

Engineering anisotropic biomimetic fibrocartilage microenvironment by bioprinting mesenchymal stem cells in nanoliter gel droplets

Umut A. Gurkan^{1,2}, Rami El Assal³, Simin E. Yildiz³, Yuree Sung³, Alexander J. Trachtenberg⁴,
Winston P. Kuo,^{4,5} , Utkan Demirci^{3,6*}*

¹ Case Biomanufacturing and Microfabrication Laboratory, Mechanical and Aerospace Engineering Department, Department of Orthopaedics, Case Western Reserve University, Cleveland, OH

² Advanced Platform Technology Center, Louis Stokes Cleveland Veterans Affairs Medical Center, Cleveland, OH

³ Bio-Acoustic-MEMS in Medicine (BAMM) Laboratory, Center for Biomedical Engineering, Renal Division and Division of Infectious Diseases, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, MA

⁴ Harvard Catalyst Laboratory for Innovative Translational Technologies, Harvard Medical School, Boston, MA

⁵ Department Developmental Biology, Harvard School of Dental Medicine, Boston, MA

⁶ Harvard-MIT Health Sciences & Technology, Massachusetts Institute of Technology, Cambridge, MA

* Authors to whom correspondence should be addressed,
umut@case.edu, udemirci@rics.bwh.harvard.edu

References
References

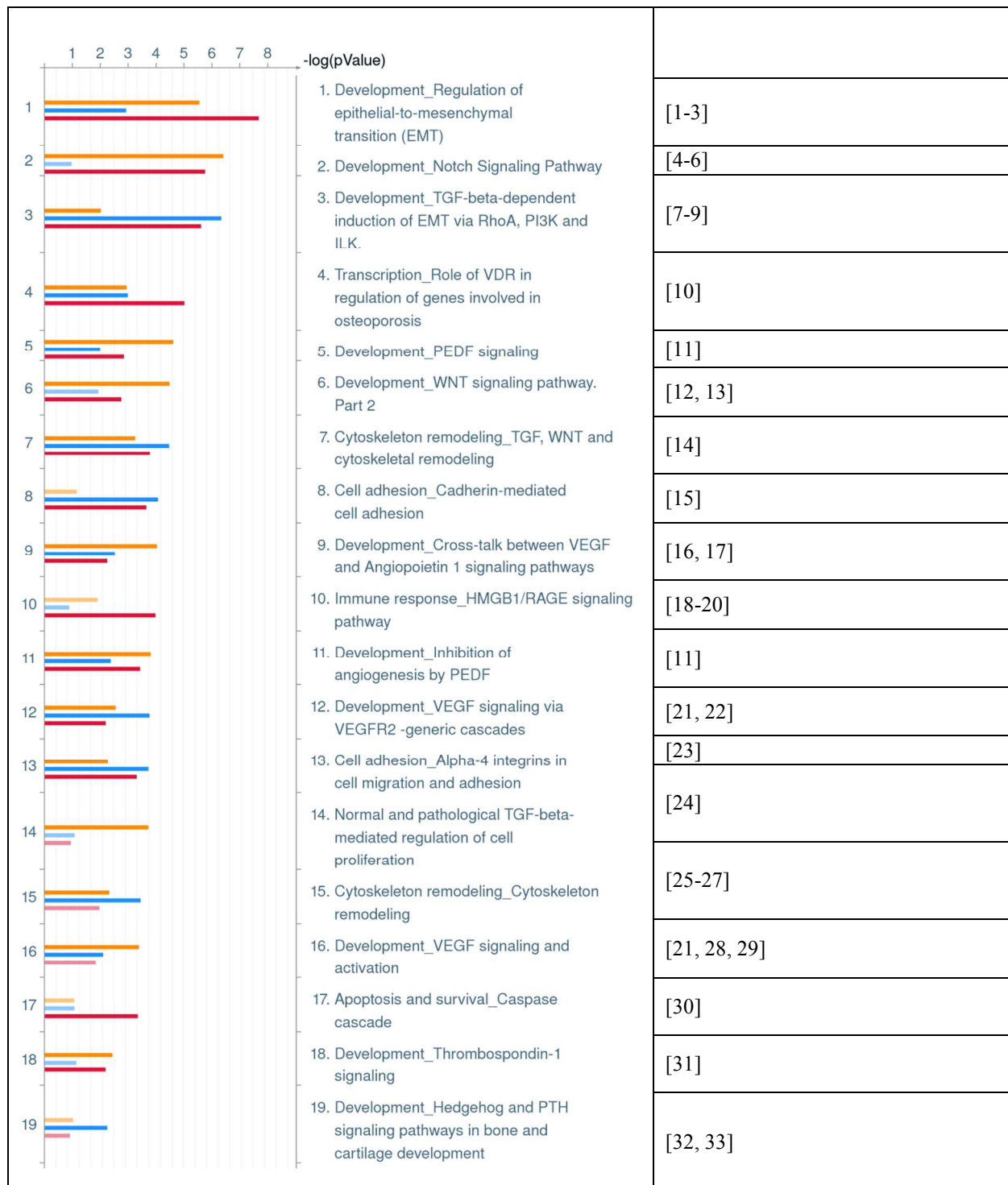


Figure S1: Pathway Maps obtained from Genego Metacore for day 14. (Bar graphs represent: single phase BMP-2, single phase TGF-β1, and multiphase TGF-β1 & BMP-2 patterning groups respectively)

References

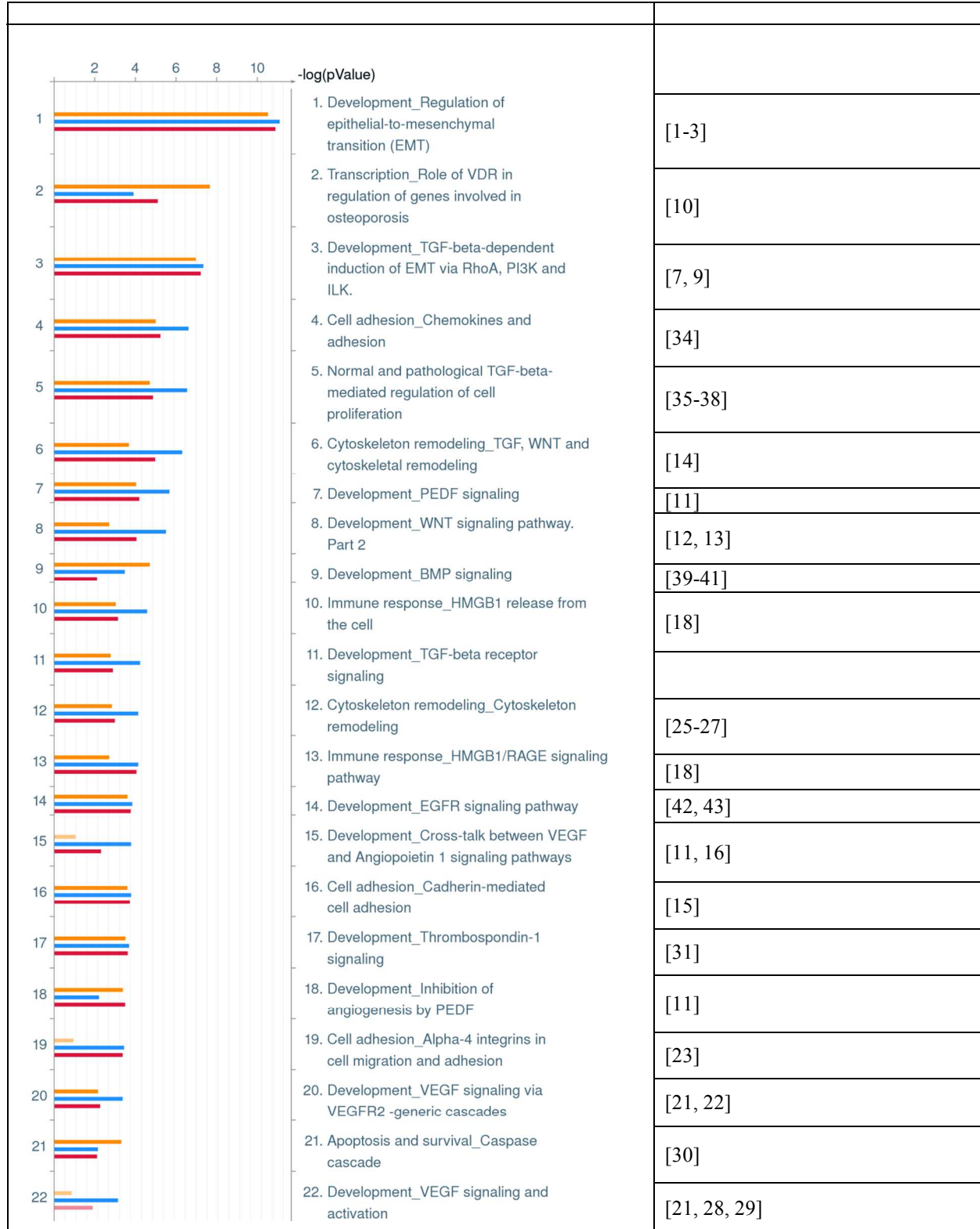


Figure S2: Pathway Maps Results from Genego Metacore for day 21. (Bar graphs represent: single phase BMP-2, single phase TGF-β1, and multiphase TGF-β1 & BMP-2 patterning groups respectively)

Supplement Materials

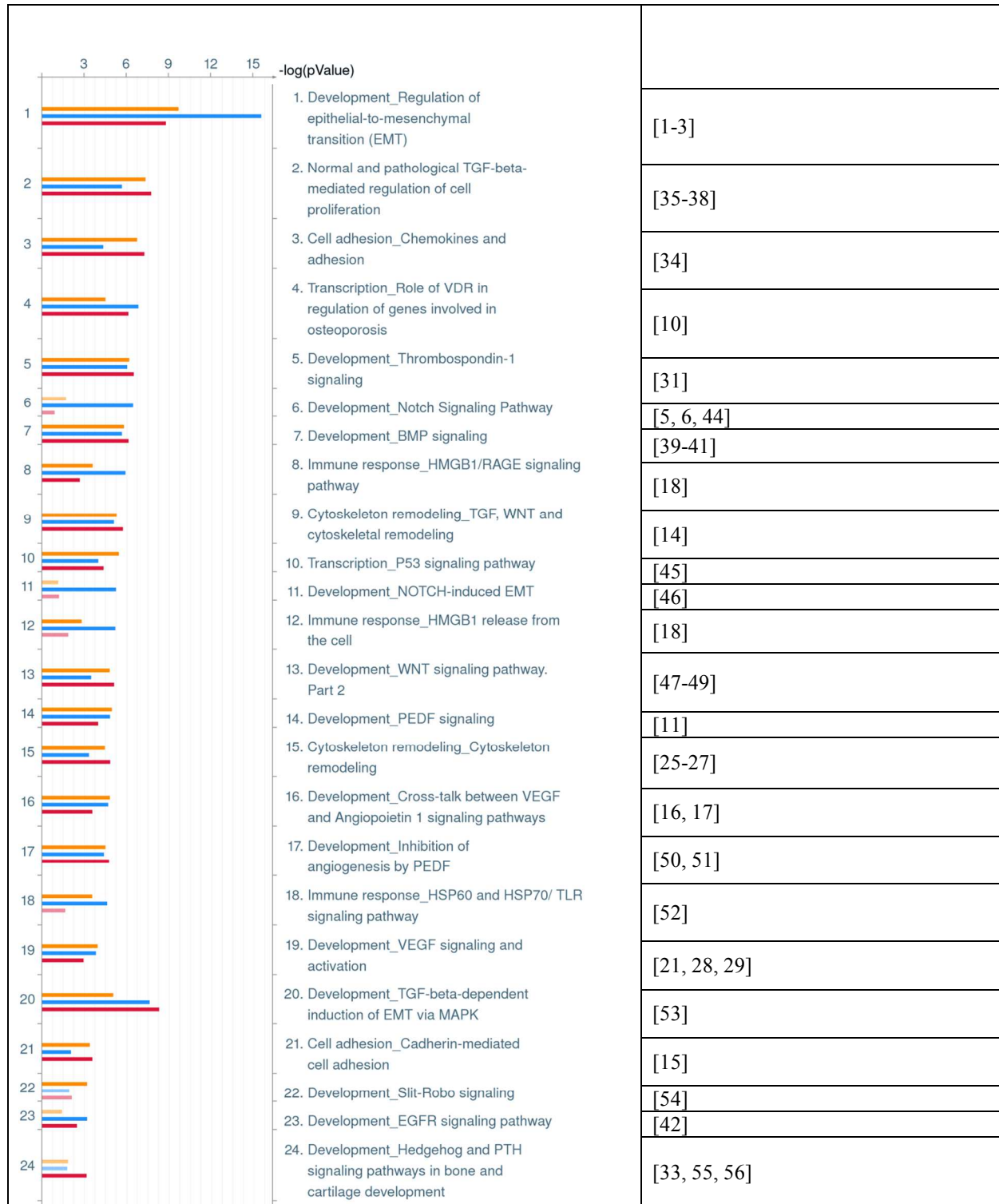


Figure S3: Pathway Map results from Genego Metacore for day 36. (Bar graphs represent: single phase BMP-2, single phase TGF- β 1, and multiphase TGF- β 1 & BMP-2 patterning groups respectively)

References

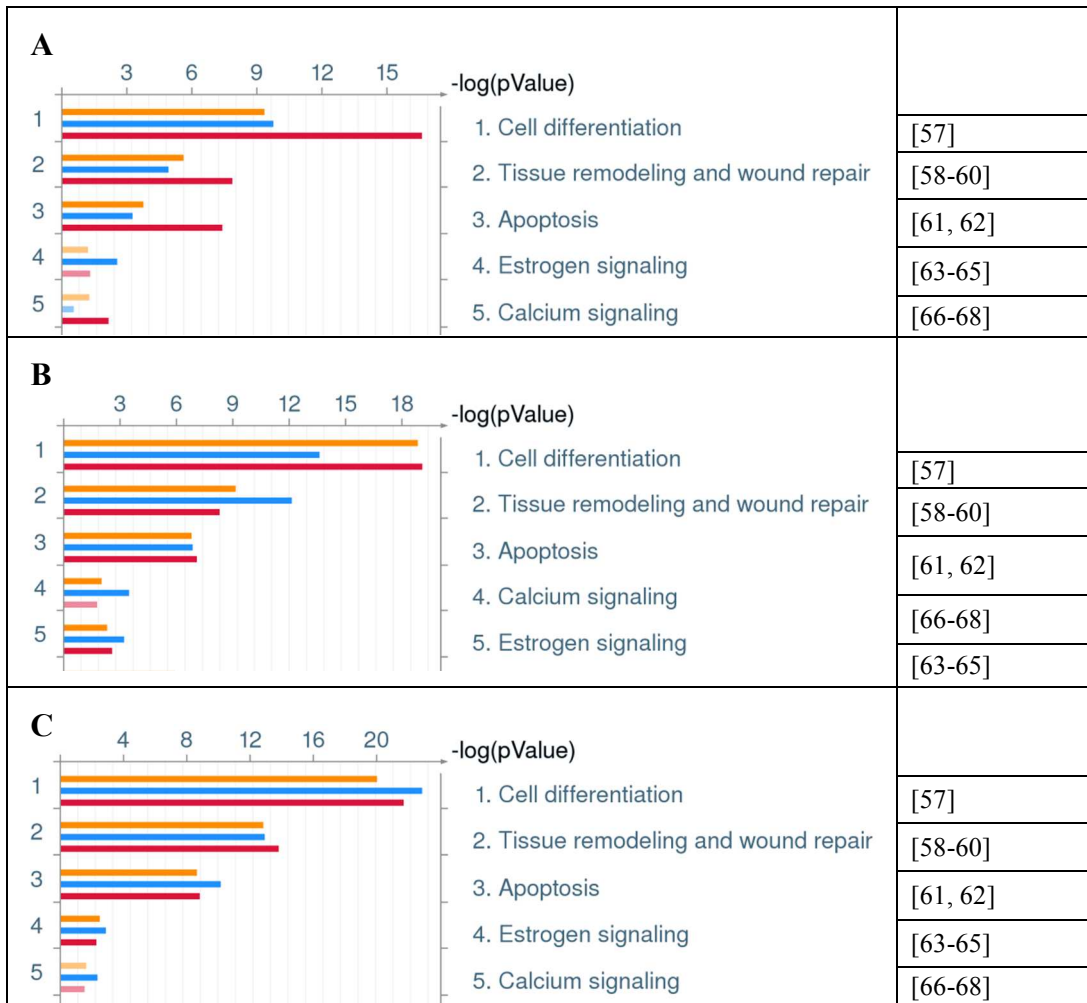


Figure S4: Map Folder results from Genego Metacore for days: (A) 14, (B) 21, and (C) 36. (Bar graphs represent: single phase BMP-2, single phase TGF- β 1, and multiphase TGF- β 1 & BMP-2 patterning groups respectively)

Supplement Materials References

1. Xu, J., S. Lamouille, and R. Derynck, *TGF-beta-induced epithelial to mesenchymal transition*. Cell Res, 2009. **19**(2): p. 156-72.
2. Batlle, R., L. Alba-Castellon, J. Loubat-Casanovas, E. Armenteros, C. Franci, J. Stanisavljevic, R. Banderas, J. Martin-Caballero, F. Bonilla, J. Baulida, J.I. Casal, T. Gridley, and A.G. de Herreros, *Snail1 controls TGF-beta responsiveness and differentiation of mesenchymal stem cells*. Oncogene, 2012.
3. Oh, J.E., R.H. Kim, K.H. Shin, N.H. Park, and M.K. Kang, *DeltaNp63alpha protein triggers epithelial-mesenchymal transition and confers stem cell properties in normal human keratinocytes*. J Biol Chem, 2011. **286**(44): p. 38757-67.
4. Kohn, A., Y. Dong, A.J. Mirando, A.M. Jesse, T. Honjo, M.J. Zuscik, R.J. O'Keefe, and M.J. Hilton, *Cartilage-specific RBPjkappa-dependent and -independent Notch signals regulate cartilage and bone development*. Development, 2012. **139**(6): p. 1198-212.
5. Nobta, M., T. Tsukazaki, Y. Shibata, C. Xin, T. Moriishi, S. Sakano, H. Shindo, and A. Yamaguchi, *Critical regulation of bone morphogenetic protein-induced osteoblastic differentiation by Delta1/Jagged1-activated Notch1 signaling*. J Biol Chem, 2005. **280**(16): p. 15842-8.
6. Watanabe, N., Y. Tezuka, K. Matsuno, S. Miyatani, N. Morimura, M. Yasuda, R. Fujimaki, K. Kuroda, Y. Hiraki, N. Hozumi, and K. Tezuka, *Suppression of differentiation and proliferation of early chondrogenic cells by Notch*. J Bone Miner Metab, 2003. **21**(6): p. 344-52.
7. Appleton, C.T., S.E. Usmani, J.S. Mort, and F. Beier, *Rho/ROCK and MEK/ERK activation by transforming growth factor-alpha induces articular cartilage degradation*. Lab Invest, 2010. **90**(1): p. 20-30.
8. Woods, A. and F. Beier, *RhoA/ROCK signaling regulates chondrogenesis in a context-dependent manner*. J Biol Chem, 2006. **281**(19): p. 13134-40.
9. Wang, G. and F. Beier, *Rac1/Cdc42 and RhoA GTPases antagonistically regulate chondrocyte proliferation, hypertrophy, and apoptosis*. J Bone Miner Res, 2005. **20**(6): p. 1022-31.
10. St-Arnaud, R. and R.P. Naja, *Vitamin D metabolism, cartilage and bone fracture repair*. Mol Cell Endocrinol, 2011. **347**(1-2): p. 48-54.
11. Broadhead, M.L., T. Akiyama, P.F. Choong, and C.R. Dass, *The pathophysiological role of PEDF in bone diseases*. Curr Mol Med, 2010. **10**(3): p. 296-301.
12. Venkatesan, J.K., M. Ekici, H. Madry, G. Schmitt, D. Kohn, and M. Cucchiaroni, *SOX9 gene transfer via safe, stable, replication-defective recombinant adeno-associated virus vectors as a novel, powerful tool to enhance the chondrogenic potential of human mesenchymal stem cells*. Stem Cell Res Ther, 2012. **3**(3): p. 22.
13. Leijten, J.C., C.A. van Blitterwijk, M. Karperien, J. Emons, S. van Gool, J.M. Wit, C. Sticht, E. Decker, G. Rappold, A. Uitterlinden, F. Rivadeneira, J. van Meurs, A. Hofman, and S. Scherjon, *GREM1, FRZB and DKK1 are key regulators of human articular cartilage homeostasis*. Arthritis Rheum, 2012.
14. Shao, M.Y., R. Cheng, F.M. Wang, H. Yang, L. Cheng, and T. Hu, *beta-Catenin and Rho GTPases as downstream targets of TGF-beta 1 during pulp repair*. Cell Biology International, 2011. **35**(2): p. 105-109.

15. Woods, A., S. Khan, and F. Beier, *C-type natriuretic peptide regulates cellular condensation and glycosaminoglycan synthesis during chondrogenesis*. *Endocrinology*, 2007. **148**(10): p. 5030-41.
16. Kishiya, M., T. Sawada, K. Kanemaru, H. Kudo, T. Numasawa, T. Yokoyama, S. Tanaka, S. Motomura, K. Ueyama, S. Harata, S. Toh, and K. Furukawa, *A functional RNAi screen for Runx2-regulated genes associated with ectopic bone formation in human spinal ligaments*. *J Pharmacol Sci*, 2008. **106**(3): p. 404-14.
17. Horner, A., S. Bord, A.W. Kelsall, N. Coleman, and J.E. Compston, *Tie2 ligands angiopoietin-1 and angiopoietin-2 are coexpressed with vascular endothelial cell growth factor in growing human bone*. *Bone*, 2001. **28**(1): p. 65-71.
18. Charoonpatrapong, K., R. Shah, A.G. Robling, M. Alvarez, D.W. Clapp, S. Chen, R.P. Kopp, F.M. Pavalko, J. Yu, and J.P. Bidwell, *HMGB1 expression and release by bone cells*. *J Cell Physiol*, 2006. **207**(2): p. 480-90.
19. Zhou, Z. and W.C. Xiong, *RAGE and its ligands in bone metabolism*. *Front Biosci (Schol Ed)*, 2011. **3**: p. 768-76.
20. Terada, C., A. Yoshida, Y. Nasu, S. Mori, Y. Tomono, M. Tanaka, H.K. Takahashi, M. Nishibori, T. Ozaki, and K. Nishida, *Gene expression and localization of high-mobility group box chromosomal protein-1 (HMGB-1) in human osteoarthritic cartilage*. *Acta Med Okayama*, 2011. **65**(6): p. 369-77.
21. Dai, J. and A.B.M. Rabie, *VEGF: an essential mediator of both angiogenesis and endochondral ossification*. *Journal of Dental Research*, 2007. **86**(10): p. 937-950.
22. Pufe, T., B. Kurz, W. Petersen, D. Varoga, R. Mentlein, S. Kulow, A. Lemke, and B. Tillmann, *The influence of biomechanical parameters on the expression of VEGF and endostatin in the bone and joint system*. *Annals of Anatomy-Anatomischer Anzeiger*, 2005. **187**(5-6): p. 461-472.
23. Ostergaard, K., D.M. Salter, J. Petersen, K. Bendtzen, J. Hvolris, and C.B. Andersen, *Expression of alpha and beta subunits of the integrin superfamily in articular cartilage from macroscopically normal and osteoarthritic human femoral heads*. *Ann Rheum Dis*, 1998. **57**(5): p. 303-8.
24. Park, J.S., H.N. Yang, D.G. Woo, H.M. Chung, and K.H. Park, *In Vitro and In Vivo Chondrogenesis of Rabbit Bone Marrow-Derived Stromal Cells in Fibrin Matrix Mixed with Growth Factor Loaded in Nanoparticles*. *Tissue Engineering Part A*, 2009. **15**(8): p. 2163-2175.
25. Gardinier, J.D., S. Majumdar, R.L. Duncan, and L.Y. Wang, *Cyclic Hydraulic Pressure and Fluid Flow Differentially Modulate Cytoskeleton Re-Organization in MC3T3 Osteoblasts*. *Cellular and Molecular Bioengineering*, 2009. **2**(1): p. 133-143.
26. Takai, E., K.D. Costa, A. Shaheen, C.T. Hung, and X.E. Guo, *Osteoblast elastic modulus measured by atomic force microscopy is substrate dependent*. *Annals of Biomedical Engineering*, 2005. **33**(7): p. 963-971.
27. Hardmeier, R., H. Redl, and S. Marlovits, *Effects of mechanical loading on collagen propeptides processing in cartilage repair*. *Journal of Tissue Engineering and Regenerative Medicine*, 2010. **4**(1): p. 1-11.
28. Carlevaro, M.F., S. Cermelli, R. Cancedda, and F.D. Cancedda, *Vascular endothelial growth factor (VEGF) in cartilage neovascularization and chondrocyte differentiation: auto-paracrine role during endochondral bone formation*. *Journal of Cell Science*, 2000. **113**(1): p. 59-69.

29. Ishijima, M., N. Suzuki, K. Hozumi, T. Matsunobu, K. Kosaki, H. Kaneko, J.R. Hassell, E. Arikawa-Hirasawa, and Y. Yamada, *Perlecan modulates VEGF signaling and is essential for vascularization in endochondral bone formation*. Matrix Biology, 2012. **31**(4): p. 234-245.
30. Gibson, G., *Active role of chondrocyte apoptosis in endochondral ossification*. Microsc Res Tech, 1998. **43**(2): p. 191-204.
31. Bailey Dubose, K., M. Zayzafoon, and J.E. Murphy-Ullrich, *Thrombospondin-1 inhibits osteogenic differentiation of human mesenchymal stem cells through latent TGF-beta activation*. Biochem Biophys Res Commun, 2012. **422**(3): p. 488-93.
32. Wang, W., N. Lian, L. Li, H.E. Moss, D.S. Perrien, F. Elefteriou, and X. Yang, *Atf4 regulates chondrocyte proliferation and differentiation during endochondral ossification by activating Ihh transcription*. Development, 2009. **136**(24): p. 4143-53.
33. Semevolos, S.A., M.L. Strassheim, J.L. Haupt, and A.J. Nixon, *Expression patterns of hedgehog signaling peptides in naturally acquired equine osteochondrosis*. J Orthop Res, 2005. **23**(5): p. 1152-9.
34. Kalwitz, G., M. Endres, K. Neumann, K. Skrinier, J. Ringe, O. Sezer, M. Sittinger, T. Haupt, and C. Kaps, *Gene expression profile of adult human bone marrow-derived mesenchymal stem cells stimulated by the chemokine CXCL7*. International Journal of Biochemistry & Cell Biology, 2009. **41**(3): p. 649-658.
35. Wildemann, B., G. Schmidmaier, S. Ordell, R. Stange, N.P. Haas, and M. Raschke, *Cell proliferation and differentiation during fracture healing are influenced by locally applied IGF-I and TGF-beta 1: Comparison of two proliferation markers, PCNA and BrdU*. Journal of Biomedical Materials Research Part B-Applied Biomaterials, 2003. **65B**(1): p. 150-156.
36. Oka, K., S. Oka, T. Sasaki, Y. Ito, P. Bringas, K. Nonaka, and Y. Chai, *The role of TGF-beta signaling in regulating chondrogenesis and osteogenesis during mandibular development*. Developmental Biology, 2007. **303**(1): p. 391-404.
37. Caterson, E.J., L.J. Nesti, W.J. Li, K.G. Danielson, T.J. Albert, A.R. Vaccaro, and R.S. Tuan, *Three-dimensional cartilage formation by bone marrow-derived cells seeded ion polylactide/alginate amalgam*. Journal of Biomedical Materials Research, 2001. **57**(3): p. 394-403.
38. Mello, M.A., A.C. Tufan, K.M. Daumer, B. Pucci, T. Lafond, D.J. Hall, and R.S. Tuan, *Regulation of chondrogenesis and cartilage maturation in vitro: Role of TGF- beta 1, thyroid hormone, and Wnt signaling*, in *Growth Plate*, I.M. Shapiro, B. Boyan, and H.C. Anderson, Editors. 2002, I O S Press: Amsterdam. p. 37-51.
39. Chen, D., M. Zhao, and G.R. Mundy, *Bone morphogenetic proteins*. Growth Factors, 2004. **22**(4): p. 233-241.
40. Shen, B.J., A.Q. Wei, H.L. Tao, A.D. Diwan, and D.D.F. Ma, *BMP-2 Enhances TGF-beta 3-Mediated Chondrogenic Differentiation of Human Bone Marrow Multipotent Mesenchymal Stromal Cells in Alginate Bead Culture*. Tissue Engineering Part A, 2009. **15**(6): p. 1311-1320.
41. Oshin, A.O. and M.C. Stewart, *The role of bone morphogenetic proteins in articular cartilage development, homeostasis and repair*. Veterinary and Comparative Orthopaedics and Traumatology, 2007. **20**(3): p. 151-158.
42. Fisher, M.C., G.M. Clinton, N.J. Maihle, and C.N. Dealy, *Requirement for ErbB2/ErbB signaling in developing cartilage and bone*. Dev Growth Differ, 2007. **49**(6): p. 503-13.

43. Zhang, X., V.A. Siclari, S. Lan, J. Zhu, E. Koyama, H.L. Dupuis, M. Enomoto-Iwamoto, F. Beier, and L. Qin, *The critical role of the epidermal growth factor receptor in endochondral ossification*. J Bone Miner Res, 2011. **26**(11): p. 2622-33.
44. Yamada, T., H. Yamazaki, T. Yamane, M. Yoshino, H. Okuyama, M. Tsuneto, T. Kurino, S. Hayashi, and S. Sakano, *Regulation of osteoclast development by Notch signaling directed to osteoclast precursors and through stromal cells*. Blood, 2003. **101**(6): p. 2227-34.
45. Rici, R.E., D. Alcantara, P. Fratini, C.V. Wenceslau, C.E. Ambrosio, M.A. Miglino, and D.A. Maria, *Mesenchymal stem cells with rhBMP-2 inhibits the growth of canine osteosarcoma cells*. BMC Vet Res, 2012. **8**: p. 17.
46. Bailey, J.M., P.K. Singh, and M.A. Hollingsworth, *Cancer metastasis facilitated by developmental pathways: Sonic hedgehog, notch, and bone morphogenic proteins*. Journal of Cellular Biochemistry, 2007. **102**(4): p. 829-839.
47. De Boer, J., H.J. Wang, and C. Van Blitterswijk, *Effects of Wnt signaling on proliferation and differentiation of human mesenchymal stem cells*. Tissue Engineering, 2004. **10**(3-4): p. 393-401.
48. Si, W.K., Q. Kang, H.H. Luu, J.K. Park, Q. Luo, W.X. Song, W. Jiang, X.J. Luo, X.M. Li, H. Yin, A.G. Montag, R.C. Haydon, and T.C. He, *CCN1/Cyr61 is regulated by the canonical Wnt signal and plays an important role in Wnt3A-induced osteoblast differentiation of mesenchymal stem cells*. Molecular and Cellular Biology, 2006. **26**(8): p. 2955-2964.
49. Im, G.I. and Z. Quan, *The Effects of Wnt Inhibitors on the Chondrogenesis of Human Mesenchymal Stem Cells*. Tissue Engineering Part A, 2010. **16**(7): p. 2405-2413.
50. Quan, G.M.Y., J. Ojaimi, Y.P. Li, V. Kartsogiannis, H. Zhou, and P.F.M. Choong, *Localization of pigment epithelium-derived factor in growing mouse bone*. Calcified Tissue International, 2005. **76**(2): p. 146-153.
51. Quan, G.M.Y., J. Ojaimi, A.P. Nadesapillai, H. Zhou, and P.F.M. Choong, *Resistance of epiphyseal cartilage to invasion by osteosarcoma is likely to be due to expression of antiangiogenic factors*. Pathobiology, 2002. **70**(6): p. 361-367.
52. Vanmuylder, N., L. Evrard, and N. Dourov, *Strong expression of heat shock proteins in growth plate cartilage, an immunohistochemical study of HSP28, HSP70 and HSP110*. Anat Embryol (Berl), 1997. **195**(4): p. 359-62.
53. Heldin, C.H., M. Vanlandewijck, and A. Moustakas, *Regulation of EMT by TGF beta in cancer*. Febs Letters, 2012. **586**(14): p. 1959-1970.
54. Sun, H., K. Dai, T. Tang, and X. Zhang, *Regulation of osteoblast differentiation by slit2 in osteoblastic cells*. Cells Tissues Organs, 2009. **190**(2): p. 69-80.
55. Nishioka, K., S. Itoh, H. Suemoto, S. Kanno, Z. Gai, M. Kawakatsu, H. Tanishima, Y. Morimoto, I. Hatamura, M. Yoshida, and Y. Muragaki, *Trps1 deficiency enlarges the proliferative zone of growth plate cartilage by upregulation of Pthrp*. Bone, 2008. **43**(1): p. 64-71.
56. Kugimiya, F. and Y. Tei, *[Regulation of chondrogenesis by PTH/PTHrP signaling]*. Clin Calcium, 2003. **13**(1): p. 19-24.
57. Hutmacher, D.W., *Scaffolds in tissue engineering bone and cartilage*. Biomaterials, 2000. **21**(24): p. 2529-2543.
58. Lall, S.P. and L.M. Lewis-McCrea, *Role of nutrients in skeletal metabolism and pathology in fish - An overview*. Aquaculture, 2007. **267**(1-4): p. 3-19.

59. Chung, R., B.K. Foster, A.C.W. Zannettino, and C.J. Man, *Potential roles of growth factor PDGF-BB in the bony repair of injured growth plate*. Bone, 2009. **44**(5): p. 878-885.
60. Ting, K., H. Ramachandran, K.S. Chung, N. Shah-Hosseini, B.R. Olsen, and I. Nishimura, *A short isoform of Col9a1 supports alveolar bone repair*. American Journal of Pathology, 1999. **155**(6): p. 1993-1999.
61. Goldring, M.B., *Chondrogenesis, chondrocyte differentiation, and articular cartilage metabolism in health and osteoarthritis*. Ther Adv Musculoskelet Dis, 2012. **4**(4): p. 269-85.
62. Abbott, R.D., A.K. Howe, H.M. Langevin, and J.C. Iatridis, *Live free or die: stretch-induced apoptosis occurs when adaptive reorientation of annulus fibrosus cells is restricted*. Biochem Biophys Res Commun, 2012. **421**(2): p. 361-6.
63. Karimian, E. and L. Savendahl, *Estrogen Signaling in Growth Plate Cartilage*, in *Cartilage and Bone Development and Its Disorders*, C. CamachoHebner, O. Nilsson, and L. Savendahl, Editors. 2011, Karger: Basel. p. 42-51.
64. Engdahl, C., C. Jochems, S.H. Windahl, A.E. Borjesson, C. Ohlsson, H. Carlsten, and M.K. Lagerquist, *Amelioration of Collagen-Induced Arthritis and Immune-Associated Bone Loss Through Signaling via Estrogen Receptor alpha, and not Estrogen Receptor beta or G Protein-Coupled Receptor 30*. Arthritis and Rheumatism, 2010. **62**(2): p. 524-533.
65. Sniekers, Y.H., H. Weinans, G. van Osch, and J. van Leeuwen, *Oestrogen is important for maintenance of cartilage and subchondral bone in a murine model of knee osteoarthritis*. Arthritis Research & Therapy, 2010. **12**(5).
66. Dormer, N.H., Y. Qiu, A.M. Lydick, N.D. Allen, N. Mohan, C.J. Berkland, and M.S. Detamore, *Osteogenic Differentiation of Human Bone Marrow Stromal Cells in Hydroxyapatite-Loaded Microsphere-Based Scaffolds*. Tissue Engineering Part A, 2012. **18**(7-8): p. 757-767.
67. Conigrave, A.D., *Regulation of Calcium and Phosphate Metabolism*. Diseases of the Parathyroid Glands, ed. A.A. Licata and E.V. Lerma. 2012, New York: Springer. 13-51.
68. Robinson, L.J., S. Mancarella, D. Songsawad, I.L. Tourkova, J.B. Barnett, D.L. Gill, J. Soboloff, and H.C. Blair, *Gene disruption of the calcium channel Orail results in inhibition of osteoclast and osteoblast differentiation and impairs skeletal development*. Laboratory Investigation, 2012. **92**(7): p. 1071-1083.