
</tr>
<tr>
<td></td>
<td>Rev GGTTGTATAGGATGGCGAGGCT</td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>Fwd CTTTTGCGCTCGCCAAG</td>
<td>NM_002046</td>
</tr>
<tr>
<td></td>
<td>Rev TTGATGGCCAACAATATCCAC</td>
<td></td>
</tr>
<tr>
<td>SOX9</td>
<td>Fwd CTCTGGAGACTTCTGAACG</td>
<td>NM_000346</td>
</tr>
<tr>
<td></td>
<td>Rev AGATGTGGGTGCTCCTC</td>
<td></td>
</tr>
<tr>
<td>Gene</td>
<td>Sequence (5’-3’)</td>
<td>Accession number</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------</td>
<td>------------------</td>
</tr>
<tr>
<td>ACAN</td>
<td>Fwd: TGCGGGTCAACAGTGCTATC, Rev: CACGATGCCTTTCAACCACGAC</td>
<td>NM_001135</td>
</tr>
<tr>
<td>COL2a1</td>
<td>Fwd: GAAGAGTGGAGACTACTGG, Rev: CAGATGTGTTTCTTCTCCTTG</td>
<td>NM_033150</td>
</tr>
<tr>
<td>OPN (SPP1)</td>
<td>Fwd: GACCAAGGAAAACCTCACTAC, Rev: CTGTATAACTGATGCCAC</td>
<td>NM_001251829</td>
</tr>
<tr>
<td>RUNX2</td>
<td>Fwd: AAGCTTGATGACTCTAAACC, Rev: TCTGTAATCTGACTCTCTGTC</td>
<td>NM_001015051</td>
</tr>
<tr>
<td>COL10a1</td>
<td>Fwd: GCTAGTATCTTTGAACTTGG, Rev: CCTTTACTCTTTATGTTAGG</td>
<td>NM_000493</td>
</tr>
<tr>
<td>VEGFA</td>
<td>Fwd: AATGTGAATGCGACAGCAAGAG, Rev: GACTTATACCGGGATTTCTTG</td>
<td>NM_001204384</td>
</tr>
<tr>
<td>COL6a1</td>
<td>Fwd: ACAGTGACGAGGCTGAGATCA, Rev: GATAGCAGTCGCTAGG</td>
<td>NM_001848</td>
</tr>
</tbody>
</table>
4.3.12 MicroCT angiography

The segmental defect surgery was repeated in 14 week-old male RNU rats (Charles River Labs) under isoflurane anesthesia. In each rat a loaded limb, (early or delayed) was paired with a contralateral control limb, stabilized by a stiff fixation plate. Two animals were lost in the delayed group due to plate complications. Contrast enhanced microCT angiography was performed at week 3 in the early loading group and week 7 in the delayed group. After perfusion, limbs were excised and scanned via microCT (as described above) with both bone and contrast agent intact. Limbs were then decalcified with Cal-Ex II fixative/decalcifier (Fisher) for 2 weeks, scans were repeated, and subtraction was used to distinguish between bone and vessel parameters in a 5 mm diameter defect ROI and 7 mm diameter total ROI. Three representative samples from each group, chosen based on average microCT-computed vessel volume, were processed for histology.

4.3.13 Statistical analysis

Individual sample sizes and details of statistical analyses are specified in each figure caption. Briefly, bridging rates were assessed by chi-square test for trend and comparisons between groups were assessed with individual chi-squared tests and Bonferroni correction for multiple comparisons. Comparisons between groups were assessed by one- or two-way analysis of variance (ANOVA) with Tukey’s multiple comparison tests, as appropriate. Where necessary and appropriate, data were log transformed to ensure normality and homoscedasticity prior to ANOVA. Normality of dependent variables and residuals were verified by D’Agostino-Pearson omnibus and
Brown-Forsythe tests, respectively. For mechanical property regressions, we used a best-subsets algorithm to determine the best predictors of maximum torque and stiffness from a subset of morphologic parameters measured, which included minimum or average polar moment of inertia ($J_{\text{min}}$ or $J_{\text{avg}}$), bone volume, bridging (binary score), and mineral density based on Akaike’s information criterion (AIC) \textsuperscript{224}. The lowest AIC selects the best model while giving preference to models with less parameters. Finally, the overall best model for each predicted mechanical property was compared to its measured value using type II general linear regression. The sample sizes for microCT, mechanical testing, and contrast enhanced angiography analyses were determined with G*Power software \textsuperscript{225} based on a power analysis using population standard deviations and estimated effect sizes from our prior studies \textsuperscript{162,226}. The power analysis assumed a two-tailed alpha of 0.05, power of 0.8, and effect sizes of ranging from 0.1 to 0.3.

4.4 Results

4.4.1 Engineered mesenchymal condensations

Human bone marrow stromal cells, also termed mesenchymal stem cells (hMSCs), were self-assembled into sheets containing local gelatin microsphere-mediated presentation of TGF-β1 (Fig. 4.1A, left), which were then formed into engineered mesenchymal condensations with cylindrical shape for \textit{in vivo} implantation\textsuperscript{193} (Fig. 4.1A, right). In this study, hMSC sheets were cultured for two days prior to either \textit{in vitro} analysis or mesenchymal condensation assembly and \textit{in vivo} transplantation into critical-sized (8 mm) bone defects (Fig. 4.1B). Bone defects were surgically created in femora of
athymic Rowett nude (RNU) rats, as described previously\textsuperscript{162,227}. This 8 mm segmental defect model represents a challenging test-bed for regenerative strategies, being 60% larger than the minimum gap size necessary to prevent spontaneous repair (5 mm)\textsuperscript{197,227}.

First, to verify that local TGF-β1 presentation induced chondrogenic lineage commitment, condensations with empty or TGF-β1-containing microspheres were evaluated at days 2 and 23 of in vitro culture in standard chondro-inductive medium (Fig. 4.1C). Subsequently, condensations assembled after 2 days of culture were transplanted in vivo for evaluation of bone regeneration, neovascularization, and endochondral ossification over 12 weeks (Fig. 4.1D). At day 2, the condensations exhibited homogeneous cellular organization without histologically detectable sulfated glycosaminoglycan (sGAG) deposition or bone formation; by day 23, characteristically shaped chondrocytes and substantial sGAG matrix deposition were apparent (Fig. 4.1E). Local TGF-β1 presentation upregulated and sustained messenger ribonucleic acid (mRNA) expression of Sry-box 9 (SOX9), Aggrecan (ACAN), and Collagen 2a1 (COL2A1) (Fig. 4.1F), with minimal expression of osteogenic markers [Runt-related transcription factor-2 (RUNX2), Alkaline phosphatase (ALP), or Collagen 1a1 (COL1a1)]. Osterix (OSX) expression was upregulated by TGF-β1 at day 2, but suppressed in differentiated chondrocytes at day 23 (Fig. 4.1F). TGF-β1 also increased phosphorylation of the chondrogenic transcription factor SMAD3 at day 2 \textit{in vitro} (Fig. 4.1G,H). These data demonstrate chondrogenic lineage priming consistent with the known dynamics of TGF-β1-SMAD signaling and downstream gene expression in the early developing limb (E11.5-12.5)\textsuperscript{62,228,229}. TGF-β1 presentation transiently upregulated transcription of the Yes-associated protein (YAP) target gene cysteine-rich angiogenic
inducer 61 (CYR61) (Fig. 4.1F) and increased YAP protein content at day 2 (Fig. 4.1I, top row). However, TGF-β1-induced chondrogenic lineage commitment by day 23 abrogated YAP expression and reduced matrix, but not intracellular, immunolocalization of CYR61 (Fig. 4.1F, I, bottom row).
Figure 4.1: Engineered mesenchymal condensations. (A) Photograph of hMSC sheets containing TGF-β1-loaded gelatin microspheres self-assembled for two days on Transwell inserts (left) prior to combination into engineered mesenchymal condensations for implantation (right). Each condensation was assembled from three sheets and enclosed within a perforated electrospun nanofiber mesh tube of polycaprolactone. (B) Photograph of a critically-sized (8 mm) segmental bone defect created in the femora of an RNU nude rat. Engineered mesenchymal condensations were implanted into bone defects. (C, D) Timelines of in vitro and in vivo analyses. (C) hMSC sheets were evaluated in vitro at 2 days (the time point of transplantation) or at 23 days of culture in chondrogenesis-supportive medium. (D) Bone regeneration, neovascularization, and endochondral ossification was evaluated over 3-12 weeks after transplantation. (E) Safranin-O/fast green staining of hMSC sheets with empty or 600 ng TGF-β1-containing gelatin microspheres, cultured for two or 23 days in vitro. Red indicates sGAGs; fast green counterstain shows cells and other matrix. (F) Messenger RNA expression of chondrogenic, osteogenic, and YAP pathway genes at day 2 and day 23 in vitro; qRT-PCR results normalized to GAPDH and expressed as fold-change over empty microsphere control sheets (n = 3 sheets/group). (G) Immunoblot of phosphorylated-SMAD3 activity at day 2 in vitro with β-actin control and (H) band intensity of p-SMAD3/SMAD3 ratio expressed as fold change over sheets without growth factor. (I) Immunostaining for YAP and CYR61 at days 2 and 23 in vitro. DAB peroxidase was used to produce a brown reaction product at locations of immunolabeled antigens. Right: negative control isotype IgG (rabbit, top; mouse, bottom). *P < 0.05, **P < 0.01, ***P < 0.001 TGF-β1-treated vs. empty, unpaired two-tailed Student’s t-test for each independent gene. Data shown are mean ± s.d. Scale bars, 100 μm.
4.4.2 Mechanical regulation of bone regeneration in vivo

Mechanical stimuli promote proper endochondral ossification during both bone development and fracture healing\(^{16,20,22}\), but the effects of in vivo mechanical loading on transplanted cell-mediated bone repair are unknown. Here, using a critical-size rat bone defect model, we modulated ambulatory load transfer using internal fixation plates of variable stiffness. The plates allow axial deformation after unlocking (Fig. 4.2A, Fig. 4.3)\(^{144,162,195}\). The timing and magnitude of mechanical forces imparted to the defects were controlled in three groups: stiff (control, \(n = 11\)); early loading (compliant plates unlocked at implantation to allow immediate loading, \(n = 9\)); and delayed loading (compliant plates unlocked to initiate loading at week 4, \(n = 9\)) (Fig. 4.2B). The multimodal mechanical behavior of the plates was assessed by ex vivo mechanical testing (stiff: \(k_{\text{axial}} = 260 \pm 28\) N/mm, locked compliant: \(k_{\text{axial}} = 250 \pm 35\) N/mm, unlocked compliant: \(k_{\text{axial}} = 8.0 \pm 3.5\) N/mm; mean ± s.d.; Fig. 4.3). Published data on femoral loading during the rat gait cycle\(^{230}\) and rule-of-mixtures theory were used to estimate load-sharing between the fixation plates and the defect tissue. These calculations indicated that interfragmentary strains at day 0 reached 2-3% in the stiff and delayed groups, and up to 10-15% in the early group. A recent in vivo strain sensor study using a modified version of the stiff plates described here confirmed these numbers within one percent for the stiff group\(^{231}\). The amount of strain induced over time is a function of the load sharing, and therefore dependent on the amount, composition, and kinetics of tissue ingrowth; however, accounting for load sharing by ingrowing bone, we estimated strains of 5-10% upon plate unlocking at week 4 in the delayed group, with all groups converging on 0.5-3% by week 12.
Figure 4.2: Mechanical loading enhanced endochondral bone regeneration. (A) Schematic of fixation plate configurations for dynamic control of ambulatory load transfer. (B) Schematic of loading timeline. Early loading features compliant plate actuation at implantation; delayed loading features unlocking at week 4. (C) Representative in vivo microcomputed tomography (microCT) reconstructions at week 4. (D) Safranin-O/fast green staining of sagittal histological sections at week 4 (left) in comparison to the native rat distal femur growth plate (right). Bottom row: magnification of boxed areas. (E) Longitudinal microCT quantification of bone volume at week 4 [n = 11, 11, 9, and 8 for stiff, early, delayed, and BMP-2/collagen (stiff), respectively], week 8 (n = 10, 9, 8, 8) and 12 (n = 10, 8, 8, 8). Repeated significance indicator letters (a,b,c) signify P > 0.05 (not significant); groups with distinct indicators (a vs b) signify P < 0.05 at each time point. (F) Representative 3D microCT reconstructions at week 12. (G) Local trabecular thickness mapping on transverse sections, indicated by boxed arrows in (F), in comparison to the native bone of the ipsilateral femoral head (H). (I) MicroCT quantification of trabecular thickness (Tb.Th), number (Tb.N), and spacing (Tb.Sp) in reference to that of the ipsilateral femoral head (femoral head mean ± s.d. shown as dotted line and shaded pink region). (J) H&E-stained histological sections at week 4 (representative sample from n = 1 per group). (K) Representative 3D microCT reconstruction of BMP-1/collagen group at week 12. (L) Trabecular thickness mapping on the section indicated in (K) illustrating heterotopic bone. All scale bars, 100 µm. Data shown with mean ± s.e.m. *p<0.05, ** p<0.01, **** p<0.0001, NS = not significant, one or two-way ANOVA with Tukey’s post-hoc analysis.
Figure 4.3: Fixation plate characterization. (A, B) Axial compression testing of fixation plates prior to implantation with fixed-fixed or fixed-free boundary conditions, as illustrated. (C, E) Four-point bend testing of fixation plates prior to implantation was performed in three directions, as illustrated, with supports and load applied to maintain constant bending moment on the plate. Statistical comparisons were evaluated by one-way ANOVA on log-transformed data to ensure normality of residuals followed by Tukey’s post hoc test, where groups sharing a letter are not significantly different. Summary data shown as individual data points with mean ± s.d.
Bone regeneration progressed through endochondral ossification, exhibiting zonal cartilage and woven bone mimetic of the native growth plate by week 4 (Fig. 4.2C,D). Both early and delayed loading significantly enhanced bone as measured by bone volume (Fig. 4.2E) and bone volume fraction (Fig. 4.4A). Loading significantly elevated bone accumulation rate between weeks 4 and 8 (*p < 0.05; Fig. 4.4B). This coincided with the timing of load initiation in the delayed group and the tissue differentiation stage, namely chondrocyte hypertrophy and endochondral transition, in all three groups. Though early loading increased bone formation (Fig. 4.2E,F), the bone volume fraction response in this group was highly variable (Fig. 4.4A) and exhibited a significantly lower bridging rate compared to delayed loading (2/8 vs. 6/8, *p < 0.05 Chi-square test, Fig. 4.4D,E) due to persistent regions of non-mineralized cartilage (Fig. 4.2C,D,F), similar to the pseudarthroses induced by large-deformation cyclic bending. In contrast, delayed loading induced robust bone formation (Fig. 4.2E,F), with consistent bridging rate (Fig. 4.4D,E). Region of interest analyses (Fig. 4.5A,B) and quantitative densitometry (Fig. 4.5C) revealed mineral concentration at the defect periphery, indicative of a cortical shell, in all three groups. The bone formed within this cortex exhibited well-defined trabecular architecture, which was quantitatively similar to native femoral head trabecular bone as assessed by microcomputed tomography (microCT) morphometry (Fig. 4.2G-I, Fig. 4.4) and histology (Fig. 4.2J).
Figure 4.4: Bone accumulation and bridging rates. (A) Bone volume fraction (BV/TV) over time where bone volume is normalized to total volume of the defect area (8mm length x 5mm diameter). Individual data points shown with mean ± s.d. Samples with bony bridging are shown in shaded data points, while open data points indicate non-bridged. (B) Bone volume accumulation rate, defined as bone volume accrual over each 4-week interval where BV(t) = bone volume over time, t ≥ 4 weeks, t-4 = time 4 weeks prior to t. Box plots display median as horizontal line, inter-quartile range as boxes, and min/max range as whiskers. Mean values are indicated by +. *p<0.05, **p<0.01, ***p<0.001, two-way ANOVA with Tukey’s post-hoc analysis. (C) High resolution (20 μm voxel size) microCT reconstructions of excised femurs at week 12 showing best- and worst-case regeneration for each group. (D) Representative x-ray images for each group at 4, 8, and 12 weeks illustrating bridged (filled circles) and non-bridged (open circles) samples. (E) Longitudinal analysis of bone bridging in vivo at 4 (n = 11, 11, 9, for stiff, early, and delayed, respectively), 8 (n = 10, 9, 8) and 12 weeks (n = 10, 8, 8) determined via x-ray as mineral fully traversing the defect. Significance of trend was analyzed by chi-square test for trend (**P < 0.001) while differences between groups were determined by chi-square test at each time point with Bonferroni correction (*P < 0.05).
Figure 4.5: MicroCT region of-interest analysis demonstrating formation of cortical and trabecular bone compartments. (A) Region of interest (ROI) analyses of bone volume in total, defect, and periphery ROIs, defined by regions either inside a 5mm diameter cylinder (defect) or annulus with 7 mm outer diameter and 5 mm inner diameter (periphery, n = 10, 8, 8 for stiff, early, and delayed respectively) at 12 weeks. (B) ROI analysis of bone volume fraction, BV/TV. (C) mean mineral density, (D) Proximal vs. distal region of interest analysis, expressed as ratio of bone volume in proximal to distal halves of the defect. (E) The location of minimum pMOI for each sample. In panel E, filled data points indicate bridged samples and open data points indicate non-bridged samples. Box plots show interquartile range with whiskers at minimum and maximum values, center lines at median, and + symbols at the mean. Bar graphs show data with mean ± s.d., where groups with shared indicator letters have no significant differences and *P < 0.05, **P < 0.01, ****P < 0.0001, one or two-way ANOVA with Tukey’s post-hoc analysis.
As a clinically-relevant positive control, a healing dose of recombinant human BMP-2 (5 μg/defect)\(^1\), delivered on absorbable collagen sponge with stiff fixation, was also evaluated (n = 8) (“BMP-2/collagen,” dashed line in Fig. 4.2E). This treatment produced rapid bone accumulation until week 4, and reduced bone formation rate thereafter (Fig. 4.2E). As reported clinically, BMP-2 treatment induced extensive heterotopic bone formation (Fig. 4.2K,L). Surgeries for the BMP-2-treated samples were performed at a separate time and therefore were not compared statistically with the other groups.

Transplanted MSCs commonly exhibit rapid cell death due to lack of vascular and nutrient supply\(^2\); however, in studies using an endochondral paradigm, viable donor cells have been observed up to several weeks after implantation\(^126,159,233,234\). Therefore, to test whether the transplanted cells functionally contributed to bone repair, we prepared identical mesenchymal condensations (6 x 10^6 cells and 1.8 μg of TGF-β1 per construct) for implantation after devitalization by freeze-thaw cycling (Fig. 4.6A). Using stiff plates, devitalized condensations elicited substantially reduced bone formation compared to live cell controls (Fig. 4.6B,C), with fibrotic and adipocytic tissue filling the defect (Fig. 4.6D). Comparisons between live and devitalized samples were not assessed statistically due to surgical operation at a separate time, but suggest a functional role of the transplanted cells.
Figure 4.6: Transplanted cell function. (A) Timeline depicting devitalization of mesenchymal condensations performed day 2, prior to transplantation. (B-C) MicroCT analysis of bone formed at week 12 in live and devitalized groups (n = 10, 5 for live and devitalized, respectively). Dotted lines illustrate the location of the native bone ends. (D) Representative Safranin-O/fast green staining at the center of the defects at week 12 (n = 1-3 per group). Representative samples selected based on mean bone volume. Scale bar, 100 µm. (E) Human nuclear antigen (HuNu) staining of live and devitalized samples (stiff plates) at week 12. DAB peroxidase produced a brown reaction product at locations of immunolabeled antigens. Dotted lines indicate the edges of the native cortical bone at the distal end of the defect. Devitalized samples exhibited some matrix-associated non-specific staining, as shown in IgG controls. (F) Immunostaining of HuNU, YAP, and CYR61 in defects of the delayed loading group (live cells). In each case, DAB peroxidase was used to produce a brown reaction product at locations of immunolabeled antigens. Isotype-matched IgG controls were used to demonstrate specificity. Bottom row shows magnification of boxed areas. Scale bars, 100 µm.
To further elucidate transplanted cell fate and function, live human cells were immunolocalized by human nuclear antigen (HuNu) staining\(^2^{27}\) (Fig. 4.6E,F). Viable human cells, morphologically identifiable as mature and hypertrophic chondrocytes, were actively engaged in endochondral ossification as late as week 12 \textit{in vivo} (Fig. 4.6E,F). In contrast, devitalized samples exhibited some non-specific background staining, illustrated by Immunoglobulin G (IgG) controls, but no live cells were detected. In live samples, human hypertrophic chondrocytes also exhibited nuclear-localized YAP protein and expression of the downstream angiogenic matricellular growth factor, CYR61 (Fig. 4.6F). We did not observe HuNu+ osteoblasts, which stained CYR61+ but HuNu- (Fig. 4.6F, top).

4.4.3 Endochondral matrix formation

To determine the extent to which loading regulated endochondral lineage progression and matrix organization\(^1^{95,2^{32}}\), we performed histological staining at weeks 3, 4, 7, and 12 (Fig. 4.7, A.1, A.2). The endochondral regenerate contained distinct bands of Safranin-O-positive cartilage featuring mature and hypertrophic chondrocytes, producing woven bone. Extensive sGAG staining was observed at early time points (3 and 4 weeks), and calcified cartilage and bone at weeks 7 and 12 (Fig. 4.7). Both early and delayed loading enhanced and prolonged the chondral phase of endochondral ossification, as indicated by Safranin-O staining intensity (Fig. 4.7A-C). Local collagen organization was evaluated by polarized light analysis of picrosirius red-stained sections at weeks 4 and 12. There were no qualitative differences in collagen birefringence at week 4 (B.1), but both early and delayed loading qualitatively decreased collagen organization compared to the
stiff controls at week 12 (Fig. 4.7D, B.1). This suggests that loading increased the proportion of immature woven bone through either increased de novo woven bone deposition or altered remodeling\textsuperscript{162,195}. 
Figure 4.7: Endochondral matrix formation. Tile-scan images of Safranin-O/fast green-stained histological sections of representative samples from stiff, early, and delayed loading groups at (A) week 4 and (B) week 12. Scale bar: 3mm. All samples oriented distal (left) to proximal (right). Dotted lines in top-left indicate the native cortical bone ends. Labels “dh” indicate location of fixation plate drill holes. Scale bar, 3 mm. (C) Magnified images of dotted boxed regions in (B) showing endochondral cartilage remnants at week 12. Scale bar, 100 µm. Bottom row: magnification of boxed regions in upper row. (D) Polarized light microscopy of picrosirius red-stained histological sections at week 12. Increased birefringent intensity indicates increased collagen fibril organization associated with bone matrix remodelling to lamellar bone; reduced birefringence indicates greater amounts of woven bone. Scale bar, 100 µm. Bottom row: magnification of boxed regions in upper row. All images were taken from a representative sample that most closely matched the average in vivo microCT morphometry of that group.
4.4.4 Recovery of mechanical properties

As the principal test of any engineered tissue needs to be its functionality\textsuperscript{188}, we evaluated the degree of restoration of bone mechanical properties by torsion testing to failure at week 12, in comparison with age-matched intact femurs (Fig. 4.8). Despite enhanced bone formation, early loading failed to restore mechanical properties, whereas delayed loading significantly increased torsional stiffness and maximum torque at failure compared to stiff plate controls (*p < 0.05; Fig. 4.8A,B) and restored the torsional stiffness to that of intact limbs (dotted line/gray shading indicate mean ± s.d.). The ability of materials to resist deformation and failure is determined by intrinsic material properties as well as the material amount and distribution, which for torsional loading can be measured as the polar moment of inertia (pMOI). Using microCT, we quantified the minimum and average pMOI for each limb; each was significantly elevated by delayed loading (Fig. 4.8C,D). Mechanical behavior was variable, particularly in the early group. To explain this we performed a best-subsets regression analysis to optimize the combination of factors that best correlate with mechanical behavior. The parameter set included: average pMOI, minimum pMOI, bone volume, binary bridging score (indicated by shaded vs. open data points), and average mineral density. The optimal model was determined by minimization of the Akaike’s information criterion (AIC)\textsuperscript{224}. We then performed Type II multivariate regression analyses to determine the amount of variability in the measured stiffness and maximum torque that is explained by the selected predictors (R\textsuperscript{2}). For both maximum torque and torsional stiffness, minimum pMOI and bridging score were the best combined predictors from among the top 5 models (Fig. 4.8E,F). Together, these data indicate that mechanical loading regulated the
amount and functionality of regenerated bone, formed through endochondral ossification of transplanted mesenchymal condensations, but the mechanisms are unclear.
Figure 4.8: Restoration of mechanical function. Structural mechanical properties were measured by torsion to failure at week 12. Age-matched intact bone properties are shown as dotted line/gray shading indicating mean ± s.d. Samples with full defect bridging are shown in filled data points; open data points indicate non-bridged samples. (A) Analysis of torsional stiffness, (B) maximum torque at failure, (C) minimum polar moment of inertia (pMOI), and (D) average pMOI (n = 8, 7, 7 for stiff, early, and delayed, respectively). Best subsets regression analysis with lowest AIC value for measured and predicted torsional stiffness (E) and maximum torque at failure (F) indicating significant contributions of minimum pMOI (Jmin) and binary bridging score. Error bars show mean ± s.d. with individual data points. Statistical comparisons between groups for each measure were performed by one-way ANOVA with Tukey’s post-hoc analyses, * P < 0.05; † P < 0.05 vs. intact bone.
4.4.5 Dual contrast-enhanced microCT imaging of vasculature and cartilage

Angiogenesis is known to enhance and initiate cartilage callus ossification\textsuperscript{123,235,236}. Therefore, we next hypothesized that mechanical cues regulate endochondral bone formation in part by modulating neovascularization and subsequent cartilage template remodeling. To test this, we performed a second set of in vivo studies to quantify vascular invasion of the cartilage anlage by microCT angiography combined with contrast-enhanced cartilage imaging three weeks after the onset of loading for both early (n = 10) and delayed (n = 8) conditions. In this study, each animal was randomly assigned a loaded limb and a contralateral stiff plate control. Thus, animals in the early loading group were evaluated at week 3 and the delayed group at week 7 (Fig. 4.9A,I). These time points were selected to capture the transient vascular network response to the dynamic mechanical environment\textsuperscript{162,236}. 

90
Figure 4.9: Mechanical control of neovascularization. (A) Schematic of stiff versus early loading featuring compliant plate actuation at implantation and contrast agent perfused at week 3. (B-C) Representative microCT reconstructions of bone (B) and blood vessels with local vessel diameter mapping (C) under stiff and early loading conditions at week 3. (D) Quantification of bone volume. (E-H) 3D vascular network morphometry quantifying vascular volume (E), connectivity (F), and vessel orientation and distribution, as measured by degree of anisotropy (G) and the angle with respect to the bone-axis of the maximum principal eigenvector (H2) of the mean intercept length (MIL) tensor (H), indicating the dominant direction of vessel orientation. Degree of anisotropy represents the ratio of the longest and shortest MIL eigenvalues; DA = 1 indicates isotropy. (I) Schematic of stiff versus delayed loading featuring compliant plate unlocking at week 4 and contrast agent perfused at week 7. (J-L) Representative microCT reconstructions of bone (J) and blood vessels with local vessel diameter mapping (K) under stiff and early loading conditions at week 7. (L) Quantification of bone volume. (M-P) Vascular network morphometry measured by vascular volume (M), connectivity (N), degree of anisotropy (O), and maximum principal vector angle (P). Quantification is in a 5-mm ROI, paired data shown either as mean ± s.e.m. or superimposed on box plots displaying median as horizontal line, inter-quartile range as boxes, and min/max range as whiskers. Mean values are indicated by +. Comparisons between groups evaluated by paired two-tailed Student’s t-tests (* P < 0.05).
MicroCT angiography was performed by perfusing the vasculature with a lead chromate-based contrast agent (Microfil MV-122) to attenuate X-rays in the vasculature\textsuperscript{223,237,238} for three-dimensional (3D) quantification of blood vessel networks in and around the defect (Fig. 4.9, fig. 3.10). Sequential microCT scanning of the perfused limbs before and after bone decalcification enabled independent quantification of bone formation and vascularization in the same samples\textsuperscript{162}. In the defect region of interest (5mm Ø ROI), early loading did not alter bone formation at week 3 (Fig. 4.9B,D), consistent with the independent prior results at week 4 (Fig. 4.3E). Early loading significantly inhibited vascular ingrowth, blunting the predominantly axial orientation of the vessel network observed in the stiff group and producing a more isotropic distribution of orientations (Fig. 4.9C,E-H). In contrast, delayed loading enhanced bone formation at week 7 (Fig. 4.9J,L), consistent with the independent prior results at week 8 (Fig. 4.3E). However, delayed loading did not alter vascular morphometry parameters other than reduced vessel anisotropy (Fig. 4.9K,M-P). Loading did not alter the vascular volume of the peripheral muscle (7 mm Ø ROI), indicating a local effect of loading on vessel recruitment by the anlage, endothelial cell invasion, or neovessel integrity (Fig. 4.10).
Figure 4.10: Vasculature in defect periphery. Vessel volume, connectivity, and anisotropy of (A) early loaded limbs compared to contralateral stiff controls in a 7mm region of interest that included vasculature from peripheral muscle (*$P < 0.05$, **$P < 0.01$, two-way paired student’s t-test). Paired individual data points are superimposed on box plots displaying median as horizontal line, inter-quartile range as boxes, and min/max range as whiskers. Mean values are indicated by . (B) Region of interest analysis in the 5mm diameter defect region compared to the 7mm-5mm peripheral region that included surrounding muscle (inset; two-way ANOVA with Tukey’s post-hoc comparisons). Box plots display median as horizontal line, inter-quartile range as boxes, and min/max range as whiskers. Mean values are indicated by . (C) Histograms of vessel diameter bins indicating similar vessel thickness distribution between groups, and (D) representative microCT angiography vessel thickness mapping. Vessel volume, connectivity, and anisotropy of (E) delayed loaded limbs ($n = 8$) compared to contralateral stiff controls in the same 7mm with (F) region of interest analysis. (G) Histograms of vessel diameter bins and (H) representative microCT angiography vessel thickness mapping.
Next, to quantify 3D cartilage distribution, we equilibrated the vascular contrast agent-perfused samples with the cartilage contrast agent CA4+, which partitions at equilibrium with the negatively charged sulfated glycosaminoglycans (sGAGs) and attenuates X-rays proportional to sGAG concentration\textsuperscript{239}. Bone, vessel, and cartilage volumes were then assessed using various region of interest (ROI) analyses. We evaluated vascularization and cartilage formation within 1.5mm Ø core and 5mm-1.5mm annular regions of interest. Consistent with cartilage observed at 4 weeks, the core region had lower vascular volume in all groups (Fig. 4.11A,B,D,E), although the sGAG-positive tissue distribution was not significantly different between regions (P = 0.84, Fig. 4.11C,F,G,H). Differences in cartilage volume between the stiff plate and early loading groups in annular and core regions were not statistically significant for either loading regimen (P = 0.53, Fig. 4.11C,F).
Figure 4.11: (A) Axial view of 3D neovessel diameter mapping under stiff and early loading conditions at week 3, n = 10. (B) Region of interest analysis to quantify vascular volume fraction in a 1.5 mm diameter core region compared to a 5mm-1.5mm annular region (inset). (C) Cationic (CA4+) cartilage contrast agent-enhanced microCT quantification of cartilage in annulus and core regions. n = 5-6. (D) Axial view of 3D neovessel diameter mapping under stiff and delayed loading conditions at week 7, n = 8. (E) ROI analysis of vascular volume fraction. (F) Cartilage contrast agent-enhanced microCT quantification of cartilage in annulus and core regions at week 7. (G) Representative image of co-registered contrast agent-enhanced cartilage with microCT angiography of neovasculature. Cartilage is shaded blue and vessels are red. (H) Safranin-O/fast green-stained histological sections of vascular contrast agent-perfused tissues (3 weeks). Residual contrast agent exhibits dark dots in vessel lumens. Scale bar, 100 μm. Data shown either as mean ± s.e.m. with individual data points or with box plots displaying median as horizontal line, inter-quartile range as boxes, and min/max range as whiskers. Mean values on box plots are indicated by +. (* P < 0.05, two-way ANOVA with Tukey’s post-hoc comparisons)
4.4.6 Temporal effects of load on progenitor lineage specification

Taken together, these data suggest that the effects of mechanical loading regulate endochondral bone regeneration in a manner dependent on load timing and endochondral cell state at the time of load initiation. Therefore, to explore the temporal effects of mechanical loading on chondrogenic lineage commitment (gene expression and matrix production) we performed in vitro bioreactor experiments using hMSCs encapsulated in fibrin hydrogels exposed to 10% dynamic compression. The fibrin hydrogels were used to provide a matrix capable of in vitro loading, which was applied continuously for 5 weeks (early), for 2 weeks following a 3 week free swelling period (delayed), or for 2 weeks prior to a 3 week free swelling period (reversed), and compared to 5 week free swelling (FS) controls (Fig. 4.12A,B). Loading increased DNA content, indicating increased proliferation and/or maintenance of viability (Fig. 4.12C), but did not alter sGAG per DNA, and reduced total collagen per DNA content (Fig. 4.12C). Alcian blue staining revealed a large, rounded cell morphology and increased pericellular sGAG staining in response to loading (Fig. 4.12D), especially in early and reversed groups where loading was applied immediately after encapsulation, suggesting load-induced pericellular matrix (PCM) deposition. To test this, we immunostained for PCM-exclusive collagen 6 and found that all loaded groups exhibited increased collagen 6 at the cell periphery, particularly in the groups loaded immediately, whereas free swelling controls were nearly devoid of collagen 6 (Fig. 4.12E). Collagen 6a1 (COL6a1) mRNA expression was significantly increased in early and delayed groups (*p < 0.0001; Fig. 4.12F), suggesting that mechanical load is needed to initiate and maintain COL6a1 expression, resulting in COL6a1 accumulation in groups loaded immediately. Collagen 6
is prevalent in the PCM of articular chondrocytes, and mediates load-induced proliferation and chondrogenic gene expression\textsuperscript{240}. 
Figure 4.12: In vitro analysis of mechanical load on chondrogenic lineage progression. (A) Photograph of hMSC-laden hydrogel and schematic of custom-made bioreactor applying dynamic compression. (B) Timeline of the four loading groups evaluated: free-swelling (FS) controls, early loading (continuous for 5 weeks), delayed loading (free-swelling for 3 weeks followed by 2 weeks of loading), and reversed loading (loading for 2 weeks followed by 3 weeks free-swelling). C, Quantification of DNA, sGAG, and total collagen content (n = 5). (D) Alcian blue and (E) pericellular COL6a1 immunostaining. DAB peroxidase was used to produce a brown reaction product at locations of immunolabeled antigen. Images shown at 20x with 10x insets, scale bars 100 µm. (F-G) Quantitative PCR at week 5 (n = 4-5 per group) of (F) COL6a1 and (G) SOX9, COL10a1, OPN, and VEGF. Relative expression was calculated as fold change over free swelling controls. Data are shown as mean ± s.d. with individual data points. Statistical comparisons between groups for each measure were performed by one-way ANOVA with Tukey’s post-hoc analyses, where groups sharing a letter (a,b,c) are not statistically different.
Consistent with this observation, mRNA expression of the master chondrogenic transcription factor SOX9 increased in all loaded groups, but was only significant when applied continuously (early loading, \(^{*}p < 0.0001\); Fig. 4.12G). This indicates a maintenance of chondrogenic gene expression that may explain the cartilage persistence seen in vivo in response to early loading. Similar trends were observed for SOX9-target genes aggrecan and collagen 2a1 (Fig. 4.13A,B). Consistent with the downregulation of YAP and downstream target CYR61 observed with TGF-β1-mediated chondrogenesis (Fig. 4.1), these genes are were also significantly decreased with chondrogenic lineage commitment induced by loading (\(^{*}p < 0.05\); Fig. 4.13B). Loading decreased collagen 10a1 and osteopontin expression in groups where load was being applied at harvest (early and delayed), but not in the reversed group, suggesting that loading delayed chondrocyte hypertrophy (Fig. 4.12G). Vascular endothelial growth factor (VEGF) is an important factor for vascular invasion during endochondral healing, and is necessary for remodeling of the fracture callus\(^{241}\). Delayed loading significantly increased VEGF expression (\(^{*}p < 0.01\); Fig. 4.12G), consistent with our finding that delayed loading supported neovascularization and enhanced endochondral bone growth.
Figure 4.13: mRNA expression of hMSCs in dynamically compressed hydrogels. A, COL2a1 deposition in samples after 5 weeks in vitro with hematoxylin counterstain (10x images, scale bars 100µm, n=2). B, Samples analyzed at 5 weeks for mRNA expression of ACAN, COL2a1, RUNX2, Col1a1, YAP, and CYR61 via qPCR (n=4-5) calculated as fold change over free swelling controls. Data are shown as mean ± s.d. with individual data points. One-way ANOVA and multi comparison by tukey’s post hoc was used to determine significance (*P < 0.05) where groups sharing a letter are not statistically different.

4.5 Discussion

In development, mechanical forces caused by fetal motion in the womb are critical for proper bone and joint morphogenesis. In adults, mechanical stability at a fracture site determines the mode of repair (intramembranous vs. endochondral). We therefore reasoned that mechanical cues would also be important for recapitulating endochondral bone development for regeneration of large bone defects. Here, we
evaluated the capacity of engineered mesenchymal condensations with local morphogen presentation to induce endochondral bone regeneration under varying conditions of in vivo mechanical loading. We found that mechanical loading enhanced endochondral bone formation in vivo and was essential to restore function to limbs with critical sized bone defects. Mechanistically, loading regulated chondrogenesis and pericellular matrix formation in vitro, and controlled cartilage persistence and neovascularization in vivo, dependent on load timing. Together, these data demonstrate the importance of mechanical cues for biomimetic bone tissue engineering and may have implications for other tissues whose form and function are dictated by mechanical stimuli during development and homeostasis. Previous reports by our group \textsuperscript{162,195} and others \textsuperscript{111,243} investigated the effects of in vivo mechanical loading on recombinant BMP-2-mediated bone defect repair. In these BMP studies, loading maximally induced an 18-20% increase in bone formation compared to stiff plate controls. By contrast, here we used an endochondral regeneration approach to show that delayed mechanical loading increased bone volume by 181% compared to stiff controls, representing an order-of-magnitude greater mechano-response than that under BMP-2 treatment. Further, the response to loading was greatest between weeks 4 and 8, especially when loading was initiated at week 4. Histologically, this corresponded with the timing of transition from hypertrophic cartilage to bone, suggesting that this stage of endochondral ossification is particularly responsive to mechanical forces. Functionally, mechanical loading was required to restore bone biomechanical properties, but mechanical outcomes exhibited high variability. Multivariate regression analysis \textsuperscript{244} revealed that this variability was determined both by whether the defects were fully bridged with bone and the distribution
of that bone in the defect. It further explains differences in mechanical outcomes between the early and delayed loading groups; although both loading conditions enhanced bone volume, the early loading failed to induce functional repair as a result of a low bridging rate due to pseudarthrotic cartilage. Together, these data demonstrate that mechanical cues are critical for restoration of bone form and function by endochondral ossification of engineered mesenchymal condensations.

Although many MSC transplantation studies observe rapid cell death \(^{227}\), functional donor cell incorporation has been observed in studies investigating endochondral ossification \(^{126,159,233,234}\). Here, the lack of bone formation after cell devitalization and immunolocalization of human antigens suggested that the transplanted human cells both participated in endochondral lineage progression and exhibited mechanosensitive gene activity upon hypertrophy. Recent studies demonstrate that hypertrophic chondrocytes can also transdifferentiate into osteoblasts during both developmental and reparative endochondral ossification \(^{135,136,245,246}\). Although we observed HuNu\(^+\) proliferative and hypertrophic chondrocytes, we did not detect HuNu\(^+\) osteoblasts or evidence of hypertrophic chondrocyte-to-osteoblast transformation.

Vascularization and anlage maturation are linked at both cellular and molecular scales and influence one another during endochondral ossification \(^{123,235,236}\). For example, inhibition of angiogenesis can promote phenotypic stability of MSC-derived chondrocytes in vivo \(^{161}\), whereas chondrocyte hypertrophy is in part responsible for neovessel recruitment \(^{133}\). Further, we found previously that angiogenesis is influenced by mechanical conditions during bone defect repair \(^{162}\) and can be modulated by scaffold architecture \(^{247}\). Here, we found that mechanical loading did not affect vascular supply in
the peripheral musculature, but both early and delayed loading increased the isotropy of the new vessel networks, particularly by decreasing the degree of axial orientation of the invading vasculature. Early loading reduced vascular volume and connectivity and in some samples caused pseudarthrotic non-union, whereas delayed loading did not alter vascular volume or connectivity and enhanced defect bridging. Together, we speculate that loading may differentially regulate invasion by two distinct sources of angiogenic vessels: one from within the cortex, and one from the surrounding musculature. Thus, loading may disproportionally impair or delay vascular invasion from the endocortical space while allowing transverse invasion from the surrounding musculature. These effects of loading on angiogenesis and cartilage transformation could be mediated by either mechanobiological or physical inhibition of neovessel network formation resulting in cartilage maintenance, or conversely, loading may delay chondrocyte hypertrophy and subsequent neovessel recruitment.

Further research will be required to dissect the distinct and/or interacting effects of loading on endochondral lineage progression vs. angiogenesis; one approach is to study the tissues in isolation. Our bioreactor data, devoid of endothelium or blood supply, show that mechanical loading was needed to initiate and maintain COL6a1 expression and regulated both chondrogenesis and angiogenic growth factor expression. Collagen 6 is prevalent in the PCM of articular chondrocytes and functions to resist cellular deformation during cartilage matrix compression, mediating load-induced proliferation and chondrogenic gene expression. Because collagen 6 protein has a slower degradation rate than its mRNA, the immunostain represents the accumulation of matrix-embedded collagen 6 over the tissue history. Thus, early loading and reversed
loading induced collagen 6 expression and deposition in the PCM, whereas neither stiff
nor delayed loading induced Col6a1 deposition by week 5. Supporting a transient
induction of COL6a1 by loading, only the groups that had been loaded immediately prior
to the time of assay at week 5 (early and delayed loading groups) showed upregulation of
COL6a1 message.

The molecular mechanisms that control endochondral ossification remain
incompletely understood. Recent evidence from our laboratory and others implicates the
transcriptional co-activator YAP as a mechanosensitive \(^{249,250}\), TGF-β1-responsive \(^{251}\)
regulator of progenitor cell lineage specification, promoting endochondral bone
development \(^{244}\) but inhibiting chondrogenesis \(^{252}\). Consistent with these reports, we
observed that YAP was consistently down-regulated with chondrogenic lineage
commitment across multiple experiments, corroborating recent evidence that YAP
inactivation is necessary to maintain the chondrogenic phenotype \(^{253,254}\). Our data further
indicate that mechanical loading promotes chondrogenesis independent of YAP induction
or transcriptional activation; however, potential roles of YAP as a mechanosensor in
cartilage remain poorly understood. We conclude that early loading, prior to substantial
matrix deposition, may have caused large cellular deformation, inducing production of a
protective PCM and promoting and sustaining chondrogenic differentiation, leading to
pseudarthrosis formation and inhibition of functional regeneration, whereas delayed
loading supported angiogenesis and induced endochondral bone regeneration.
4.5.1 Limitations

The goals of this study were to mimic the cellular organization and local morphogen environment of the early developing limb for endochondral regeneration of large bone defects, and to investigate the effects of in vivo mechanical cues on cell function and regenerative outcome. We found that mechanical cues are key mediators of endochondral regeneration, but the study had several limitations. First, although the use of hMSCs as a cell source provides translational potential, these cells are developmentally and functionally distinct from the mesenchymal cells that comprise the early limb bud. Further work will be necessary to test the functional consequences of these distinctions and/or identify other cell types better capable of reproducing the embryonic niche. Second, MSCs are known to exhibit donor-donor variability; we have directly investigated these effects in prior studies, but this study was not designed to evaluate differences between donors and all experiments were performed using a single donor. We used athymic (RNU) rats to facilitate xenogeneic cell transplantation and assessment of functional hMSC engraftment, but this model may miss some immunomodulatory functions of the transplanted cells. However, potential translational application of these findings to the clinic will require further testing in a larger animal model, but would likely involve autologous cell transplantation or immune-matched allogeneic cells which would not illicit a T-cell response. Clinical implementation of this mechanical stimuli-induced, development-inspired, approach will require further research, beyond the scope of this study; however, these data support continued work on the roles of mechanical cues in development-mimetic tissue regeneration and may impact clinical management of challenging bone augmentation.
cases in the future. Further work will be necessary to identify the precise cellular, biochemical, and mechanical cues necessary for efficient regeneration and clinical translation in humans.
5.1 Abstract

Endochondral ossification during long bone development and natural fracture healing initiates by mesenchymal cell condensation and is directed by local morphogen signals and mechanical cues. Here, we aimed to mimic these developmental conditions for regeneration of large bone defects. We hypothesized that engineered human mesenchymal condensations with in situ presentation of transforming growth factor-β1 (TGF-β1) and/or bone morphogenetic protein-2 (BMP-2) from encapsulated microparticles would promote endochondral regeneration of critical-sized rat femoral bone defects in a manner dependent on the in vivo mechanical environment. Mesenchymal condensations induced bone formation dependent on morphogen presentation, with dual BMP-2 + TGF-β1 fully restoring mechanical bone function by week 12. In vivo ambulatory mechanical loading, initiated at week 4 by delayed unlocking of compliant fixation plates, significantly enhanced the bone formation rate in

---

the four weeks after load initiation in the dual morphogen group. *In vitro*, local presentation of either BMP-2 alone or BMP-2 + TGF-β1 initiated endochondral lineage commitment of mesenchymal condensations, inducing both chondrogenic and osteogenic gene expression through SMAD3 and SMAD5 signaling. *In vivo*, however, extensive endochondral cartilage formation was evident in the BMP-2 + TGF-β1 group and was enhanced by mechanical loading. The degree of bone formation was comparable to BMP-2 soaked on collagen but without the ectopic bone formation that limits the clinical efficacy of BMP-2/collagen. In contrast, mechanical loading had no effect on autograft-mediated repair. Together, this study demonstrates a biomimetic template for recapitulating developmental morphogenetic and mechanical cues *in vivo* for tissue engineering.

5.2 Introduction

Endochondral ossification is an indirect mode of bone formation that occurs during long bone development and natural fracture repair whereby mesenchymal progenitor cells form a cartilage anlage that is replaced by bone. In both development and repair, mechanical cues are essential for proper endochondral ossification. For example, experimental fetal paralysis significantly decreases bone mass *in ovo* and motion *in utero* is important for normal bone and joint development. Likewise, during fracture repair, the amount and mode of interfragmentary strain determines whether a fracture will heal through endochondral or intramembranous ossification. Though bone fractures heal with 90-95% success rates by forming a cartilaginous callus, large bone defects greater than 3 cm in length cannot
form a callus and exhibit high complication rates, representing a significant clinical burden\textsuperscript{262}. Current standard treatments for large bone defects include autologous bone grafting and delivery of high-dose recombinant human bone morphogenetic protein-2 (BMP-2) soaked on a collagen sponge carrier; however, these treatments are limited by donor-site morbidity and ectopic bone formation, respectively.

Cell-based tissue engineering strategies may provide a promising alternative to bone grafts. One proposed strategy combines osteogenic/progenitor cells with materials that mimic the structural properties of mature bone. However, poor cell engraftment and viability due to insufficient vascular supply limit the efficacy of osteogenic cell delivery\textsuperscript{263–265}, and the rigidity of mature bone matrix-like scaffolds can impede stimulatory mechanical loads\textsuperscript{144}. As the cartilage anlage is mechanically compliant, avascular, and capable of naturally recruiting neovascularure and endogenous progenitors and osteoblasts, this approach may overcome key limitations for the regeneration of challenging bone defects. Here, we sought to recapitulate the (1) mesenchymal condensation, (2) sequential morphogenetic cues, and (3) mechanical cues that mediate developmental endochondral ossification for regeneration of critical-sized bone defects in adult rats.

Mesenchymal cell condensation and chondrogenic differentiation in the developing limb bud are regulated by sequential morphogenic signals, including TGF-\textbeta\textsuperscript{266} and BMP\textsuperscript{69}, which mediate cell condensation and induce the master chondrogenic transcription factors SRY-Box (SOX) 5, 6, and 9\textsuperscript{58,267}. Recent studies have shown that avascular cartilage templates derived from human mesenchymal stem cell (hMSC)
aggregates are capable of progressing through endochondral ossification, producing mineralized matrix, vasculature, and a bone marrow hematopoietic stem cell niche, but these required extended pre-culture with exogenous growth factors in vitro for chondrogenic priming. To address this problem, we developed scaffold-free mesenchymal condensations, formed through self-assembly of bone marrow-derived hMSC sheets incorporated with transforming growth factor-β1 (TGF-β1)-releasing gelatin microspheres for in situ chondrogenic priming. These formed robust cartilage tissue in vitro and induced endochondral bone defect regeneration after implantation in vivo. Further, sustained individual or co-delivery of BMP-2 in mesenchymal condensations induced both chondrogenesis and osteogenesis in vitro and endochondral regeneration of calvarial defects in vivo. Local morphogen presentation circumvents the need for lengthy exogenous supplementation of inductive signals and enables in vivo implantation of the cellular constructs in a timely and cost-efficient manner, thereby providing a promising system to investigate the progression of mesenchymal condensation through endochondral ossification in vivo.

The mechanical environment considerably influences bone development, homeostasis, and regeneration. To investigate the roles of mechanical cues in large bone defect regeneration, we developed a critically-sized rat femoral bone defect model in which ambulatory load transfer can be controlled by dynamic modulation of axial fixation plate stiffness. We previously showed that load initiation, delayed to week four (after the onset of regeneration and bony bridging), significantly enhanced bone formation, biomechanical properties, and local tissue adaptation mediated by BMP-2-releasing hydrogel. Recently, we showed that in vivo loading of...
engineered mesenchymal condensations, containing TGF-β1-releasing gelatin microparticles, restored bone function through endochondral ossification.

However, these studies focused on single morphogen presentation, and bone development features an intricate and tightly coordinated sequence of both morphogenetic and mechanical cues. Here, we investigated the combinatorial roles of controlled temporal presentation of TGF-β1 and/or BMP-2, to mimic events in the developing limb bud, with in vivo mechanical loading. To control local morphogen presentation, we engineered mesenchymal condensations incorporated with gelatin or mineral microparticles for local release of TGF-β1 to drive chondrogenesis and BMP-2 to promote remodeling of the cartilaginous anlage to bone, respectively. To regulate in vivo mechanical conditions, we utilized the same custom fixation plates described previously that modulate fixation plate stiffness through elective unlocking, allowing increased ambulatory load-sharing between the defect and fixation plate (see Materials and Methods for stiffness values of the plates). We previously estimated that interfragmentary strains of 2-3% exist in the stiff and delayed loading groups at day 0, 5-10% in the delayed group after plate unlocking, and 0.5-3% in all groups at week 12.

Variables such as the tissue composition, amount, and growth kinetics temporally regulate the strain state in the defects.

We found that morphogen co-delivery and in vivo mechanical loading combinatorially regulated bone regeneration and directed ossification mode, with combined treatment inducing full functional restoration of bone mechanical properties.
5.3 Materials and Methods

5.3.1 Study design

The objective of this work was to mimic the cellular, biochemical, and mechanical environment of the endochondral ossification process during early limb development via *in situ* morphogen priming of high-density mesenchymal condensations and controlled *in vivo* mechanical cues upon implantation in large bone defects. We employed the established critical-size rat femoral segmental defect model in 12-week-old male Rowett nude (RNU) rats with custom internal fixation plates that allow controlled transfer of ambulatory loads *in vivo*. The sample size was determined with G*Power software\(^{225}\) based on a power analysis using population standard deviations and estimated effect sizes from our prior studies\(^{162,270}\). The power analysis assumed a two-tailed alpha of 0.05, power of 0.8, and effect sizes of ranging from 0.1 to 0.3. A minimum sample number of \(N = 6\) per group was computed, with an ideal sample number of \(N = 12\) for all non-destructive and destructive analyses per time point. An \(N = 10\) was selected for all non-destructive and destructive analyses per time point, accommodating a 5-10% complication rate consistent with our prior studies. Animals were randomly assigned to the treatment groups for both studies. Where indicated, limbs were excluded from the analysis based on radiographic evidence of fixation plate failure. Data collection occurred at predetermined time points informed by previous studies. All analyses were performed by examiners blinded to the treatment group.
5.3.2 Experiments and study groups.

**Initial studies:** For the first initial study, the experimental design featured one treatment group with two mechanical loading conditions. Defects received morselized autograft prepared by mincing the excised cortical biopsy in sterile phosphate buffered saline (PBS) [Autograft] contained within an electrospun, perforated PCL nanofiber mesh tube. Limbs were stabilized with stiff [Stiff] or axially-compliant [Compliant] fixation plates initially implanted in a locked configuration to prevent loading ($k_{axial} = 250 \pm 35 \text{ N/mm}$), but after four weeks the plates were surgically unlocked to enable load transfer ($k_{axial} = 8.0 \pm 3.5 \text{ N/mm}$) ($N = 6-8$ per group). For the second initial study, the experimental design featured three treatment groups with one mechanical loading condition. Defects received 1) mesenchymal condensations comprised of three microparticle-containing hMSC sheets for a total of $6.0 \times 10^6$ cells with bone morphogenetic protein-2 (BMP-2)-loaded mineral-coated hydroxyapatite (MCM) microparticles (1.9 µg) and unloaded gelatin microspheres (GM) [BMP-2 (hMSCs)], 2) BMP-2 (1.9 µg) soaked onto 8 mm pre-cut absorbable collagen sponge (INFUSE™ Bone Graft; Medtronic, Memphis, TN) 15 min prior to implantation [BMP-2 (collagen)], or 3) morselized autograft in sterile PBS [Autograft], each contained within an electrospun, perforated PCL nanofiber mesh tube. Limbs were stabilized with stiff fixation plates [Stiff] that limit load transfer ($k_{axial} = 260 \pm 28 \text{ N/mm}$) ($N = 7-10$ per group), modified from prior studies.\textsuperscript{162,192,195} **Main study:** The experimental design featured three treatment groups with two mechanical loading conditions. Defects received mesenchymal condensations comprised of three microparticle-containing hMSC sheets for a total of $6.0 \times 10^6$ cells incorporated with 1) unloaded MCM and GM [Empty/Control], 2) BMP-
2-loaded MCM (1.9 µg) and unloaded GM [BMP-2], or 3) BMP-2-loaded MCM (1.9 µg) and transforming growth factor-β1 (TGF-β1)-loaded GM (1.8 µg) [BMP-2+TGF-β1] contained within an electrospun, perforated PCL nanofiber mesh tube. Limbs were stabilized with stiff [Stiff] or axially-compliant [Compliant] fixation plates initially implanted in a locked configuration to prevent loading (k_{axial} = 250 ± 35 N/mm), but after four weeks the plates were surgically unlocked to enable load transfer (k_{axial} = 8.0 ± 3.5 N/mm) (N = 3-10 per group).

5.3.3 hMSC isolation and expansion.

Human bone marrow-derived mesenchymal stromal/stem cells (hMSCs) were derived from the posterior iliac crest of two healthy male donors (26 and 41 years of age) using a protocol approved by the University Hospitals of Cleveland Institutional Review Board. Cells were isolated using a Percoll gradient (Sigma-Aldrich, St. Louis, MO) and cultured in low-glucose Dulbecco’s modified Eagle’s medium (DMEM-LG; Sigma-Aldrich, St. Louis, MO) containing 10% fetal bovine serum (FBS; Sigma-Aldrich), 1% penicillin/streptomycin (P/S; Fisher Scientific), and 10 ng/ml fibroblast growth factor-2 (FGF-2, R&D Systems, Minneapolis, MN) \(^{269,271}\).

5.3.4 Hydroxyapatite microparticle mineral coating and BMP-2 loading.

MCM were kindly provided by Dr. William L. Murphy (University of Wisconsin, Madison, WI). Their preparation using low carbonate (4.2 mM NaHCO\(_3\)) coating buffer and detailed characterization has been reported previously\(^{271}\). Lyophilized MCM from the same batch as used in these prior studies, and our own\(^{64}\), were loaded with a 100 µg/ml solution of recombinant human (rh) BMP-2 (Dr. Walter Sebald, Department of
Developmental Biology, University of Würzburg, Germany; 6.4 μg/mg) in PBS for 4 h at 37°C. BMP-2-loaded MCM were then centrifuged at 800xg for 2 min and washed 2x with PBS. Unloaded MCM without growth factor were incubated with PBS only and treated similarly.

5.3.5 Gelatin microsphere synthesis and TGF-β1 loading.

Gelatin microspheres (GM)\textsuperscript{52,131,220,271} were synthesized from 11.1% (w/v) gelatin type A (Sigma-Aldrich) using a water-in-oil single emulsion technique and crosslinked for 4 h with 1% (w/v) genipin (Wako USA, Richmond, VA). Hydrated GM were light blue in color and spherical in shape with an average diameter of 52.9±40.2 μm and a crosslinking density of 25.5 ± 7.0%. Growth factor-loaded microspheres were prepared by soaking crosslinked, UV-sterilized GM in 80 μg/ml solution of rhTGF-β1 (Peprotech, Rocky Hill, NJ) in PBS for 2 h at 37°C. Unloaded microspheres without growth factor were hydrated similarly using only PBS.

5.3.6 Nanofiber mesh production.

Nanofiber meshes were formed by dissolving 12% (w/v) poly-(ε-caprolactone) (PCL; Sigma-Aldrich) in 90/10 (v/v) hexafluoro-2-propanol/dimethylformamide (Sigma-Aldrich). The solution was electrospun at a rate of 0.75 ml/h onto a static aluminum collector. 9 mm x 20 mm sheets were cut from the product, perforated with a 1 mm biopsy punch (VWR, Radnor, PA), and glued into tubes around a 4.5 mm mandrel with UV glue (Dymax, Torrington, CT). Perforated PCL nanofiber mesh tubes were sterilized by 100% ethanol evaporation under UV light over-night and washed 3x with sterile PBS before use.
5.3.7 Preparation of microparticle-incorporated mesenchymal condensations.

Expanded hMSCs (2.0×10^6 cells/construct; passage 4) were thoroughly mixed with BMP-2-loaded MCM (1.6 µg/mg; 0.4 mg/sheet) and TGF-β1-loaded GM (0.4 µg/mg; 1.5 mg/construct) in chemically defined medium [DMEM-HG (Sigma-Aldrich), 1% ITS+ Premix (Corning), 1 mM sodium pyruvate (HyClone), 100 µM non-essential amino acids (Lonza), 100 nM dexamethasone (MP Biomedicals, Solon, OH), 0.05 mM L-ascorbic acid-2-phosphate (Wako), and 1% P/S (Fisher Scientific)]^{233,269}. Five hundred microliter of the suspension were seeded onto the pre-wetted membrane of transwell inserts (3 µm pore size, 12 mm diameter; Corning) and allowed to self-assemble into hMSC sheets for 2 days. After 24 h, medium in the lower compartment was replaced. Control constructs containing either unloaded MCM and/or GM were prepared and cultured in a similar fashion. After 48 h, three microparticle-incorporated hMSC sheets per group were combined into a sterile perforated PCL mesh tube to form the mesenchymal condensations for implantation.

5.3.8 Surgical procedure

Critical-sized (8 mm) bilateral segmental defects were created in the femora of 12-week-old male Rowett nude (RNU) rats (Taconic Biosciences Inc., Hudson, NY) under isoflurane anesthesia^{197}. Limbs were stabilized by custom internal fixation plates that allow controlled transfer of ambulatory loads in vivo^{192} and secured to the femur by four bi-cortical miniature screws (J.I. Morris Co, Southbridge, MA). Animals were given subcutaneous injections of 0.04 mg/kg buprenorphine every 8 h for the first 48 h postoperative (post-op) and 0.013 mg/kg every 8 h for the following 24 h, with or without
4-5 mg/kg carprofen every 24 h for 72 h. In addition, 5 ml of 0.9% NaCl were administered subcutaneously to aid in recovery. All procedures were performed in strict accordance with the NIH Guide for the Care and Use of Laboratory Animals, and the policies of the Institutional Animal Care and Use Committee (IACUC) at Case Western Reserve University (Protocol No. 2015-0081) and the University of Notre Dame (Protocol No. 14-05-1778).

5.3.9 In vivo X-ray and microCT.

In vivo X-rays were obtained using an Xtreme scanner (Bruker, Billerica, MA) at 45 kVp, 0.4 mA, and 2 s exposure time. A binary bridging score was assigned by two independent, blinded observers, and determined as mineralized tissue fully traversing the defect. In vivo microCT scans were performed at or 4, 8, and 12 weeks to assess longitudinal defect healing. For initial studies, animals were scanned using an Inveon microPET/CT system (Siemens Medical Solutions, Malvern, PA) at 45 kVp, 0.2 mA, and 35 μm isotropic voxels. Data were reconstructed using system-default parameters for analyzing bone and accounting for the metal in the fixation plates. DICOM-exported files were processed for 3-D analysis (CTAn software, Skyscan; Bruker) using a gauss filter at 1.0 pixel radius and a global threshold range of 28-255 for all samples. Bone volume was determined in a standard region of interest (ROI) spanning the length of the defect. For the main study, animals were scanned using an Albira PET/SPECT/CT system (Bruker) at 45 kVp, 0.4 mA, and 125 μm voxel size. A global threshold was applied for each data set, and bone volume determined in a standard ROI spanning the length of the defect.
5.3.10 Ex vivo microCT.

After 12 weeks, the animals were euthanized by CO₂ asphyxiation and hind limbs were excised for high resolution microCT analysis. Data were acquired using a Skyscan 1172 microCT scanner (Bruker) with a 0.5 mm aluminum filter at 75 kVp and 0.1 mA. Femora wrapped in gauze were placed in a plastic sample holder with the long axis oriented parallel to the image plane, and scanned in PBS at 20 µm isotropic voxels, 560 ms integration time, rotation step of 0.5°, and frame averaging of 5. All samples were scanned within the same container using the same scanning parameters. All scans were then reconstructed using NRecon software (Skyscan) with the same reconstruction parameters (ring artifact reduction of 5, beam hardening correction of 20%). For 3-D analysis (CTAn software, Skyscan), a gauss filter at 1.0 pixel radius and a global threshold range of 65-255 were used. This segmentation approach allowed viewing of the normal bone architecture in the binary images as seen in the original reconstructed images. Three hundred and twenty-five slices in the center of each defect were analyzed in a standard ROI using a 10 mm (total) or 5 mm (defect) diameter circle centered on the medullary canal. Bone volume, bone volume fraction, polar moment of inertia (pMOI), and the morphometric parameters trabecular number, trabecular thickness, trabecular separation, structure model index, degree of anisotropy, and connectivity density were calculated. Trabecular morphometry and pMOI of three age-matched femora were analyzed in the same manner for comparison. Proximal and distal total bone volume were calculated by halving the slice number in each sample and separately segmenting each half for comparison. All representative images were chosen based on average bone volume values.
5.3.11 Biomechanical testing

Femora excised at 12 weeks were biomechanically tested in torsion to failure. Limbs were cleaned of soft tissue and fixation plates were carefully removed. Bone ends were potted in Wood’s metal (Alfa Aesar; Fisher Scientific), mounted on a Mark-10 TSTM-DC test stand with MR50-12 torque sensor (1.35 N-m) and 7i torque indicator (Mark-10 Corp., Copiague, NY) using custom fixtures, and tested to failure at a rate of 3%. For each sample, maximum torque at failure was recorded and torsional stiffness computed as the slope of the linear region in the torque-rotation curve. Samples were compared to 3 age matched, un-operated femurs.

5.3.12 Histological analysis

Day 2 hMSC sheets (N = 3/group) were fixed in 10% neutral buffered formalin (NBF) for 24 h at 4°C before switching to 70% ethanol. One representative femur per group was taken for histology at weeks 4 and 12 post-surgery, chosen based on microCT-calculated average bone volumes. Femora were fixed in 10% NBF for 72 h at 4°C and then transferred to 0.25 M ethylenediaminetetraacetic acid (EDTA) pH 7.4 for 14 d at 4°C under mild agitation on a rocker plate, with changes of the decalcification solution every 3-4 days. Following paraffin processing, 5 µm mid-sagittal sections were cut using a microtome (Leica Microsystems Inc., Buffalo Grove, IL) and stained with hematoxylin & eosin (H&E) and Safranin-O/Fast-green (Saf-O). Light microscopy images were captured using an Olympus BX61VS microscope (Olympus, Center Valley, PA) with a Pike F-505 camera (Allied Vision Technologies, Stadtroda, Germany).
5.3.13 Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis.

Day 2 hMSC sheets (N = 3/group) were homogenized in TRI Reagent (Sigma-Aldrich) for subsequent total RNA extraction and cDNA synthesis (iScript™ kit; Bio-Rad, Hercules, CA). One hundred nanograms of cDNA were amplified in duplicates in each 40-cycle reaction using a Mastercycler (Eppendorf, Hauppauge, NY) with annealing temperature set at 60°C, SYBR® Premix Ex Taq™ II (Takara Bio Inc., Kusatsu, Shiga, Japan), and custom-designed qRT-PCR primers (Table 5.1; Life Technologies, Grand Island, NY). Transcript levels were normalized to GAPDH and gene expression was calculated as fold-change using the comparative C_T method^{221}. 

123
<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence (5’-3’)</th>
<th>Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOX9</td>
<td>Fwd CACACAGCTCACTCGACCTTG</td>
<td>NM_000346.3</td>
</tr>
<tr>
<td></td>
<td>Rev TTCGGTTATTTTTAGGATCATCTCG</td>
<td></td>
</tr>
<tr>
<td>ACAN</td>
<td>Fwd TGCGGGTCAACAGTGCCATAC</td>
<td>NM_001135.3</td>
</tr>
<tr>
<td></td>
<td>Rev CACGATGCCTTTCCACCACGAC</td>
<td></td>
</tr>
<tr>
<td>COL2A1</td>
<td>Fwd GGGACTTTTGTGCCCAAGATG</td>
<td>NM_001844.4</td>
</tr>
<tr>
<td></td>
<td>Rev TCACCAGGTTCACCAGGATTGC</td>
<td></td>
</tr>
<tr>
<td>RUNX2</td>
<td>Fwd ACAGAAACCACAAGTGCGGTGCAA</td>
<td>NM_001015015.3</td>
</tr>
<tr>
<td></td>
<td>Rev TGGCTGCTAGTGACCTCGGA</td>
<td></td>
</tr>
<tr>
<td>ALP</td>
<td>Fwd CCACGTCTTCAACATTTGGTG</td>
<td>NM_000478.4</td>
</tr>
<tr>
<td></td>
<td>Rev GCAGTGAGGCTTCTTGTC</td>
<td></td>
</tr>
<tr>
<td>COL1A1</td>
<td>Fwd GATGGATTCCAGTTCAGATATG</td>
<td>NM_000088.3</td>
</tr>
<tr>
<td></td>
<td>Rev GTTTGGGTGCTTCTGGTGT</td>
<td></td>
</tr>
<tr>
<td>OSX</td>
<td>Fwd TGGCTAGGTGTTGGGCCAGG</td>
<td>NM_001173467.2</td>
</tr>
<tr>
<td></td>
<td>Rev TGGGCAGCTGGGGGTTCAGT</td>
<td></td>
</tr>
<tr>
<td>BMPRIA</td>
<td>Fwd CAGAGATTGGGAATCCGCCTGC</td>
<td>NM_004329.2</td>
</tr>
<tr>
<td></td>
<td>Rev ATCGGGCCGTGGCATCT</td>
<td></td>
</tr>
<tr>
<td>BMPRIB</td>
<td>Fwd GCAAGCCTGCCATAAGTGAG</td>
<td>NM_001203.2</td>
</tr>
<tr>
<td></td>
<td>Rev CACAGGCAACACAGAGTCAT</td>
<td></td>
</tr>
<tr>
<td>BMPRII</td>
<td>Fwd CTGCAAATGGCCAAGCATGT</td>
<td>NM_001204.6</td>
</tr>
<tr>
<td></td>
<td>Rev ATGGTTGTAGCGATGCGCTCC</td>
<td></td>
</tr>
<tr>
<td>TGFBR1</td>
<td>Fwd ACCCTGCCTAGTGCAAGTTAC</td>
<td>NM_001130916.2</td>
</tr>
<tr>
<td></td>
<td>Rev AAGCCAAGTTTTTCCACCCCA</td>
<td></td>
</tr>
<tr>
<td>TGFBR2</td>
<td>Fwd GTTGGCGAGGAGTTTCCTGTT</td>
<td>NM_001024847.2</td>
</tr>
<tr>
<td></td>
<td>Rev GTCTATTACAGCTGGGGCA</td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>Fwd GGGGCGTGCAATGGCCCTCAA</td>
<td>NM_002046.5</td>
</tr>
<tr>
<td></td>
<td>Rev GGCTGGTGAGCTGGGTACCT</td>
<td></td>
</tr>
</tbody>
</table>
5.3.14 Immunoblotting

Day 2 hMSC sheets (N = 3/group) were homogenized in CellLytic™ MT lysis buffer (Sigma-Aldrich) supplemented with Halt™ protease and phosphatase inhibitor cocktail (Thermo Scientific). Equal amounts (15 µg) of protein lysates, determined by standard BCA protein assay kit (Pierce; Thermo Fisher Scientific), were subjected to SDS-PAGE using 10% NuPAGE® Bis-Tris gels (Invitrogen; Thermo Fisher Scientific) and transferred to 0.45 µm PVDF membranes (Millipore, Billerica, MA). Membranes were blocked with 5% bovine serum albumin (BSA) in standard TBST. The phosphorylation of intracellular SMAD3 and SMAD5 was detected using specific primary antibodies (anti-phospho-SMAD3 [ab52903]; anti-phospho-SMAD5 [ab92698]: Abcam, Cambridge, MA) followed by HRP-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA). Subsequently, the blots were stripped (Western Blot Stripping Buffer, Pierce; Thermo Fisher Scientific) and re-probed for the detection of the respective total protein (anti-SMAD3 [ab40854]; anti-SMAD5 [ab40771]: Abcam) and loading control (anti-β-Actin [A1978]: Sigma-Aldrich) with respective HRP-conjugated secondary antibodies (Jackson ImmunoResearch). Bound antibodies were visualized with the ECL detection system (Pierce; Thermo Fisher Scientific) on autoradiography film (Thermo Fisher Scientific). The intensity of immunoreactive bands was quantified using ImageJ software (National Institutes of Health, Bethesda, MD).

5.3.15 Statistical analysis

Differences in bone volume and bone volume accumulation rate by in vivo microCT at weeks 4, 8, and 12 were determined by two-way analysis of variance
(ANOVA) with Tukey’s *post hoc* test. Defect bridging was determined by chi-square test for trend in each group; comparisons between groups were assessed with individual chi-square tests and Bonferroni correction for multiple comparisons. *Ex vivo* microCT bone volume, bone volume fraction, and 3-D morphometry were assessed by one- or two-way ANOVA with interaction and Tukey’s *post hoc* test. Biomechanical properties were analyzed by two-way ANOVA with interaction and Tukey’s *post hoc* test. Mechanical property regressions were performed using an exhaustive best-subsets algorithm to determine the best predictors of maximum torque and torsional stiffness from a subset of morphologic parameters measured, including minimum or mean polar moment of inertia ($J_{\text{min}}$ or $J_{\text{mean}}$), bone volume fraction (BV/TV), binary bridging score (yes or no), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp), trabecular number (Tb.N), degree of anisotropy (DA), and connectivity density (Conn.D) based on Akaike’s information criterion (AIC)$^{224}$. The lowest AIC selects the best model while giving preference to models with less parameters. Finally, the overall best model for each predicted mechanical property was compared to its measured value using type II general linear regression. All data are shown with mean ± SD, some with individual data points, or as box plots showing median as horizontal line, mean as +, and 25th and 75th percentiles as boxes with whiskers at minimum and maximum values. Fold-changes in mRNA expression and ratios of phosphorylated SMADs/total SMADs were analyzed by one-way ANOVA with Tukey’s multiple comparison *post hoc* test. The significance level was set at $p<0.05$ or lower. Groups with shared letters have no significant differences. GraphPad Prism software v6.0 (GraphPad Software, La Jolla, CA) was used for all analyses.
5.4 Results

5.4.1 Effects of in vivo mechanical loading on autograft-mediated bone regeneration

Previously, we and others\textsuperscript{111,162,195,243,270}, found that \textit{in vivo} mechanical loading can enhance the regeneration of large bone defects. First, to test whether loading can enhance autograft-mediated bone regeneration, we treated critical-sized (8 mm) defects in Rowett nude (RNU) rat femora with morselized cortical bone autografts in two groups: control (stiff fixation plates) and delayed loading (compliant plates, unlocked to allow ambulatory load transfer at week 4) (Fig. 5.1A). Bone formation was evaluated over 12 weeks by longitudinal microcomputed tomography (microCT) imaging. Mechanical loading did not affect autograft-mediated bone formation (Fig. 5.1B,C). Since stiff load-bearing scaffolds can impede load-induced bone repair\textsuperscript{144}, we next tested whether non-load bearing mesenchymal condensations containing local presentation of BMP-2 could promote bone defect repair.
Figure 5.1: Effects of morselized autografts and in vivo mechanical loading on longitudinal bone formation and bone accumulation rate. (A) Stiff and compliant fixation plate configurations for dynamic control of ambulatory load transfer, and loading timeline with compliant plate unlocking at week 4. (B) Longitudinal quantification of bone volume at weeks 4, 8, and 12 by in vivo microCT (N = 6-8 per group). (C) Bone volume accumulation rate, defined as bone volume accrual over each 4-week interval. Data shown with mean ± SD. Box plots display median as horizontal line, mean as +, inter-quartile range as boxes, and min/max range as whiskers. Comparisons between groups were evaluated by two-way ANOVA with Tukey’s post-hoc tests. Repeated significance indicator letters (a,b,c) signify p > 0.05, while groups with distinct indicators signify p < 0.05 at each time point.
5.4.2 Comparison of BMP-2-containing mesenchymal condensations with the current standard of care in absence of mechanical loading

To this end, we compared the bone formation capacity of BMP-2-containing mesenchymal condensations with either autograft or BMP-2-loaded collagen sponge controls, without mechanical loading (i.e., stiff fixation). Mesenchymal condensations were assembled with mineral-coated hydroxypatite microparticles for in situ controlled presentation of 2 μg BMP-2\textsuperscript{220,233,273,271}. The BMP-2/collagen group received the same dose of BMP-2 (2 μg), adsorbed onto lyophilized collagen sponges, and the autograft group featured morselized cortical bone.

High-resolution ex vivo microCT analysis was performed at week 12 to evaluate bone formation and architecture. Both BMP-2/collagen and morselized autograft produced significantly greater bone volume fraction and trabecular number, and smaller trabecular separation compared to BMP-2-containing mesenchymal condensations (Fig. 5.2A-C). However, ectopic bone formation (i.e., bone extending beyond the 5-mm defect diameter) was significantly greater in defects treated with BMP-2 delivered on collagen compared to BMP-2-containing mesenchymal condensations (~3-fold) or autografts (~4-fold; Fig. 5.2B,C). While the development-mimetic mesenchymal condensations induced bone formation with less ectopic bone than BMP-2/collagen, their ultimate regenerative capacity was inferior.
Figure 5.2: Effects of BMP-2-primed engineered mesenchymal condensations and routine clinical therapies on new bone quantity and architecture in absence of mechanical cues. (A) Representative 3-D microCT defect reconstructions of mid-shaft transverse (top) and sagittal (bottom) sections at week 12, selected based on mean bone volume. Dashed circles show 5 mm defect region. Rectangular boxes illustrate transverse cutting planes. Scale bar, 1 mm. (B) Morphometry analysis of bone volume fraction, (C) trabecular number, (D) trabecular thickness, (E) trabecular separation, shown with native femoral head properties (N = 3; dotted lines with gray shading: mean ± SD; †p<0.05 vs. femoral head), and (F) ectopic bone formation (i.e., bone extending beyond the 5-mm defect diameter) at week 12 (N = 7-10 per group). Individual data points shown with mean ± SD. Comparisons between groups were evaluated by two-way ANOVA with Tukey’s post-hoc tests. Repeated significance indicator letters (a,b,c)
5.4.3 Controlled combinatorial morphogen presentation with in vivo mechanical load transfer

However, natural bone development and fracture repair occur through endochondral ossification in response to combined chondrogenic, osteogenic, and mechanical cues. Therefore, we hypothesized that these factors would be required in a combinatorial fashion to induce bone regeneration in a manner that reproduces natural bone formation. To this end, we next treated defects with mesenchymal condensations containing local presentation of TGF-β1 and/or BMP-2, with or without in vivo mechanical loading.

We previously showed that delayed in vivo mechanical loading, initiated at week 4 by compliant fixation plate unlocking, moderately enhanced (18%) bone regeneration by cell-free hydrogel-delivered BMP-2 and substantially enhanced (180%) endochondral regeneration by TGF-β1-incorporated mesenchymal condensations. Here, we investigated the interactions of mechanical loading with morphogen presentation. TGF-β1 was presented in gelatin microparticles for early release and BMP-2 from hydroxyapatite microparticles for sustained release. Three morphogen conditions were evaluated: mesenchymal condensations with empty microparticles (empty/controls), condensations with BMP-2-releasing microparticles, and condensations with BMP-2 + TGF-β1-releasing microparticles. Each was implanted in vivo with either stiff plates (control) or compliant plates unlocked at week 4 (delayed loading), for a total of six groups (Table 5.2).
### TABLE 5.2

**EXPERIMENTAL DESIGN**

<table>
<thead>
<tr>
<th>Group</th>
<th>Morphogen Condition</th>
<th>Mechanical Loading Condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Empty/Control</td>
<td>Stiff</td>
</tr>
<tr>
<td>2</td>
<td>BMP-2</td>
<td>Stiff</td>
</tr>
<tr>
<td>3</td>
<td>BMP-2 + TGF-β1</td>
<td>Stiff</td>
</tr>
<tr>
<td>4</td>
<td>Empty/Control</td>
<td>Delayed (compliant plate unlocked at week 4)</td>
</tr>
<tr>
<td>5</td>
<td>BMP-2</td>
<td>Delayed (compliant plate unlocked at week 4)</td>
</tr>
<tr>
<td>6</td>
<td>BMP-2 + TGF-β1</td>
<td>Delayed (compliant plate unlocked at week 4)</td>
</tr>
</tbody>
</table>

5.4.4 In vivo radiography and microCT analyses

Longitudinal X-ray radiography and microCT analyses were performed at weeks 4, 8, and 12. BMP-2-containing mesenchymal condensations enhanced bone regeneration relative to empty/controls at weeks 8 and 12 with both loading regimens (Fig. 5.3A,B red lines/boxes, C). Some instances of bridging were observed (stiff: 3/9; compliant: 4/7; Fig. 5.4). The BMP-2-mediated regenerative effects were significantly enhanced by TGF-β1 co-delivery (Fig. 5.3A,B blue lines/boxes, C). Bridging was achieved in nearly all dual-morphogen samples (stiff: 9/11; compliant: 9/10; Fig. 5.4).
Figure 5.3: Effects of morphogen priming of engineered mesenchymal condensations and \textit{in vivo} mechanical loading on longitudinal bone formation and bone accumulation rate. (A) Longitudinal quantification of bone volume at weeks 4, 8, and 12 by \textit{in vivo} microCT (N = 4-11 per group). Data shown with mean ± SD. (B) Bone volume accumulation rate, defined as bone volume accrual over each 4-week interval. Box plots display median as horizontal line, mean as +, inter-quartile range as boxes, and min/max range as whiskers. (C) Representative 3-D microCT reconstructions showing bone formation per group over time. Representative samples were selected based on mean bone volume at week 12. Scale bar, 3 mm. Comparisons between groups were evaluated by two-way repeated measures ANOVA with Tukey’s post-hoc tests. Repeated significance indicator letters (a,b,c) signify p > 0.05, while groups with distinct indicators signify p < 0.05 at each time point. Comparisons between time points were not assessed.
Mechanical loading significantly elevated the bone volume accumulation rate during the four weeks immediately after compliant plate unlocking in BMP-2 + TGF-β1-presenting mesenchymal condensations compared to all other groups and time intervals (Fig. 5.3B). New bone formation was negligible in empty/control samples, regardless of mechanical loading (Fig. 5.3A,B black lines/boxes, C), and none achieved bridging by week 12 (stiff: 0/8; compliant: 0/4; Fig. 5.4).

Figure 5.4: Effects of morphogen priming of engineered mesenchymal condensations and in vivo mechanical loading on defect bridging. (A) Longitudinal determination of defect bridging by in vivo radiography, defined as mineral fully traversing the defect (N = 4-11 per group). (B) Representative radiography images at 4, 8 and 12 weeks showing defect bridging per group over time, selected based on mean bone volume at week 12 (Figure 3). Significance of trend was analyzed by chi-square test (**p<0.01, ***p<0.001, ****p<0.0001). Differences between groups were determined by chi-square test at each time point with Bonferroni correction (p<0.01, correction factor of 5. Repeated significance indicator letters (a,b,c) signify p > 0.05, while groups with distinct indicators signify p < 0.05.
Thus, transplanted mesenchymal condensations induced bone regeneration dependent on morphogen identity, and mechanical loading influenced the rate of bone formation during the four weeks following load initiation in samples containing both BMP-2 and TGF-β1.

5.4.5 Ex vivo microCT analysis

Tissue composition and organization was then evaluated at high-resolution by ex vivo microCT analysis at week 12. Empty/control mesenchymal condensations without morphogen presentation failed to induce healing regardless of mechanical loading, with new bone formation merely capping the exposed medullary canals, predominantly on the proximal end (Fig. 5.5, Fig. 5.6). Both BMP-2 and BMP-2 + TGF-β1 presentation enhanced bone regeneration compared to empty/control mesenchymal condensations (Fig. 5.5A,B). New bone within the defects exhibited an approximately uniform proximal-to-distal distribution (Fig. 5.6A), and lacked notable ectopic bone (Fig. 5.6B), in contrast to BMP-2 delivery on collagen sponge (Fig. 5.2). Dual morphogen presentation and mechanical loading together produced regenerated bone with a trabecular internal architecture contained within a cortical shell, quantitatively similar in structure to native trabecular/cortical bone architecture (Fig. 5.5C-E; Fig. 5.6C-F). These data show that bone distribution and architecture were determined primarily by presented morphogen identity.
Figure 5.5: Effects of morphogen priming of engineered mesenchymal condensations and *in vivo* mechanical loading on new bone quantity and architecture. (A) Representative 3-D microCT reconstructions, with bone formation illustrated at mid-shaft transverse (top) and sagittal (bottom) sections at week 12, selected based on mean bone volume. Dashed circles show 5 mm defect region. Rectangular boxes illustrate transverse cutting planes. Note, due to minimal bone regeneration, additional transverse sections for stiff and compliant no growth factor controls were derived from the proximal end of the defect (small dashed circles and arrows). Scale bar, 1 mm. (B) Morphometric analysis of bone volume fraction, (C) trabecular number, (D) trabecular thickness, and (E) trabecular separation at week 12 (N = 4–11 per group), shown with corresponding measured parameters of femoral head trabecular bone (N = 3; dotted lines with gray shading; mean ± SD; † p<0.05 vs. femoral head). Individual data points shown with mean ± SD. Comparisons between groups were evaluated by two-way ANOVA with Tukey’s post-hoc tests. Repeated significance indicator letters (a,b,c) signify p > 0.05, while groups with distinct indicators signify p < 0.05.
Figure 5.6: Effects of morphogen priming of engineered mesenchymal condensations and in vivo mechanical loading on new bone distribution and architecture. (A) Morphometry analysis of proximal vs. distal bone volume distribution (N = 4-11 per group; p<0.05), and (B) ectopic bone formation (i.e., bone extending beyond the 5-mm defect diameter) shown with BMP-2 soaked on collagen data (N = 9; dotted line with green shading: mean ± SD). (C) Representative 3-D microCT reconstructions of native femoral head transverse (top) and sagittal (bottom) sections, selected based on mean bone volume. Dashed circles show 5 mm defect region. Rectangular box illustrates transverse cutting plane. Scale bar, 1 mm. (D) Morphometry analysis of connectivity density, (E) degree of anisotropy, and (F) structure model index within the defect region (N = 4-11 per group), shown with native femoral head properties (N = 3; dotted lines with gray shading: mean ± SD; †p<0.05 vs. femoral head). Individual data points shown with mean ± SD. Comparisons between groups were evaluated by two-way ANOVA with Tukey’s post-hoc tests. Repeated significance indicator letters (a,b,c) signify p > 0.05, while groups with distinct indicators signify p < 0.05.
5.4.6 Restoration of mechanical bone function

Next, we evaluated the restoration of limb mechanical properties by torsion testing to failure at week 12, in comparison to age-matched intact femurs. BMP-2 + TGF-β1-containing mesenchymal condensations enhanced stiffness and failure torque compared to empty/controls. Mechanical loading did not significantly alter mechanical properties compared to corresponding stiff plate controls for each group, but significantly increased the mean polar moment of inertia (a measure of structural cross-sectional geometry) and fully restored functional mechanical properties in the BMP-2 + TGF-β1 group (Fig. 5.7A-C), with statistically equivalent torsional stiffness and maximum torque at failure compared to age-matched intact femurs (Fig. 5.7A,B cf. gray band).

To identify the key structural predictors of mechanical behavior, we performed a type II multivariate best-subsets regression analysis with model predictors selected by minimization of the Akaike’s information criterion (AIC). For torsional stiffness, bone volume fraction, trabecular separation, and minimum pMOI were the best combined predictors (Fig. 5.7E). For maximum torque, bone volume fraction and minimum pMOI were the best combined predictors (Fig. 5.7F). Thus, the mechanical properties were determined by the amount, distribution, and trabecular organization of the regenerate bone.

Together, these data indicate that restoration of biomechanical competence was dependent on the identity of presented morphogens and induced full functional repair only by dual morphogen presentation with in vivo mechanical loading.
Figure 5.7: Effects of morphogen priming of engineered mesenchymal condensations and *in vivo* mechanical loading on functional defect regeneration. (A) Torsional stiffness, (B) maximum torque at failure, (C) mean polar moment of inertia (pMOI), and (D) minimum pMOI. Best subsets regression analysis ($R^2$) with lowest Akaike’s information criterion (AIC) value for measured and predicted (E) torsional stiffness and (F) maximum torque at failure indicating significant contributions of bone volume fraction (BV/TV), trabecular separation (Tb.Sp), and minimum pMOI ($J_{\text{min}}$). Individual data points shown with mean ± SD (N = 3-10 per group). Comparisons between groups were evaluated by two-way ANOVA with Tukey’s post-hoc tests. Repeated significance indicator letters (a,b,c) signify $p > 0.05$, while groups with distinct indicators signify $p < 0.05$.

Biomechanical and structural parameters are shown with age-matched intact bone properties, with pMOI obtained from the same midshaft ROI as used for the defects (N = 3; dotted lines with gray shading: mean ± SD; †,#$p<0.05$ vs. intact bone).
5.4.7 In vitro signaling and differentiation analyses

We hypothesized that the cellular organization into condensations and the development-mimetic morphogen presentation would induce endochondral bone formation. TGF-β superfamily ligands bind to type I and II serine/threonine kinase receptor complexes and transduce signals via SMAD proteins\textsuperscript{275}. In the developing limb, TGF-β signaling has been shown to occur early during the chondrogenic cascade, prior to the BMPs\textsuperscript{58,266,267}. Further, a recent study proposed that transient activation of the TGF-β pathway may be required to promote a chondrogenic response to BMP signaling during later stages of chondrogenesis\textsuperscript{276}. Therefore, we next tested the effects of combinatorial morphogen presentation on chondrogenic and osteogenic activity of microparticle-containing hMSC sheets used to form the mesenchymal condensations \textit{in vitro}. As prepared for \textit{in vivo} transplantation, TGF-β1 was presented in an early manner by release from gelatin microspheres, while BMP-2 was released in a more sustained manner from mineral-coated hydroxyapatite microparticles.

After two days \textit{in vitro} culture (coinciding with the time of \textit{in vivo} transplantation), the engineered hMSC sheets exhibited homogeneous cellular organization across groups, with uniformly distributed microspheres and no detectable GAG or mineral deposition (Fig. 5.8A). Transcript analysis of key differentiation markers revealed that either TGF-β1 or BMP-2 presentation alone significantly induced mRNA expression of genes indicative of both chondrogenic (SOX9, aggrecan (ACAN), and collagen type 2A1 (COL2A1)) and osteogenic (alkaline phosphatase (ALP)) priming, relative to growth factor-free controls (Fig. 5.8B). BMP-2 + TGF-β1 co-delivery further
increased expression of SOX9, ACAN, COL2A1, ALP and osterix (OSX) mRNA (Fig. 5.8B). Lastly, BMP-2 presentation significantly increased both SMAD3 and SMAD5 phosphorylation relative to empty controls without growth factor, and was significantly potentiated by TGF-β1 co-delivery (Fig. 5.8C,D). These in vitro data suggest that presentation of either BMP-2 or BMP-2 + TGF-β1 induced chondrogenic lineage priming via both SMAD3 and SMAD5 signaling at the time on implantation.
Figure 5.8: Effects of morphogen priming of engineered mesenchymal condensations on \textit{in vitro} chondrogenic lineage specification at the time of implantation. Histological Safranin-O/Fast green staining of representative microparticle-containing hMSC sheets at the time of implantation (2 days; N = 3 per group). Scale bars, 100 μm (10x: top; 40x: bottom, magnification of dotted squares). (B) Normalized mRNA fold-change over control of key chondrogenic or osteogenic markers by qRT-PCR (N = 3 per group; *p<0.05, **p<0.01, ***p<0.001 vs. empty/control; $p<0.05$ vs. BMP-2-containing hMSC sheets). (C) Representative immunoblots and (D) relative quantification of p-SMAD5/SMAD5 and (E) p-SMAD3/SMAD3 in lysates of day 2 hMSC sheets (N = 3 per group). β-Actin served as loading control. Individual data points shown with mean ± SD. Comparisons between groups were evaluated by one-way ANOVA with Tukey’s post-hoc tests. Repeated significance indicator letters (a,b,c) signify $p > 0.05$, while groups with distinct indicators signify $p < 0.05$.
5.4.8 In vivo tissue differentiation and composition

Next, to test the combinatorial effects of morphogen presentation and mechanical loading on local tissue differentiation, endochondral lineage progression, and matrix organization in vivo, we performed histological analyses of defect tissues at weeks 4 and 12. Empty/control mesenchymal condensations exhibited predominantly fibrous and adipose tissue spanning the defects, and bone formation was apparent only capping the diaphyseal ends (Fig. 5.9A,B; A.3, A.4). BMP-2-containing mesenchymal condensations induced the formation of primary woven bone and lamellar bone with lacunae-embedded osteocytes surrounded by marrow-like tissue by week 4, and increased lamellar bone by week 12 (Fig. 5.9A). Aside from stiff control at 4 weeks, empty/control and BMP-2-containing groups exhibited minimal Safranin-O-stained glycosaminoglycan (GAG) matrix at both time points. (Fig. 5.9A,B; A.3, A.4). Co-delivery of BMP-2 + TGF-β1 induced robust bone formation exhibiting lacunae-embedded osteocytes in well-defined trabeculae with peripheral positive GAG-staining, evidence of prior cartilaginous template transformation. Mechanical loading of BMP-2 + TGF-β1-containing mesenchymal condensations promoted formation of growth plate-like, transverse cartilage bands that featured zonal organization of mature and hypertrophic chondrocytes with prominent Safranin-O-stained GAG matrix embedded in trabecular bone and aligned orthogonal to the principal ambulatory load axis (Fig. 5.9A,B). Hypertrophic chondrocytes and new bone formation at the interface were indicative of active endochondral bone formation in the dual morphogen group with mechanical loading at both 4 and 12 weeks (Fig. 5.9B).
Together, these data suggest that, though both BMP-2 and BMP-2 + TGF-β1 induced chondrogenic priming prior to implantation, endochondral ossification \textit{in vivo} was most strongly apparent with morphogen co-presentation. Further, \textit{in vivo} mechanical cues potentiated cartilage formation and prolonged endochondral ossification.

Figure 5.9: Effects of morphogen priming of engineered mesenchymal condensations and \textit{in vivo} mechanical loading on tissue-level bone regeneration. Representative histological (A) H&E and (B) Safranin-O/Fast green staining of defect tissue at week 4 (left) and week 12 (right), with stiff (top) and compliant fixation (bottom), selected based on mean bone volume. Scale bars, 100 μm (40x).
5.5 Discussion

The aim of this study was to replicate the cellular, biochemical, and mechanical environment present during limb development for functional regeneration of large segmental bone defects. Specifically, we used (i) engineered mesenchymal condensations formed by cellular self-assembly, that contained (ii) microparticle-mediated growth factor presentation to activate specific morphogenetic pathways \textit{in situ}, and which, upon implantation, were exposed to (iii) \textit{in vivo} mechanical loading. We tested the hypothesis that TGF-\(\beta\)1 and/or BMP-2 presentation from encapsulated microparticles within engineered mesenchymal condensations would promote endochondral regeneration of critical-sized femoral defects in a manner dependent on the \textit{in vivo} mechanical environment. While both BMP-2 and BMP-2 + TGF-\(\beta\)1 presentation induced chondrogenic priming at the time of \textit{in vivo} transplantation, endochondral ossification was most strongly evident in the dual morphogen group and was enhanced by mechanical loading. Specifically, \textit{in vivo} ambulatory mechanical loading significantly enhanced the rate of bone formation in the four weeks after load initiation in the dual morphogen group, improved bone distribution in the callus, and fully restored mechanical bone function. In contrast, mechanical loading had no effect on bone regeneration in empty/control mesenchymal condensations without morphogen presentation, and likewise had no effect on autograft-mediated repair.

Multiple reports have described self-assembled hMSC aggregates to form cartilage templates that can undergo hypertrophy and progress through endochondral ossification \textit{in vivo}\textsuperscript{126,128,130,156,157,159,268}. In these studies, chondrogenic priming was achieved by means of exogenously supplied morphogens, involving \textit{in vitro} pre-culture...
of 3 weeks and longer\textsuperscript{126,128,130,156,157,159,268}. This requires time and associated costs and limits the precision of morphogen spatial distribution control. Further, few studies to date have achieved function-restoring bone formation in orthotopic models using this strategy\textsuperscript{157,234,277}. We previously demonstrated that sequential \textit{in situ} morphogen presentation to mesenchymal condensations, utilizing mineral-coated hydroxyapatite microparticles\textsuperscript{273} and crosslinked gelatin microspheres\textsuperscript{269} to control the bioavailability of BMP-2 and TGF-β1, respectively, facilitates both chondrogenic and osteogenic differentiation \textit{in vitro}\textsuperscript{218}, and promotes calvarial bone regeneration via endochondral ossification \textit{in vivo}\textsuperscript{233}. We also found in mesenchymal condensations with local TGF-β1 presentation that devitalization of the cells after condensation assembly but prior to transplantation abrogated bone formation, suggesting that both the presented morphogens and cells are important for the endochondral bone formation response\textsuperscript{270}. Here, we show that this spatiotemporally controllable and localized morphogen delivery strategy, inspired by early limb development, eliminates the need for time- and cost-ineffective pre-differentiation of the cellular constructs and achieved mechanically-functional regeneration without the ectopic bone formation associated with BMP-2/collagen.

In addition to their efficacy in morphogen presentation, the mesenchymal condensations facilitated endochondral healing by providing a non-structural, immature intermediate, much like a callus in fracture healing or cartilage anlage in limb development. We showed previously that structural scaffolds that mimic the material properties of mature bone shield tissue from the stimulatory and beneficial effects of mechanical load during healing\textsuperscript{144}, suggesting that having a flexible intermediate structure is a more suitable graft material for mechanical regulation of bone regeneration.
Here, we found that in vivo mechanical loading via compliant fixation exerted stimulatory effects on defect healing, particularly in the period of plate actuation (4-8 wks), which resulted in complete functional bone regeneration (i.e., restoration of biomechanical competency comparable to un-operated, intact limbs). These data indicate the importance of morphogen presentation for stem cell-mediated regeneration of bone defects, and potentially imply that the high stiffness of autograft bone may interfere with load-induced bone formation.

Compressive interfragmentary motion is necessary for cartilaginous callus formation and endochondral ossification during fracture healing\textsuperscript{23,170}, and here the presence of growth plate-like cartilage structures, exhibiting zonal organization of mature and hypertrophic chondrocytes embedded in marrow-containing trabecular bone, suggests that BMP-2 + TGF-β1-containing mesenchymal condensations facilitated defect healing chiefly through endochondral ossification. This was consistent with a recent study demonstrating that a chondrogenic response to BMP-4 is dependent upon transient activation of TGF-β signaling in the early limb bud\textsuperscript{276}. In vitro analysis confirmed robust chondrogenic priming of the cellular constructs at the time of surgery. While this was also the case with BMP-2-only presenting mesenchymal condensations, upon defect implantation these constructs stimulated overall inferior bone regeneration compared to dual morphogen groups independent of the in vivo mechanical environment.

Nevertheless, no ectopic bone formation, as seen with BMP-2 soaked on collagen at ~2 μg\textsuperscript{194}, was observed, similar to autografts as the other clinical standard we initially tested our technology against. This suggests an improved safety profile in the context of BMP-2 delivery from scaffold-free, self-assembled cellular constructs. The mechanical
environment also influences neovascularization during endochondral ossification, and we recently showed that mechanical forces regulate angiogenesis and vascular remodeling during endochondral regeneration of large bone defects by TGF-β-containing mesenchymal condensations\textsuperscript{270}. Future studies will explore the interactions of morphogen identity and presentation with mechanical cues for large bone defect regeneration.

In conclusion, this study presents a human progenitor cell-based bone tissue engineering approach that recapitulates certain aspects of the normal endochondral cascade in early limb development. Implantation of chondrogenically-primed high-density mesenchymal condensations, achieved through \textit{in situ} morphogen presentation rather than lengthy pre-culture, in large bone defects that would otherwise not heal if left untreated, re-established biomechanical competency in limbs stabilized with custom compliant fixation plates with elective actuation at 4 weeks, after stable fixation to initiate bone regeneration.

Further studies may elucidate the role of elective actuation timing in this regenerative strategy. Here, all mechanically loaded plates were unlocked at 4 weeks regardless of growth factor treatment. However, this does not take into account the combination of tissues in each defect that were shown to differ with growth factor treatment, and may affect their different responses to mechanical load. Though challenging for this particular animal model, future studies could assess more radiographic time points in order to determine the most appropriate point of unlocking for each group or animal. This would in fact be more akin to clinical bone repair approaches in which sufficient tissue growth is observed before stresses can be applied to the affected bone. Alternatively, in this model, the maturity of the mesenchymal condensation could
be modulated before implantation, to understand how the cell state at the time of implantation influences the endochondral response to mechanical load. Our findings are of clinical relevance and advance the current understanding in the growing field of developmental engineering. Furthermore, the system described herein can be used to study the complex biophysical mechanisms that govern tissue regeneration in health and disease.
CHAPTER 6:
THE TIMING OF MECHANICAL LOADING MODULATES ENDOCHONDRAL OSSIFICATION OF CHONDROGENICALLY PRIMED MSCS

6.1 Introduction

Endochondral ossification is a mode of secondary bone formation that occurs during development and fracture healing, whereby condensing mesenchymal stem cells form a cartilage template that is remodeled to bone with invading vasculature, and has recently emerged as an attractive tissue engineering option to regenerate large bone defects. Current methods typically incorporate harvesting readily available donor mesenchymal stem cells (MSCs) to scale up tissue engineered grafts. After chondrogenic priming, MSC-derived chondrocytes tend to progress to hypertrophy despite attempts to maintain a stable cartilage phenotype, making them a poor choice for cartilage engineering, but an advantageous choice for bone engineering through endochondral ossification\(^\text{80}\). However, endochondral approaches have achieved limited success recapitulating this developmental process, in part due to our incomplete understanding of chemical and mechanical cues that direct MSC lineage progression.

In both skeletal development and fracture healing, mechanical loading is essential to induce endochondral ossification. *In utero*, chemical or surgical induction of muscular paralysis inhibits bone growth through reduced chondrocyte proliferation and delayed
ossification, while, in fracture repair, some compressive motion is necessary for callus formation and endochondral healing. The effects of loading in fractures and cells in vitro depend on the mode, rate, magnitude as well as gap size and time of applied load. For example, early loading of high strain to a fracture callus produces a robust cartilage response and possible pseudoarthrosis and rarely results in full bone healing. Similarly, high strain compressive loads in vitro have been shown to increase cartilage matrix production while suppressing expression of hypertrophic markers. Conversely, loading that is delayed until after a degree of chondrogenic hypertrophy in fracture or critically sized defect models may be more beneficial to ossification. Claes et al, observed greater fracture healing in osteotomies dynamized late than those loaded early, due to a stiffer callus decreasing interfragmentary strains.

In vitro, delayed loading promotes more bone matrix synthesis in chondrogenically-primed MSCs, indicating that some chondrogenic differentiation may be necessary before loading is beneficial for bone healing.

We reported previously that mechanical loading was essential for stem-cell mediated endochondral regeneration of critical-sized defects in vivo, but this was also dependent on load timing. Mechanical loading initiated in defects after the onset of chondrocyte hypertrophy and osteogenesis (week 4) exhibited the greatest extent of bone regeneration, while immediate loading enhanced bone formation but prolonged chondrogenesis, inhibited early vessel invasion, and caused pseudoarthrosis. Together, these data suggest a significant interaction between temporal mechanical loading and lineage specification of progenitor cells during endochondral ossification, but the
influence of the timing of load initiation during chondrogenic priming of mesenchymal stem cells (MSCs) remains unclear.

In this study we applied dynamic compression to fibrin-encapsulated human mesenchymal stem cells (hMSC) after different degrees of chondrogenic priming in a custom-made bioreactor, and investigated endochondral gene expression and matrix production. Fibrin hydrogels were chosen as an encapsulation material that is easily mechanically manipulated in vitro, and best mimics the tissue in the fracture environment. Here we hypothesized that early loading in vitro would promote chondrogenesis of MSCs and delay or arrest endochondral ossification, while delayed loading would promote chondrocyte maturation, hypertrophy, and ossification.

6.2 Methods and Materials

6.2.1 Study design

Part 1: Dynamic cyclic unconfined compression (DC) was applied to hMSC laden fibrin hydrogels for 2 weeks after different degrees of chondrogenic priming and compared to time-matched free swelling (FS) controls. The hydrogels were collected at the end of their loading cycle for analysis. These groups are labeled throughout the study according to their priming time (Table 6.1).

Part 2: Dynamic cyclic unconfined compression (DC) was applied to hMSC laden fibrin hydrogels for 2 weeks in chondrogenic media without growth factor or supplemented with 1ng/mL TGF-β3.
TABLE 6.1

STUDY DESIGN

<table>
<thead>
<tr>
<th>Part 1: Media +10ng/mL TGF-β3</th>
<th>0 weeks</th>
<th>0 weeks of priming + 2 weeks of loading compared to free swelling for 2 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 weeks</td>
<td>2 weeks</td>
<td>2 weeks of priming + 2 weeks of loading compared to free swelling for 4 weeks</td>
</tr>
<tr>
<td>4 weeks</td>
<td>4 weeks</td>
<td>4 weeks of priming + 2 weeks of loading compared to free swelling for 6 weeks</td>
</tr>
<tr>
<td>6 weeks</td>
<td>6 weeks</td>
<td>6 weeks of priming + 2 weeks of loading compared to free swelling for 8 weeks</td>
</tr>
<tr>
<td>Part 2: Media +/- 1ng/mL TGF-β3</td>
<td>Repeat 0 weeks</td>
<td>0 weeks priming + 2 weeks loading</td>
</tr>
</tbody>
</table>

6.2.2 Cell culture

Human bone marrow derived mesenchymal stem cells (hMSCs, P3) (Lonza) were expanded in high glucose Dulbecco’s modified eagles medium (4.5 mg/mL glucose, 200 mM l-glutamine, hgDMEM) that was supplemented with 10% fetal bovine serum (FBS), 1% Penstrep, and 5 ng/mL FGF-2. The cultures were expanded to passage 3 and maintained in a humidified environment at 37 C, 5% CO2, and 5% O2.

6.2.3 Fibrin gel preparation and chondrogenic culture

At 80% confluency hMSCs were trypsinized and resuspended (P3) in a 10,000KIU/mL aprotinin solution (Nordic Pharma) with 19 mg/mL sodium chloride and 100mg/mL bovine fibrinogen (Sigma-Aldrich). This was combined 1:1 with a solution of 5U/mL thrombin and 40mM CaCl₂ for a final solution of 50mg/mL fibrinogen, 2.5U/mL thrombin, 5000KIU/mL aprotinin, 17mg/mL sodium chloride, 20mM CaCl₂, and 15x10⁶ cells/mL. Gel solution was pipetted into 5mm diameter x 2mm thickness cylindrical
agarose molds to create uniform constructs with a total cell volume of approximately 600,000 each.

Throughout the study, culture was maintained in chondrogenic media consisting of hgDMEM supplemented with 1% Penstrep, 100 KIU/mL aprotinin, 100 μg/mL sodium pyruvate, 40 μg/mL L-proline, 1.5 mg/mL bovine serum albumin, 4.7 μg/mL linoleic acid, 1x insulin-transferrin-selenium, 50 μg/mL L-ascorbic acid-2-phosphate, 100 nM dexamethasone (all Sigma-aldrich), and 10ng/mL or 1ng/mL TGF-β3 (ProSpec-Tany TechnoGene Ltd., Israel). Fresh media was supplied every 3 days and culture was maintained in a humidified environment at 37°C, 5% CO₂, and 5% O₂.

6.2.4 Dynamic compression

Dynamic unconfined compressive loading was applied to the constructs using a custom-made bioreactor (Fig. 6.1A,B). Load was applied 2 hours per day, 5 days a week at 1Hz and 10% strain after a .01N preload was applied using an in-house Matlab code.

6.2.5 Biochemical analysis

All constructs were harvested and analyzed at the end of their loading cycle for biochemical content (n=5). Samples were digested overnight at 60°C in a solution of 125 μg/mL papain, 0.1M sodium acetate, 5mM L-cysteine, 0.05M EDTA (all Sigma-Aldrich). DNA content was quantified with Hoechst Bisbenzimide 33258 dye assay (Sigma-Aldrich) as described previously²⁷⁹, and sulfated glycosaminoglycan content was quantified using the dimethylmethylene blue dye-binding assay (Blyscan; Biocolor Ltd.). Ortho-hydroxyproline was measured by dimethylaminobenzaldehyde and chloramine T
assay. Hydroxyproline to collagen ration of 1:7.69 was used to represent total collagen content.

6.2.6 RNA isolation and qPCR

Total RNA was extracted from fibrin/hMSC constructs after each loading cycle using RNeasy mini kit (Qiagen). RNA (300ng) was reverse transcribed using into cDNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Gene expression changes relative to free swelling controls were quantified via real-time reverse transcription-polymerase chain reaction (qRT-PCR). Reactions were carried out in triplicate 20µL volumes of 10µL Sybr Green Master Mix (Applied Biosystems), 30ng cDNA, 400nM Sigma Kicqstart forward and reverse primers, on an ABI 7500 real-time PCR system (Applied Biosystems) with a profile of 95°C for 10 min, and 40 cycles of denaturation at 95°C for 15 sec, and annealing/amplification at 60°C for 1min. Quantification of target genes (Table 6.2) was determined against housekeeping reference gene GAPDH as fold change over free swelling controls using the delta CT method.
### TABLE 6.2

**QPCR TARGET GENES**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence 5'-3'</th>
<th>Accession Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>Fwd CTTTTGCGTCGCCAG Rev TTGATGGCAACCAATATCCAC</td>
<td>NM_002046</td>
</tr>
<tr>
<td>SOX9</td>
<td>Fwd CTCTGGAGACTTCTGAACG Rev AGATGTGCGTCTGCTC</td>
<td>NM_000346</td>
</tr>
<tr>
<td>ACAN</td>
<td>Fwd TGCGGGTCAACAGTCCTATC Rev CACGATGCCTTTTACCACAGC</td>
<td>NM_001135</td>
</tr>
<tr>
<td>Col2a1</td>
<td>Fwd GAAGAGTGAGAGACTACTGG Rev CAGATGTGTITTCTCTCCTTGG</td>
<td>NM_033150</td>
</tr>
<tr>
<td>OPN (SPP1)</td>
<td>Fwd GACCAAGGAAGACTACTAC Rev CTGTTAAGGTGATGCGA</td>
<td>NM_001251829</td>
</tr>
<tr>
<td>RUNX2</td>
<td>Fwd AAGCTTGATGACTTAAACC Rev TCTGTAACACTGTCTGCC</td>
<td>NM_001015051</td>
</tr>
<tr>
<td>Col10a1</td>
<td>Fwd GCTAGATCCTTGAACTTGG Rev CTTTTACTCTTTATGGTGTAGG</td>
<td>NM_000493</td>
</tr>
<tr>
<td>VEGFA</td>
<td>Fwd AATGTGAAATGAGACACAAAG Rev GACTTACCCGGGATTTCTTG</td>
<td>NM_001204384</td>
</tr>
</tbody>
</table>

#### 6.2.7 Histology and immunohistochemistry

Hydrogels were fixed in 4% paraformaldehyde (n=2) overnight at 4°C. Constructs were halved and paraffin embedded cut surface down, and sectioned at 5µm to provide a cross-section of the hydrogel center. Sections were cleared in xylene, rehydrated in graded alcohols, and stained for sGAG in 1% Alcian blue with 0.1% Nuclear Fast Red counter stain.

Deposition of col2a1 (Santa Cruz) and col6a1 (Abcam) were identified through immunohistochemistry. Antigen retrieval was either heat mediated (col6a1, Innovex Unitrieve) or enzymatic (col2a1, chondroitinase ABC, Sigma-Aldrich). Slides were blocked with Innovex Background Buster and incubated with primary antibody, diluted in PBS, overnight at 4°C. Slides were washed with PBS and incubated for 10 mins with 160
universal rabbit IgG secondary antibody, followed by 10 mins with HRP enzyme, and DAB substrate for 5 mins (all Innovex). Sections were counterstained in Hematoxylin (VWR), and mounted with Cytoseal XYL.

6.2.8 Mechanical testing

Hydrogels were mechanically tested in unconfined compression (Zwick Roell Z005, Herefordshire, UK) between two steel platens and a 5N load cell. Hydrogels were hydrated in a PBS bath at room temperature. Equilibrium modulus was obtained via a stress relaxation test where a 10% strain was applied and maintained until equilibrium was reached. Dynamic modulus was determined after 10% strain was applied for 10 cycles at 1Hz and at 0.1Hz. In both tests a preload of 0.01N was applied to ensure contact between the hydrogel and machine platens.

6.2.9 Statistics

All loaded groups are compared to their free swelling controls via either two-tailed student t-tests or one-way ANOVA with multi comparison by tukey’s post hoc where significance was determined as p < 0.05.

6.3 Results part 1:

In order to investigate how the degree of chondrogenic priming and mechanical loading intersect in mesenchymal cell lineage progression, we utilized a custom-made bioreactor to apply dynamic compression to hMSC-laden hydrogels (Fig. 6.1A,B). Hydrogels were permitted to mature in chondrogenic media for either 0, 2, 4, or 6 weeks prior to 2 weeks of dynamic compression. All samples were harvested at the end of their
loading cycle for analysis and compared to a free swelling control that continued to mature in chondrogenic media during the 2 week loading period (Fig. 6.1C). For this reason, different timed groups cannot be statistically compared, and comparisons over time are made qualitatively based on progression of free swelling controls over the 8 weeks period, and how application of mechanical load affects that progression.

Figure 6.1: Bioreactor setup and loading timeline. (A) hMSC-laden hydrogels (P3, 600,000 cells, 50mg/mL fibrinogen) were subjected to different degrees of chondrogenic priming (0wks, 2wks, 4wks, 6wks) followed by 2 weeks of (B) dynamic compression in a custom-made bioreactor. (C) All samples were collected after their loading cycle and compared to free-swelling controls at the same time.
6.3.1 Biochemical content

Dynamic compression only significantly increased DNA content from 0-2 weeks (0wks priming; (Fig. 6.2A), and only increased sulfated glycosaminglycan (sGAG) deposition when it was applied from 2-4 weeks (2wks priming), but this effect was lost when normalized to DNA content (Fig. 6.3). There were no significant effects of dynamic compression on collagen deposition (calculated via hydroxyproline content), however, cartilage matrix deposition in all cases increased steadily over time, indicative of continual chondral priming. Alcian blue staining for sGAG matrix depict a large deposition over time with large chondrocyte like cells, though differences in content with the application of dynamic compression are not readily observable (Fig. 6.2B).
Figure 6.2: Biochemical content of mechanically stimulated hydrogels. (A) DNA, sulfated glycosaminoglycans (sGAG), and collagen content calculated after each loading cycle in comparison to free swelling controls. Data are displayed as mean ± s.d. with individual data points. Two-tailed student t-test was used to determine significance (*p<0.05) (B) Alcian blue staining for sGAG with nuclear fast red counter stain (scale bar =100µm).
Figure 6.3: Normalized biochemical content. (A) sGAG content and (B) Collagen content normalized to hydrogel DNA quantity after their loading cycle in comparison to free-swelling controls. Data are displayed as mean ± s.d. with individual data points. Two-tailed student t-test was used to determine significance (*p<0.05).

6.3.2 Chondrogenic gene expression

Mechanical loading decreased SOX9 expression at 0wks priming (Fig. 6.4A), where qPCR findings are shown as fold change of loaded groups over their free swelling controls at the time of harvest. Groups are not statistically compared to each other, but displayed together for qualitative comparison over time. Dynamic compression had no effect on message level expression of aggregan (ACAN) after 0 weeks of priming, but increased expression at every other priming time point. Expression of Col2a1 was increased at every time point by the application of dynamic compression, but this was not reflected at the protein level by immunohistochemistry (IHC) staining for Col2a1 (Fig. 6.4B).
Figure 6.4: Chondrogenic differentiation of hydrogels. (A) Samples were analyzed at the end of their loading cycle for message level expression of SOX9, ACAN, and Col2a1 calculated as fold change over free swelling controls. Data are shown as mean ± s.d. with individual data points. Two-tailed student t-test was used to determine significance (*p<0.05) (B) Immunohistochemistry for Col2a1 with hematoxylin counterstain (scale bar= 100µm).

6.3.3 Hypertrophic maturation

Loading increased Col10a1 expression after 0 weeks and 6 weeks of priming, but showed no differences at 2 or 4 weeks. VEGF, another marker of chondrocyte hypertrophy had a variable response (Fig. 6.5A). There are not large differences in Col10a1 protein deposition (Fig. 6.5B), but loading affected the distribution, where
deposition is more uniform throughout free swelling samples and appears mainly around the periphery in when dynamically loaded.

Figure 6.5: Hypertrophic maturation of hydrogels in response to mechanical load. (A) Samples were analyzed at the end of their loading cycle for message level expression of hypertrophic genes Col10a1 and VEGF as fold change over free swelling controls. Data are displayed as mean ± s.d. with individual data points. Two-tailed student t-test was used to determine significance (*p<0.05) (B) Immunohistochemistry for Col10a1 with hematoxylin counterstain (scale bar= 100µm).

6.3.4 Osteogenic expression in response to load

Loading decreased expression of osteogenic marks RUNX2 and OPN after 4 weeks, and 2 and 4 weeks priming respectively, but this suppressive effect is lost after 6
weeks of priming (Fig. 6.6A). Similarly, alizarin red staining for mineral was negative in all groups until after 4 weeks priming and harvest at 6 weeks where positive staining can be seen in free swelling controls. Loaded groups at this time point are negative. However, after 6 weeks priming and 2 weeks loading, both dynamic compression and free swelling controls exhibit positive alizarin red staining (Fig. 6.6B).
Figure 6.6: Osteogenic suppression in response to load. (A) Samples were analyzed at the end of their loading cycle for message level expression of osteogenic genes RUNX2 and OPN calculated as fold change over free swelling controls. Data are shown as mean ± s.d. with individual data points. Two-tailed student t-test was used to determine significance (*p<0.05) (B) Alizarin Red stain for hydroxyapatite (scale bar= 100µm).
6.3.5 Mechanical properties

After each loading cycle hydrogels were mechanically tested in unconfined compression. Loading did not affect equilibrium modulus in comparison to controls at each time point, but modulus did increase over time, coinciding with matrix deposition (Fig. 6.7A). Dynamic modulus increased in response to load after 4 and 6 weeks of priming (Fig. 6.7B).

Figure 6.7: Mechanical properties of hydrogels over time. Samples were tested in unconfined compression for (A) equilibrium modulus and (B) dynamic modulus at the end of the loading cycle in comparison to free swelling controls. Data are shown as displayed as mean ± s.d. with individual data points. Two-tailed students t-test was used to determine significance (*p<0.05).

6.3.6 Pericellular matrix formation

Staining for pericellular matrix (PCM) protein collagen 6 (Col6a1) did not indicate significant changes between dynamically loaded groups and controls, but staining did decrease over time in all samples, indicating a shift from pericellular matrix components to tissue matrix (Fig. 6.8).
6.4 Results part 2:

In order to decouple the effects of loading and exogenous growth factor we repeated two weeks of loading (0-2 weeks) with either a reduced 1ng/mL TGF-beta in chondrogenic media or media without TGF-beta supplementation.

6.4.1 Biochemical content

DNA and sGAG content were significantly increased in groups that were supplemented with growth factor and received dynamic compression (Fig. 6.9A), but when normalized to DNA the dynamically loaded groups without growth factor (GF-, DC+), is no longer significantly different. Alcian blue staining reveals that free swelling
samples without growth factor (GF-, DC-) were virtually devoid of sGAGs, and there were only small instances of staining when dynamic compression (GF-, DC+) was applied (Fig. 6.9B). Groups that received growth factor exhibit light staining for sGAG typical of this time point, and staining is more pronounced when load is applied.

Figure 6.9: Matrix production in response to load without the presence of growth factor. Hydrogels were cultured with or without 1ng/mL TGF-β3 (+/-GF) and were subjected to dynamic compression for 2 weeks, or allowed to free swell. (A) Gross quantity of DNA, sGAG, or sGAG normalized to DNA. Data are shown as mean ± s.d. with individual data points. Significance between groups was determined by one-way ANOVA and multi comparison by tukey’s post hoc where groups sharing letters (a,b,c) are not statistically different. (B) Alcian blue with nuclear fast red counter staining for sGAG, (scale bar = 100µm)
6.4.2 Gene expression

Dynamic compression was able to increase message level expression of col2a1 in unsupplemented media, to comparable levels (Fig. 6.10a) as those supplemented with growth factor, however this effect was not seen in IHC staining for Col2a1 protein (Fig. 6.10B). On the contrary, though DC did not have effect on message level expression in growth factor treated groups, Col2a1 protein was visibly increased in comparison to free swelling treated controls.
Figure 6.10: Chondrogenic gene expression in response to loading without the presence of growth factor. Hydrogels were cultured with or without 1ng/mL TGF-β3 (+/-GF) and were subjected to dynamic compression for 2 weeks, or allowed to free swell (+/-DC). (A) Message level expression of Sox9, ACAN, and Col2a1 calculated as fold change over free swelling samples without growth factor (GF-, DC-). Data are shown as mean ± s.d. with individual data points. Significance between groups was determined by one-way ANOVA multi comparison by tukey’s post hoc where groups sharing letters (a,b,c) are not statistically different. (B) Col2a1 immunohistochemistry (IHC) with nuclear fast red counter stain. (scale bar = 100µm)
6.5 Discussion and conclusion

In this study we investigated how the amount of priming prior to mechanical load affects lineage specification of progenitor cells, and whether or not these effects are contingent on growth factor priming. Loading significantly increased DNA content when applied from 2-4 weeks (2 weeks priming) and increased sGAG content from 4-6 weeks (4 weeks priming). However, this increase in matrix was no longer significant when normalized to DNA content, suggesting that the effect of dynamic compression was more proliferative than matrix stimulating. DNA steadily increased over time as well, further indicating proliferation rather than a maintenance of viability. This effect was also observed by the increase in DNA seen in DC groups without growth factor (GF-, DC+) compared to free swelling controls. Loading alone was able to stimulate this proliferative response, but this did not translate to increased sGAG production or noticeable Alcian blue staining in these groups. Indeed, others have seen that without mechanical or morphogenic stimulus, MSCs rapidly die\textsuperscript{112,282}.

Alcian blue staining in the initial study was very robust in all groups but slowed after 6 weeks, leading us to believe that the amount of TGF-β3 (10ng/mL) used may have been too high to fully observe the effects of loading. For this reason in Part 2, we incorporated 1ng/mL TGF- β3 in comparison 0 ng/mL TGF- β3. In this study the effects of dynamic compression on col2a1 and sGAG production (via Alcian blue staining) in groups supplemented with growth factor were readily apparent.

Similarly, message level expression of col2a1 and ACAN was increased in groups receiving dynamic compression after 2, 4, or 6 weeks of priming. This indicates that loading promotes an increase in chondrogenic gene expression. In previous \textit{in vivo}
studies, we similarly saw that mechanical loading was necessary to induce endochondral healing of tissue-engineered mesenchymal condensations placed in critically-sized rat femoral defects, exhibiting a mixture of cartilage and bone. Non-loaded limbs failed to functionally regenerate, displaying mainly fibrotic tissue, suggesting a similar role in vivo of mechanical-load mediated progenitor differentiation. However, differences in co2a1 protein were not readily apparent, indicating that in our in vitro model, mechanical load has a greater effect on gene expression than protein production. In part 2 dynamic compression was able to increase expression of col2a1 and ACAN to the same level as growth factor treated samples, but matrix production was virtually non-existent. This suggests that mechanical loading may be necessary to induce chondrogenic gene expression, but available morphogenic cues may be necessary to induce matrix production.

MSCs in culture tend to become hypertrophic without intervention\(^8\), so in addition to inducing chondrogenesis, onset of load after 2 or 4 weeks priming also either delayed or arrested endochondral progression through downregulation of osteogenic genes and delay of mineralization. Similarly, early loading in vivo exhibited persistent gaps of cartilage and possible pseudoarthrosis, suggesting that persistent early loading may work to maintain a stable cartilage phenotype, a tissue engineering method that has been proposed\(^102,103\). However, after 6 weeks, when mineralization is seen in free swelling fibrin hydrogels, dynamic compression supported both chondrogenesis and osteogenesis, and hypertrophic markers such as Col10a1 were upregulated, suggesting that late loading may also enhance hypertrophy and endochondral progression.
Together, these data demonstrate a significant temporal interaction between mechanical loading and endochondral lineage specification, and that there is an optimal priming time for cartilage tissue engineering vs. bone. Future tissue engineering strategies may utilize mechanical priming as a tool to enhance chondrogenesis and stable articular cartilage when applied early, and to support both chondrocyte maturation and osteogenesis when applied late.
CHAPTER 7:
CONCLUSIONS AND FUTURE DIRECTIONS

In conclusion, this work presents bone tissue engineering approaches that recapitulate certain aspects of endochondral ossification in early limb development. We first showed in Chapter III that mechanical load was beneficial to bone healing, but was more beneficial in non-structural scaffolds that do not impede mechanical loads. To this end, in Chapters IV and V, we implanted chondrogenically-primed high-density mesenchymal condensations into mechanically loaded defects that would mimic the endochondral cascade. We found that without mechanical loading, large bone defects would not heal through endochondral bone regeneration, and that healing was dependent on load timing; loading delayed to 4 weeks exhibited robust bone healing, while limbs loaded immediately exhibited gaps of unmineralized cartilage that prevented limb stability. However, the state of the implanted condensation prior to load actuation remains unknown in this model.

This led us to investigate the role of endochondral priming prior to mechanical load in vitro using a custom-made bioreactor system in Chapter VI. Here we found that loading initiates chondrogenic gene expression regardless of growth factor presentation, though growth factor was necessary to initiate matrix production. Loading also has a significant effect on the endochondral progression of progenitor cells, and can either
enhance or suppress the progression to hypertrophy depending on if load is applied early or late respectively.

Though this work evidences the role of mechanical load in chondrogenic gene expression and matrix production, it does not explain the mechanism involved in transducing that load, or how a cell’s interaction with their matrix components changes this response. Future studies will investigate increasing the maturity of the mesenchymal condensation prior to implantation to understand how the cell state at the time of implantation influences the endochondral response to mechanical load in vivo. Additional future studies in vitro will seek to understand whether it is the cell’s maturing environment, or the cell’s maturing phenotype that determines their temporal response to load. This will be achieved by altering the extracellular matrix of mature condensations using component-specific proteases, and by encapsulating immature cells in matrices of varying collagen content. Together these experiments will allow us to elucidate the role of mechanical loading at different cell and matrix maturation states on endochondral ossification in vivo bone repair and in vitro bioreactor stimulation.
APPENDIX A:

HEMATOXYLIN & EOSIN AND SAFRANIN-O PANELS

A.1 Full histological analysis of endochondral bone formation at week 4

Figure A.1: (A-F) Tile-scan images of H&E- (top) and Safranin-O/Fast green-stained (bottom) histological sections of representative samples from Stiff, Early, and Delayed loading groups at week 4. All samples oriented distal (left) to proximal (right). Dotted lines in panel A indicate the native cortical bone ends. Labels “dh” indicate location of fixation plate drill holes. Dotted boxes (1-3) indicate location of magnifications. Scale bar, 3mm. (A1-3-F1-3) Magnified images of sections in (A-F). Dotted boxes indicate location of magnifications. Scale bars, 100 µm. All images were taken from the sample that most closely matched the average in vivo microCT morphometry of that group.
<table>
<thead>
<tr>
<th></th>
<th>Stiff</th>
<th>Early</th>
<th>Delayed</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
</tr>
<tr>
<td>B</td>
<td><img src="image4.png" alt="Image" /></td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
</tr>
<tr>
<td>A1</td>
<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
<td><img src="image9.png" alt="Image" /></td>
</tr>
<tr>
<td>A2</td>
<td><img src="image10.png" alt="Image" /></td>
<td><img src="image11.png" alt="Image" /></td>
<td><img src="image12.png" alt="Image" /></td>
</tr>
<tr>
<td>A3</td>
<td><img src="image13.png" alt="Image" /></td>
<td><img src="image14.png" alt="Image" /></td>
<td><img src="image15.png" alt="Image" /></td>
</tr>
</tbody>
</table>

181
A.2 Full histological analysis of endochondral bone formation at week 12

Figure A.2: (A-F) Tile-scan images of H&E- (top) and Safranin-O/Fast green-stained (bottom) histological sections of representative samples from Stiff, Early, and Delayed loading groups at week 4. All samples oriented distal (left) to proximal (right). Dotted lines in panel A indicate the native cortical bone ends. Labels “dh” indicate location of fixation plate drill holes. Dotted boxes (1-3) indicate location of magnifications. Scale bar, 3mm. (A1-3-F1-3) Magnified images of sections in (A-F). Dotted boxes indicate location of magnifications. Scale bars, 100 µm. All images were taken from the sample that most closely matched the average in vivo microCT morphometry of that group.
A.3 Effects of morphogen priming of engineered mesenchymal condensations and *in vivo* mechanical loading on tissue-level bone regeneration

Figure A.3: Overview histology of bone formation at 4 weeks. (A-F) Representative histological H&E and (G-L) Safranin-O/Fast green staining of defect tissue at week 4, selected based on mean bone volume. Scale bar, 100 μm.
A.4 Effects of morphogen priming of engineered mesenchymal condensations and *in vivo* mechanical loading on tissue-level bone regeneration.

Figure A.4: Overview histology of bone formation at 12 weeks. (A-F) Representative histological H&E and (G-L) Safranin-O/Fast green staining of defect tissue at week 12, selected based on mean bone volume.
APPENDIX B:

PICRO-SIRIUS RED PANELS

B.1 Collagen organization

Figure B.1: Photomicrographs of picrosirius red staining obtained using polarized light microscopy on sections at week 4 (A-C) and 12 (D-F) (n = 1 per group at each time point, chosen by proximity to mean bone volume in vivo at 4 and 12 weeks). Under polarized light, large collagen fibers birefringe yellow and orange, while thinner fibers are green. Images are shown at 10x (top) and 40x (bottom) magnification of dotted boxes. Scale bars, 100 μm.
<table>
<thead>
<tr>
<th>Stiff</th>
<th>Early</th>
<th>Delayed</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1.png" alt="Image A" /></td>
<td><img src="image2.png" alt="Image B" /></td>
<td><img src="image3.png" alt="Image C" /></td>
</tr>
<tr>
<td><img src="image4.png" alt="Image D" /></td>
<td><img src="image5.png" alt="Image E" /></td>
<td><img src="image6.png" alt="Image F" /></td>
</tr>
</tbody>
</table>
REFERENCES


60. Bell, D. et al. SOX9 directly regulates the type-II collagen gene. *nature.com* at <https://www.nature.com/articles/ng0697-174>


118. Lanske, B. *et al.* PTH/PTHrP receptor in early development and Indian hedgehog-regulated bone growth. *science.sciencemag.org* at <http://science.sciencemag.org/content/273/5275/663.short>


180. Mason, B. N., Starchenko, A., Williams, R. M., Bonassar, L. J. & Reinhart-King, C. A. Tuning three-dimensional collagen matrix stiffness independently of


Dang, P. N. *et al.* Controlled dual growth factor delivery from microparticles incorporated within human bone marrow-derived mesenchymal stem cell
aggregates for enhanced bone tissue engineering via endochondral ossification. 


256. Solorio, L. D., Dhami, C. D., Dang, P. N., Vieregge, E. L. & Alsberg, E. Spatiotemporal regulation of chondrogenic differentiation with controlled


266. Tuli, R. *et al.* Transforming growth factor-β-mediated chondrogenesis of human mesenchymal progenitor cells involves N-cadherin and mitogen-activated protein kinase and Wnt. *ASBMB* at <http://www.jbc.org/content/278/42/41227.short>


