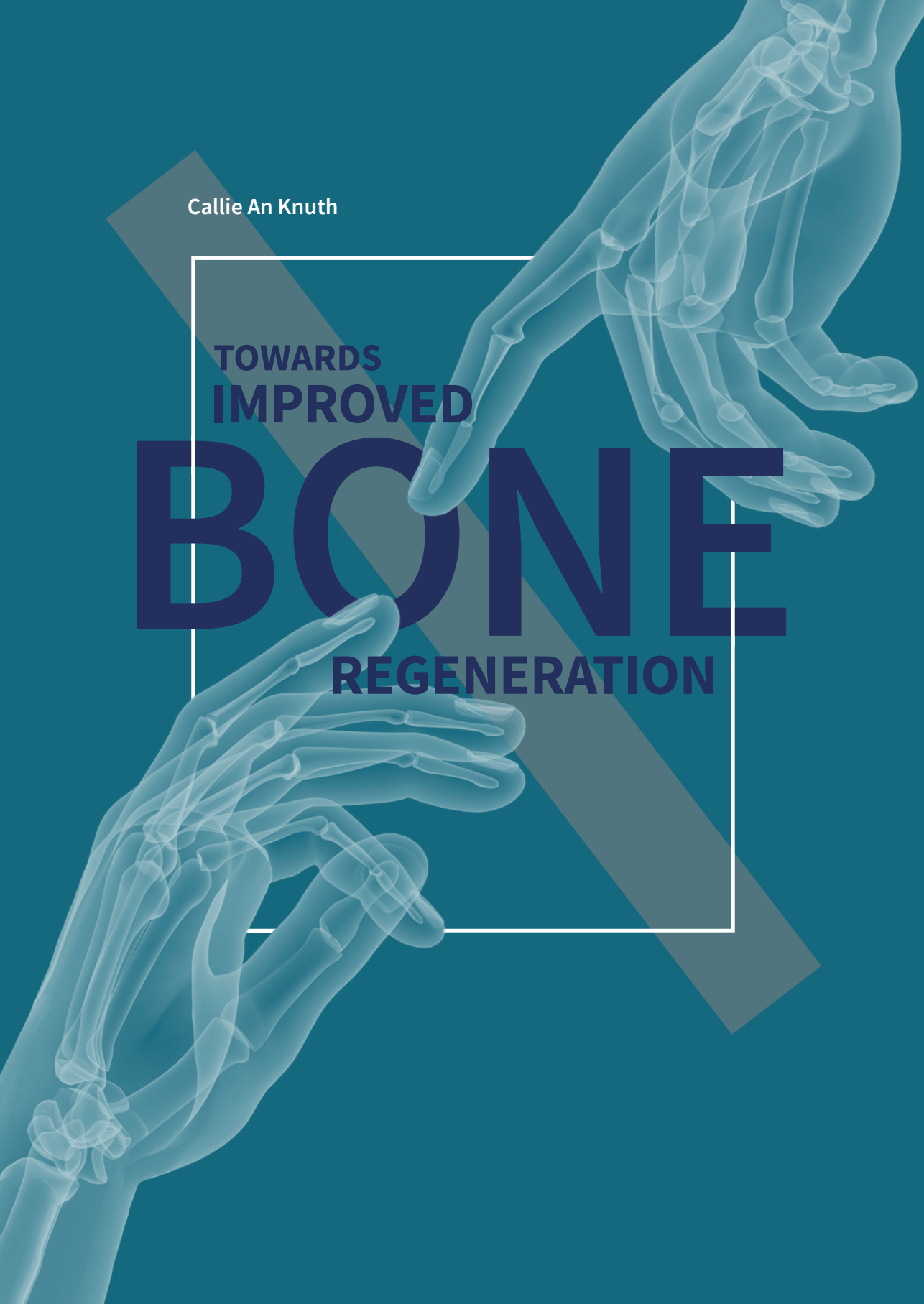
Callie An Knuth

TOWARDS IMPROVED BONE REGENERATION



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Towards Improved Bone Regeneration

Naar verbeterde botregeneratie

Thesis

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Prof. dr. R.C.M.E. Engels

And in accordance with the decision of the Doctoral Board.

The public defence ceremony shall be held on
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by

Callie An Knuth
born in Bethel Park, Pennsylvania (USA)

Erasmus University Rotterdam

Doctoral Committee

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INTRODUCTION

BACKGROUND

Bone is a unique tissue, capable of self- repair when a small defect exists (1). However, when a large defect occurs, for example following injury, trauma or surgery, which exceeds the body's natural capacity for regeneration, surgical intervention is required. Bone is one of the most commonly transplanted tissues in the world, with more than 2.2 million transplantations performed annually (2). The current gold standard treatment is use of autologous bone grafts (ABG) (3, 4), which are generally well accepted having a success rate of around 90% (5, 6). Complication rates have been reported ranging between 8.6% (for major incidents) and 20.6% (for minor incidents) (7). Although effective, harvestable material is limited and harvesting ABGs can result in donor site morbidity (6), resulting in further complications for the patient. ABG alternatives, such as the use of allogeneic or xenogenic bone graft material, are associated with other inherent risks such as disease transference and immune rejection (8) making them a less desirable treatment option. Unfortunately there are no alternatives available which are capable of regenerating bone or achieving the level of successful integration with the surrounding host bone as demonstrated by ABGs (9), indicating there is a clear and present need for alternative bone substitutes.

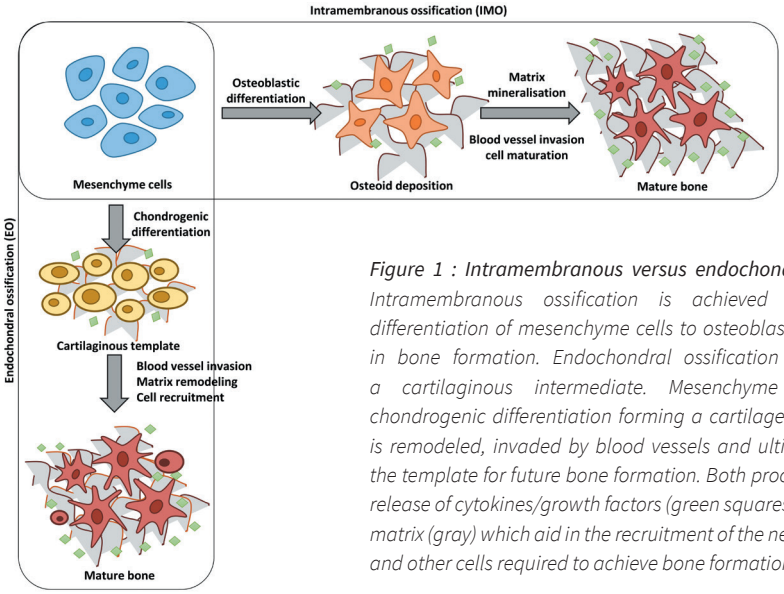


Figure 1 : Intramembranous versus endochondral ossification. Intramembranous ossification is achieved via the direct differentiation of mesenchyme cells to osteoblastic cells resulting in bone formation. Endochondral ossification is achieved via a cartilaginous intermediate. Mesenchyme cells undergo chondrogenic differentiation forming a cartilage template, which is remodeled, invaded by blood vessels and ultimately serves as the template for future bone formation. Both processes involve the release of cytokines/growth factors (green squares) and a bioactive matrix (gray) which aid in the recruitment of the nearby vasculature and other cells required to achieve bone formation.

Through the years scientists have focused on creating biologically relevant cell based bone substitutes using mesenchymal stem cells (MSCs). Typically MSC based constructs are modelled after one of the developmental pathways of bone formation, the intramembranous ossification (IMO) which involves the direct osteoblastic differentiation of MSCs, resulting in bone formation (figure 1) . Although such grafts have often shown successful bone formation *in vitro* (10), they often fail due to insufficient vascularisation within the construct, resulting in poor integration and necrosis *in vivo* (11, 12). With these limitations in mind, we and others have focused on creating tissue engineered grafts which achieve bone formation via endochondral ossification (EO).

DEVELOPMENTAL ENDOCHONDRAL OSSIFICATION

Unlike IMO, EO is achieved via a cartilage intermediate. During developmental EO, an avascular cartilage template, formed via mesenchymal condensation, is establishment. This template, often referred to as the cartilage anlagen, is composed of chondrocytes at various stages of differentiation (13): the resting, proliferative, and hypertrophic zones (figure 2) (13, 14). “Resting chondrocytes” are thought to be essential for maintaining longitudinal growth orientation. Resting chondrocytes maintain a specific cell population which serves as a source of chondrocyte “stem-cells,” which when triggered give rise to proliferative chondrocytes (15-17). Resting chondrocytes help inhibit hypertrophic differentiation of proliferative chondrocytes, maintaining them in a proliferative state when close to the resting zone border (16, 18, 19).

The proliferative chondrocytes contribute to longitudinal bone growth (21, 22). Chondrocyte proliferation is regulated by a complex feedback loop involving transforming growth factor-beta (TGF-β), parathyroid hormone-related peptide (PTHrP) and Indian hedgehog (Ihh) (22-24). This feedback loop also triggers the hypertrophic differentiation of proliferative chondrocytes when appropriate (23). As proliferative chondrocytes approach the hypertrophic zone, they will exit the cell cycle and undergo hypertrophic differentiation (17, 25). During hypertrophy, chondrocytes enlarge and ultimately contribute to longitudinal bone growth (13). During this phase the matrix is prepared for calcification. Hypertrophic chondrocytes secrete collagen type X (COLX) which accounts for more than 45% of the collagens produced during this stage (26). During hypertrophy COLX not only adds structural stability to the pericellular network (27, 28) but also helps initiate matrix mineralisation via binding with annexin V on matrix vesicles. This binding allows calcium influx into vesicles initiating biomineralisation (29-31). At the same time production of alkaline phosphatase is increased, ultimately being packaged into matrix vesicles

(32, 33). These two events are crucial for the induction of bone formation. Alkaline phosphatase plays an essential role in initiating calcification within matrix vesicles, ultimately allowing for remodeling to take place (34, 35).

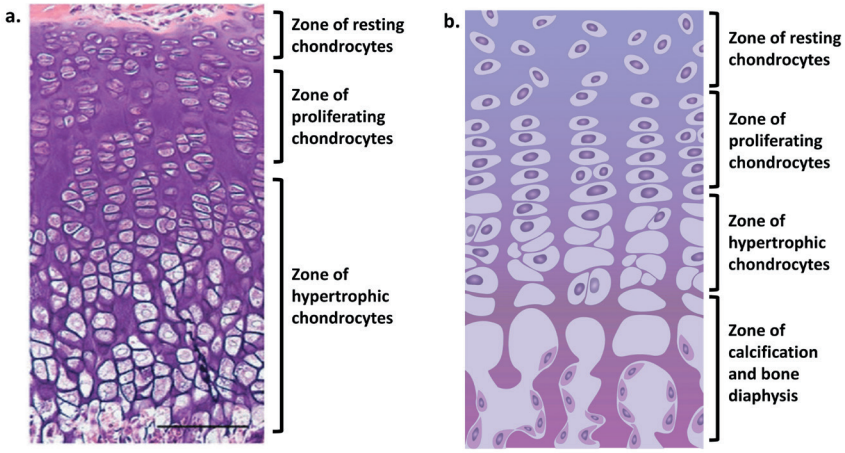


Figure 2: Chondrocyte zones within growth plate. a) Growth plate of a 4 week old mouse stained with H&E shows clearly the different zones of chondrocytes found within the growth plate (adapted from Usami, 2016) (20). b) Graphical depiction of chondrocyte zones within the growth plate. Resting zone chondrocytes display a more sporadic placement, however when proliferation is initiated becomes more elongated in distinct column patterns. Hypertrophic chondrocytes are identified by clear cell enlargement. As cells move through the different zones they contribute to longitudinal bone growth (adapted from Mgraw Company).

Preparation of the cartilage template for vessel invasion and bone formation

Following initiation of matrix remodeling and mineralization the primary ossification center is formed. At the primary ossification center, hypertrophic chondrocytes produce angiogenic factors (including VEGF, ANG-1 and PDGRα) which ultimately contribute to vascularisation of the cartilage template (36, 37). As the hypertrophic zone and primary ossification center is established the perichondrium, a thin homogenous layer of mesenchymal cells at the periphery of the template (38), begins to differentiate into the periosteum where the first cells which invade the cartilage template originate from (14, 39, 40). As the primary ossification center is established mesenchymal cells in the perichondrium undergo osteoblastic differentiation, contributing to the formation of the bone collar through calcification of the hypertrophic cartilage template prior to vascularization (40, 41). The periosteum is an essential source of osteoprogenitor cells which will initially invade and ossify the primary ossification center (42). These osteoprogenitors in combination with a specific subset of hypertrophic chondrocytes (43-45) within the cartilage template and ongoing differentiation of mesenchymal cells

results in appositional bone growth (46). Once the template has begun to undergo mineralisation, matrix remodeling begins to allow for vascular invasion and bone formation.

Release of proteolytic enzymes, including matrix metalloproteinases (MMPs) and aggrecanases, initiates matrix degradation localised around hypertrophic chondrocytes (47, 48). This degradation releases matrix bound factors including VEGF, MMPs and RANKL. While the released MMPs continue to degrade the cartilage template (49-51), VEGF (52, 53) and RANKL (54, 55) are important to initiate osteoclast recruitment. Matrix remodeling results in glycoaminoglycan (GAGs) degradation within the cartilage template (56). This decreases the matrix charge potential allowing vessels to more easily invade the cartilage template. This is because the degradation makes the net charge between the matrix and the vessels more neutral allowing for less resistance between the two (57). This decreased charge is also beneficial as endothelial cell adhesion is hindered in the presence of cartilage proteoglycans and GAGs (56, 58). Simultaneous with matrix remodeling, apoptosis of a subset of hypertrophic chondrocytes occurs (40, 59). Together these events lead to the formation of vascular channels allowing for vascular invasion of the cartilage template (60, 61).

Vascular invasion and mineralisation of the cartilage template

Angiogenic stimuli produced by hypertrophic chondrocytes and osteoblasts, including VEGF, ANG-1, and PDGRα, aid in the recruitment of the nearby vasculature from the periosteum (62-64). Vascular invasion of the primary ossification center is the result of vascular sprouting from existing capillaries in the bone collar rather than *de novo* synthesised by invading endothelial cells as once thought (65, 66). In fact blood vessels in the periosteum initiate vascularisation of the cartilage template and ultimately contribute to 70-80% of the overall blood supply to the bone cortex (40, 46, 67). The vascular network within developing bone is dense consisting of an interconnected network composed of different capillary subtypes which play different roles in maintaining endochondral bone during development and aging (68-71). Vascularisation of the cartilage template is important as the invading blood vessels bring osteoblasts further into the cartilage template further aid in calcification and bone formation (36).

Pre-osteoblastic precursors move in a pericyte-like fashion into remodeling cartilage templates, co-migrating with the invading vasculature ultimately contributing to stabilisation of the vascular network and bone formation following vessel invasion (36). Endothelial cells further contribute to ossification by secreting BMPs influencing osteoblast cell behavior and contributing to the differentiation of mesenchymal cells to osteoblasts (72, 73). This bone formation can in part be controlled by the correct zonal distribution of matrix vesicles which induce bone formation where found (74, 75). COLX is thought to regulate distribution of these matrix vesicles via interactions with annexin V located on the MV outer

surface, anchoring them within the hypertrophic zone (76, 77). Annexin V further facilitates calcium influx into vesicles which is important for the initiation of mineralisation within vesicles, in turn influencing matrix mineralisation and bone formation (75). However, the exact role of COLX is still somewhat debated in the field (78, 79) and further research is required to determine its exact role. Regardless, as this initial mineralisation begins and the matrix is remodeled, and osteoblasts from the bone collar and transdifferentiated HC within the cartilage matrix (43) lay down an osteoid matrix on the remaining cartilage template (80), maturing within the matrix eventually becoming osteocytes (81). In this way bones are formed developmentally during endochondral ossification (figure 3).

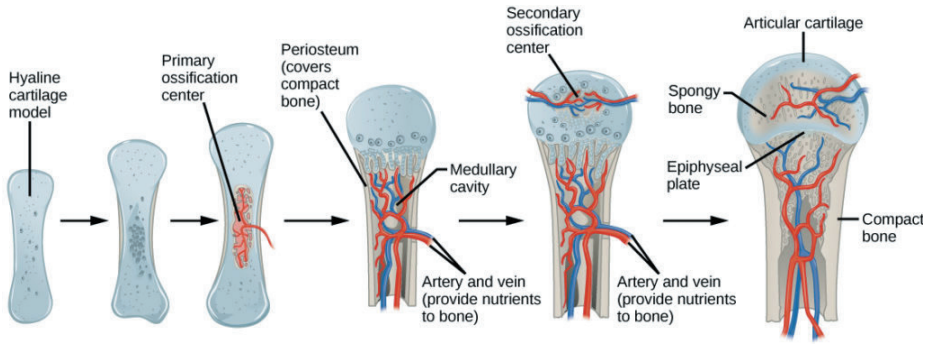


Figure 3: Process of endochondral ossification. *Following the establishment of the cartilage template, the matrix is remodeled, blood vessels invade and bone formation occurs (image modified from beyondachondroplasia.org)*

TISSUE ENGINEERED ENDOCHONDRAL OSSIFICATION

Many researchers have shown that endochondral bone formation can be achieved by chondrogenically differentiating MSCs *in vitro* and subcutaneously implanting them *in vivo* (figure 4) (82-86). Unlike TE intramembranous grafts, endochondral TE grafts rely on the use of a MSC derived cartilage intermediate to achieve bone formation which is advantageous as cartilage is well suited to survive in a hypoxic avascular defect site (87). Chondrogenically differentiated MSCs are also capable of inducing the migration of nearby vasculature greatly improving construct survival (83, 84, 87).

In addition to vascularisation, chondrogenic MSCs also trigger the migration of osteoclasts and osteoblasts via factors which are both secreted from the construct and trapped within the extracellular matrix, including but not limited to VEGF, ANG-1, PDGRα, TNF-α, TIMP-1/2 and BMP2 (82, 84, 89). This recruitment initiates matrix remodeling and bone formation as seen in the developmental situation (90-92). These constructs are

quite promising, even being shown in some instances to be capable of bridging large bone defects without the need for external biomaterials or growth factors (82-86). What is also impressive is that these constructs not only form endochondral bone following implantation but also a fully functional marrow cavity (85), highlighting a potential use for these constructs in fields outside of tissue engineering. In **chapter 2**, we review the current literature on TE MSC endochondral bone formation. We focus on the role of donor cells and extracellular matrix components in orchestrating *in vivo* EO. We review our current understanding of how these grafts have achieved bone formation as well as highlight areas others are focusing on to improve TE graft performance.

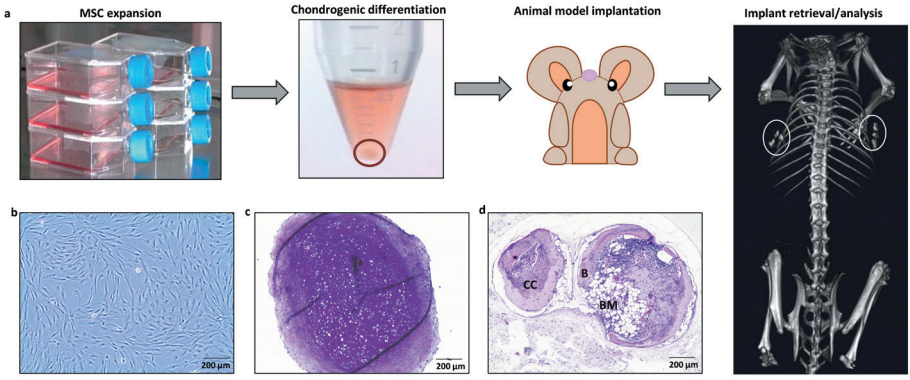


*Figure 4: Achieving tissue engineered endochondral ossification. **a)** Mesenchymal stem cells (MSCs) are expanded to reach required cell number via cell passage. MSCs are then chondrogenically differentiated, usually through the addition of TGFβ, dexamethasone and vitamin C (here a chondrogenic pellet is shown in red circle). Following differentiation the resulting chondrogenic cells are implanted in an animal model for a predetermined period of time. Following implantation the resulting construct can be retrieved and analysed (constructs in white circles) **b)** Representative MSCs during expansion phase. **c)** A representative thionine staining of MSCs chondrogenically differentiated for 21 days via pellet culture. **d)** H&E staining showing representative bone formed from chondrogenically differentiation MSCs after 8 weeks of subcutaneous implantation in nude mice (B-bone, CC-calcified cartilage, BM-bone marrow).*

IMPROVING TISSUE ENGINEERED ENDOCHONDRAL BONE FORMATION

TE MSC mediated bone formation has the potential to one day replace ABG treatment options but these constructs are in need of further development in order for this to occur. The goal of this thesis was to investigate how we could further improve construction of these TE grafts and investigate how we might be able to improve the current approach to TE endochondral bone formation to address some real world clinical applications.

In **chapter 3**, we identified and characterised a novel source of paediatric MSCs (P-MSCs). These cells compared to adult bone marrow derived MSC (A-MSCs) exhibit better expansion characteristics and were found to be a less senescent cell source. Most importantly, we found that these P-MSCs were capable of more robust and reproducible differentiation which indicate they would be a better cell source of MSC for TE applications. In this way we offer researchers an improved cell source option opposed to A-MSCs, the current "gold standard" cell source (93, 94).

In **chapter 4**, we investigated how an important extracellular matrix component, COLX, contributes to chondrogenic differentiation of MSCs and its importance in subsequent bone formation. We were able to show when COLX is significantly down regulated it not only effected chondrogenic differentiation of MSCs but also how this absence significantly hinders *in vivo* endochondral bone formation. In this way we were able to further improve our understanding of how MSC mediated EO is achieved and prove how important COLX can be to the process.

In **chapter 5**, we created a novel micropellet based construct which showed positive bone formation *in vivo*. These micropellets are advantageous as they are small enough to be optimised as an injectable bone substitute. With further optimisation these micropellets will allow for irregular shaped defects, which require tailor void filling(95) to be treated easily by clinicians. These micropellets could be used further in combinational approaches as discussed in **chapter 6** to further improve TE EO.

In **chapter 6**, we characterised the behaviour of MSCs in combination with a commercially available enamel matrix derivative (Emdogain (EMD)) used for periodontal tissue regeneration, showing EMD did not alter the chondrogenic differentiation of MSCs (96, 97). One day it could be possible to use EMD with chondrogenic MSCs to aid in the regeneration of soft tissue which is often also damaged around the bone defect site. With further development and research this line of work could create an improved construct which would potentially allow surgeons to treat both tissue types simultaneously, circumventing the need for an additional surgery improving patient treatment and recovery.

Our findings are summarised in **chapter 7**, where we discuss the future perspectives for MSC mediated EO. Although just the beginning, this thesis helps better our understanding and implementation of MSC mediated endochondral bone.

References

1. Schindeler A, McDonald MM, Bokko P, Little DG, editors. Bone remodeling during fracture repair: the cellular picture. Seminars in cell & developmental biology; 2008: Elsevier.
2. Giannoudis PV, Dinopoulos H, Tsiridis E. Bone substitutes: an update. Injury. 2005;36(3):S20-S7.
3. Lieberman JR, Friedlaender GE. Bone regeneration and repair: biology and clinical applications: Springer; 2005.
4. Frohlich M, Grayson WL, Wan LQ, Marolt D, Drobnic M, Vunjak-Novakovic G. Tissue engineered bone grafts: biological requirements, tissue culture and clinical relevance. Current stem cell research & therapy. 2008;3(4):254-64.
5. Hayden RE, Mullin DP, Patel AK. Reconstruction of the segmental mandibular defect: current state of the art. Current opinion in otolaryngology & head and neck surgery. 2012;20(4):231-6.
6. Bauer TW, Muschler GF. Bone graft materials: an overview of the basic science. Clinical Orthopaedics and Related Research®. 2000;371:10-27.
7. Finkemeier CG. Bone-grafting and bone-graft substitutes. JBJS. 2002;84(3):454-64.
8. Gómez-Barrena E, Rosset P, Lozano D, Stanovici J, Ermthaller C, Gerbhard F. Bone fracture healing: Cell therapy in delayed unions and nonunions. Bone. 2015;70:93-101.
9. Dimitriou R, Jones E, McGonagle D, Giannoudis PV. Bone regeneration: current concepts and future directions. BMC medicine. 2011;9(1):66.
10. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, et al. Multilineage potential of adult human mesenchymal stem cells. science. 1999;284(5411):143-7.
11. Chatterjea A, Meijer G, van Blitterswijk C, de Boer J. Clinical application of human mesenchymal stromal cells for bone tissue engineering. Stem cells international. 2010;2010.
12. Meijer GJ, de Bruijn JD, Koole R, van Blitterswijk CA. Cell based bone tissue engineering in jaw defects. Biomaterials. 2008;29(21):3053-61.
13. Mackie EJ, Ahmed YA, Tatarczuch L, Chen KS, Mirams M. Endochondral ossification: how cartilage is converted into bone in the developing skeleton. The international journal of biochemistry & cell biology. 2008;40(1):46-62.
14. Sharma B, Williams CG, Kim TK, Sun D, Malik A, Khan M, et al. Designing zonal organization into tissue-engineered cartilage. Tissue engineering. 2007;13(2):405-14.
15. Schrier L, Ferns SP, Barnes KM, Emons JAM, Newman EI, Nilsson O, et al. Depletion of resting zone chondrocytes during growth plate senescence. Journal of Endocrinology. 2006;189(1):27-36.
16. Abad V, Meyers JL, Weise M, Gafni RI, Barnes KM, Nilsson O, et al. The Role of the Resting Zone in Growth Plate Chondrogenesis. Endocrinology. 2002;143(5):1851-7.
17. Yang Y, Topol L, Lee H, Wu J. Wnt5a and Wnt5b exhibit distinct activities in coordinating chondrocyte proliferation and differentiation. Development. 2003;130(5):1003-15.
18. Dreier R. Hypertrophic differentiation of chondrocytes in osteoarthritis: the developmental aspect of degenerative joint disorders. Arthritis research & therapy. 2010;12(5):216.

19. Jaroszewicz J, Kosowska A, Hutmacher D, Swieszkowski W, Moskalewski S. Insight into characteristic features of cartilage growth plate as a physiological template for bone formation. Journal of Biomedical Materials Research Part A. 2016;104(2):357-66.
20. Usami Y, Gunawardena AT, Iwamoto M, Enomoto-Iwamoto M. Wnt signaling in cartilage development and diseases: lessons from animal studies. Laboratory investigation. 2016;96(2):186.
21. Chen W-H, Liu H-Y, Lo W-C, Wu S-C, Chi C-H, Chang H-Y, et al. Intervertebral disc regeneration in an ex vivo culture system using mesenchymal stem cells and platelet-rich plasma. Biomaterials. 2009;30(29):5523-33.
22. Ballock RT, O'Keefe RJ. The Biology of the Growth Plate. Jbjs. 2003;85(4):715-26.
23. Kronenberg HM. Developmental regulation of the growth plate. Nature. 2003;423(6937):332-6.
24. Chen X, Macica CM, Nasiri A, Broadus AE. Regulation of articular chondrocyte proliferation and differentiation by Indian hedgehog and parathyroid hormone–related protein in mice. Arthritis & Rheumatology. 2008;58(12):3788-97.
25. de Crombrugghe B, Lefebvre V, Nakashima K. Regulatory mechanisms in the pathways of cartilage and bone formation. Current opinion in cell biology. 2001;13(6):721-8.
26. Luvalle P, Daniels K, Hay ED, Olsen BR. Type X Collagen is Transcriptionally Activated and Specifically Localized During Sternal Cartilage Maturation. Matrix. 1992;12(5):404-13.
27. Shen G. The role of type X collagen in facilitating and regulating endochondral ossification of articular cartilage. Orthodontics & Craniofacial Research. 2005;8(1):11-7.
28. Schmid TM, Linsenmayer TF. Immunohistochemical localization of short chain cartilage collagen (type X) in avian tissues. The Journal of cell biology. 1985;100(2):598-605.
29. Kim HJ, Kirsch T. Collagen/annexin V interactions regulate chondrocyte mineralization. Journal of Biological Chemistry. 2008.
30. Kirsch T, Pfäffle M. Selective binding of anchorin CII (annexin V) to type II and X collagen and to chondrocalcin (C-propeptide of type II collagen) Implications for anchoring function between matrix vesicles and matrix proteins. FEBS letters. 1992;310(2):143-7.
31. Wu L, Genge B, Lloyd G, Wuthier R. Collagen-binding proteins in collagenase-released matrix vesicles from cartilage. Interaction between matrix vesicle proteins and different types of collagen. Journal of Biological Chemistry. 1991;266(2):1195-203.
32. Anderson HC. Molecular biology of matrix vesicles. Clinical orthopaedics and related research. 1995;314:266-80.
33. Chaudhary SC, Kuzynski M, Bottini M, Beniash E, Dokland T, Mobley CG, et al. Phosphate induces formation of matrix vesicles during odontoblast-initiated mineralization in vitro. Matrix Biology. 2016;52:284-300.
34. Yadav MC, Bottini M, Cory E, Bhattacharya K, Kuss P, Narisawa S, et al. Skeletal Mineralization Deficits and Impaired Biogenesis and Function of Chondrocyte-Derived Matrix Vesicles in Phospho1−/−and Phospho1/Pit1 Double-Knockout Mice. Journal of Bone and Mineral Research. 2016;31(6):1275-86.

35. Cui L, Houston DA, Farquharson C, MacRae VE. Characterisation of matrix vesicles in skeletal and soft tissue mineralisation. Bone. 2016;87:147-58.
36. Maes C, Kobayashi T, Selig MK, Torrekens S, Roth SI, Mackem S, et al. Osteoblast precursors, but not mature osteoblasts, move into developing and fractured bones along with invading blood vessels. Dev Cell. 2010;19(2):329-44.
37. Zheng Q, Zhou G, Morello R, Chen Y, Garcia-Rojas X, Lee B. Type X collagen gene regulation by Runx2 contributes directly to its hypertrophic chondrocyte–specific expression in vivo. The Journal of cell biology. 2003;162(5):833-42.
38. Colnot CI, Helms JA. A molecular analysis of matrix remodeling and angiogenesis during long bone development. Mechanisms of development. 2001;100(2):245-50.
39. Kim TK, Sharma B, Williams CG, Ruffner MA, Malik A, McFarland EG, et al. Experimental model for cartilage tissue engineering to regenerate the zonal organization of articular cartilage. Osteoarthritis and cartilage. 2003;11(9):653-64.
40. Colnot C, Lu C, Hu D, Helms JA. Distinguishing the contributions of the perichondrium, cartilage, and vascular endothelium to skeletal development. Developmental biology. 2004;269(1):55-69.
41. Dwek JR. The periosteum: what is it, where is it, and what mimics it in its absence? Skeletal radiology. 2010;39(4):319-23.
42. Seeman E. Periosteal bone formation—a neglected determinant of bone strength. New England Journal of Medicine. 2003;349(4):320-3.
43. Zhou X, von der Mark K, Henry S, Norton W, Adams H, de Crombrugghe B. Chondrocytes transdifferentiate into osteoblasts in endochondral bone during development, postnatal growth and fracture healing in mice. PLoS genetics. 2014;10(12):e1004820.
44. Tsang KY, Chan D, Cheah KS. Fate of growth plate hypertrophic chondrocytes: death or lineage extension? Development, growth & differentiation. 2015;57(2):179-92.
45. Park J, Gebhardt M, Golovchenko S, Branguli FP, Hattori T, Hartmann C, et al. Dual pathways to endochondral osteoblasts: a novel chondrocyte-derived osteoprogenitor cell identified in hypertrophic cartilage. Biology open. 2015:BIO201511031.
46. Roberts SJ, van Gastel N, Carmeliet G, Luyten FP. Uncovering the periosteum for skeletal regeneration: the stem cell that lies beneath. Bone. 2015;70:10-8.
47. Touaitahuata H, Cres G, de Rossi S, Vives V, Blangy A. The mineral dissolution function of osteoclasts is dispensable for hypertrophic cartilage degradation during long bone development and growth. Developmental biology. 2014;393(1):57-70.
48. Song RH, D Tortorella M, Malfait AM, Alston JT, Yang Z, Arner EC, et al. Aggrecan degradation in human articular cartilage explants is mediated by both ADAMTS-4 and ADAMTS-5. Arthritis & Rheumatology. 2007;56(2):575-85.
49. Page-McCaw A, Ewald AJ, Werb Z. Matrix metalloproteinases and the regulation of tissue remodelling. Nature reviews Molecular cell biology. 2007;8(3):221.

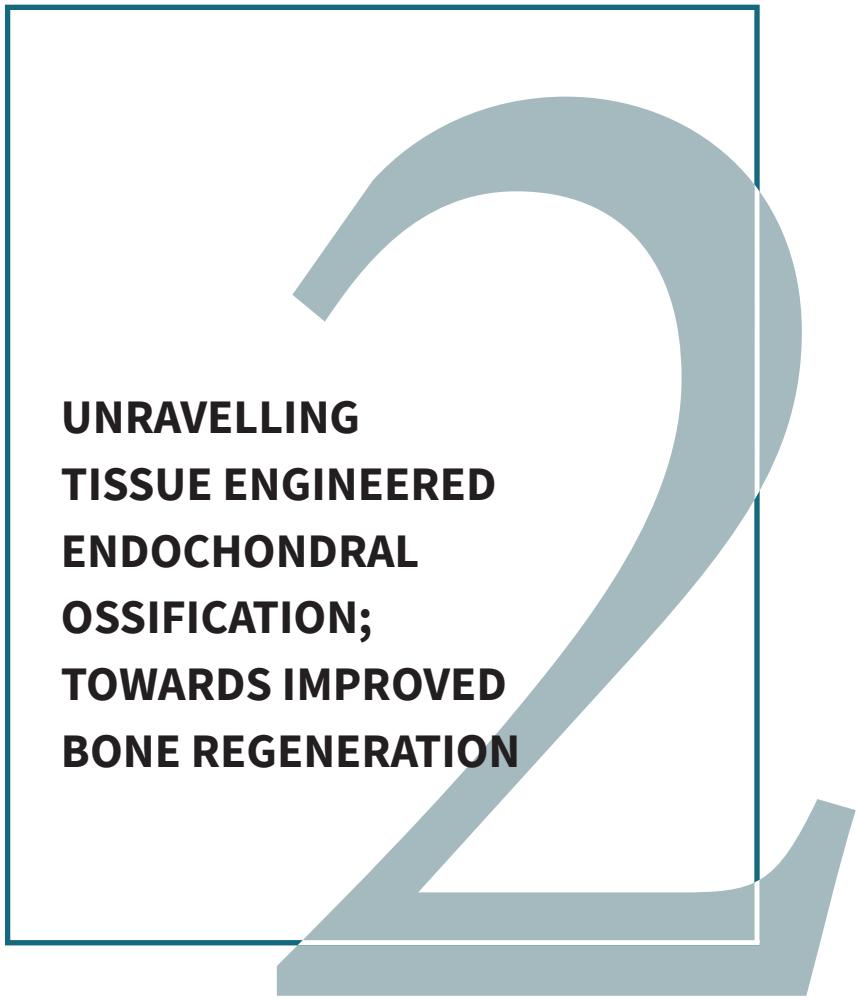
50. Nishimura R, Wakabayashi M, Hata K, Matsubara T, Honma S, Wakisaka S, et al. Osterix regulates calcification and degradation of chondrogenic matrices through matrix metalloproteinase 13 (MMP13) expression in association with transcription factor Runx2 during endochondral ossification. Journal of Biological Chemistry. 2012;287(40):33179-90.
51. Engsig MT, Chen Q-J, Vu TH, Pedersen A-C, Therkidsen B, Lund LR, et al. Matrix Metalloproteinase 9 and Vascular Endothelial Growth Factor Are Essential for Osteoclast Recruitment into Developing Long Bones. The Journal of Cell Biology. 2000;151(4):879-90.
52. Hu K, Olsen BR. Osteoblast-derived VEGF regulates osteoblast differentiation and bone formation during bone repair. The Journal of clinical investigation. 2016;126(2):509.
53. Liu Y, Olsen BR. Distinct VEGF functions during bone development and homeostasis. Archivum immunologiae et therapiae experimentalis. 2014;62(5):363-8.
54. Boyce BF, Xing L. Functions of RANKL/RANK/OPG in bone modeling and remodeling. Archives of biochemistry and biophysics. 2008;473(2):139-46.
55. Xiong J, Onal M, Jilka RL, Weinstein RS, Manolagas SC, O'Brien CA. Matrix-embedded cells control osteoclast formation. Nature medicine. 2011;17(10):1235-41.
56. Ortega N, Behonick DJ, Werb Z. Matrix remodeling during endochondral ossification. Trends in cell biology. 2004;14(2):86-93.
57. Akkiraju H, Nohe A. Role of chondrocytes in cartilage formation, progression of osteoarthritis and cartilage regeneration. Journal of developmental biology. 2015;3(4):177-92.
58. Bara JJ, Johnson WEB, Caterson B, Roberts S. Articular cartilage glycosaminoglycans inhibit the adhesion of endothelial cells. Connective tissue research. 2012;53(3):220-8.
59. Shapiro IM, Adams CS, Freeman T, Srinivas V. Fate of the hypertrophic chondrocyte: microenvironmental perspectives on apoptosis and survival in the epiphyseal growth plate. Birth Defects Research Part C: Embryo Today: Reviews. 2005;75(4):330-9.
60. Franz-Odendaal TA, Andrews D, Kumar S. Angiogenesis Meets Skeletogenesis: The Cross-Talk between Two Dynamic Systems. Physiologic and Pathologic Angiogenesis-Signaling Mechanisms and Targeted Therapy: InTech; 2017.
61. Gerber H-P, Vu TH, Ryan AM, Kowalski J, Werb Z, Ferrara N. VEGF couples hypertrophic cartilage remodeling, ossification and angiogenesis during endochondral bone formation. Nature medicine. 1999;5(6).
62. Yang Q, McHugh KP, Patntirapong S, Gu X, Wunderlich L, Hauschka PV. VEGF enhancement of osteoclast survival and bone resorption involves VEGF receptor-2 signaling and β 3-integrin. Matrix Biology. 2008;27(7):589-99.
63. Knowles HJ, Athanasou NA. Hypoxia-inducible factor is expressed in giant cell tumour of bone and mediates paracrine effects of hypoxia on monocyte–osteoclast differentiation via induction of VEGF. The Journal of pathology. 2008;215(1):56-66.

64. Nakagawa M, Kaneda T, Arakawa T, Morita S, Sato T, Yomada T, et al. Vascular endothelial growth factor (VEGF) directly enhances osteoclastic bone resorption and survival of mature osteoclasts. FEBS Letters. 2000;473(2):161-4.
65. Walzer SM, Cetin E, Grübl-Barabas R, Sulzbacher I, Rueger B, Girsch W, et al. Vascularization of primary and secondary ossification centres in the human growth plate. BMC developmental biology. 2014;14(1):36.
66. Aszódi A. The cartilaginous growth plate. Cartilage: Springer; 2016. p. 83-113.
67. Chanavaz M. Anatomy and histophysiology of the periosteum: quantification of the periosteal blood supply to the adjacent bone with 85Sr and gamma spectrometry. The Journal of oral implantology. 1995;21(3):214-9.
68. Kusumbe AP, Ramasamy SK, Adams RH. Coupling of angiogenesis and osteogenesis by a specific vessel subtype in bone. Nature. 2014;507(7492):323.
69. Qiu M, Wang C, Chen D, Shen C, Zhao H, He Y. Angiogenic and Osteogenic Coupling Effects of Deferoxamine-Loaded Poly (lactide-co-glycolide)-Poly (ethylene glycol)-Poly (lactide-co-glycolide) Nanoparticles. Applied Sciences. 2016;6(10):290.
70. Ramasamy SK, Kusumbe AP, Wang L, Adams RH. Endothelial Notch activity promotes angiogenesis and osteogenesis in bone. Nature. 2014;507(7492):376.
71. Kusumbe AP, Ramasamy SK, Itkin T, Mäe MA, Langen UH, Betsholtz C, et al. Age-dependent modulation of vascular niches for haematopoietic stem cells. Nature. 2016;532(7599):380.
72. Maes C, Carmeliet P, Moermans K, Stockmans I, Smets N, Collen D, et al. Impaired angiogenesis and endochondral bone formation in mice lacking the vascular endothelial growth factor isoforms VEGF 164 and VEGF 188. Mechanisms of development. 2002;111(1):61-73.
73. Deckers MML, Van Bezooijen RL, Van Der Horst G, Hoogendam J, van der Bent C, Papapoulos SE, et al. Bone morphogenetic proteins stimulate angiogenesis through osteoblast-derived vascular endothelial growth factor A. Endocrinology. 2002;143(4):1545-53.
74. Anderson HC. Matrix vesicles and calcification. Current rheumatology reports. 2003;5(3):222-6.
75. Anderson HC, Garimella R, Tague SE. The role of matrix vesicles in growth plate development and biomineralization. Front Biosci. 2005;10(1):822-37.
76. Yang Y-Q, Tan Y-Y, Wong R, Wenden A, Zhang L-K, Rabie ABM. The role of vascular endothelial growth factor in ossification. International journal of oral science. 2012;4(2):64-8.
77. Golub EE. Role of matrix vesicles in biomineralization. Biochimica et Biophysica Acta (BBA)-General Subjects. 2009;1790(12):1592-8.
78. Rosati R, Horan GS, Pinero GJ, Garofalo S, Keene DR, Horton WA, et al. Normal long bone growth and development in type X collagen-null mice. Nature genetics. 1994;8(2):129.
79. Sweeney E, Campbell M, Watkins K, Hunter C, Jacenko O. Altered endochondral ossification in collagen X mouse models leads to impaired immune responses. Developmental Dynamics. 2008;237(10):2693-704.
80. Caplan AI. Bone development and repair. Bioessays. 1987;6(4):171-5.

81. Marie PJ. Transcription factors controlling osteoblastogenesis. Archives of biochemistry and biophysics. 2008;473(2):98-105.
82. van der Stok J, Koolen M, Jahr H, Kops N, Waarsing J, Weinans H, et al. Chondrogenically differentiated mesenchymal stromal cell pellets stimulate endochondral bone regeneration in critical-sized bone defects. Eur Cell Mater. 2014;27(137):e48.
83. Farrell E, Both SK, Odörfer KI, Koevoet W, Kops N, O'Brien FJ, et al. In-vivo generation of bone via endochondral ossification by in-vitro chondrogenic priming of adult human and rat mesenchymal stem cells. BMC musculoskeletal disorders. 2011;12(1):31.
84. Scotti C, Tonnarelli B, Papadimitropoulos A, Scherberich A, Schaeren S, Schauerte A, et al. Recapitulation of endochondral bone formation using human adult mesenchymal stem cells as a paradigm for developmental engineering. Proceedings of the National Academy of Sciences. 2010;107(16):7251-6.
85. Scotti C, Piccinini E, Takizawa H, Todorov A, Bourgine P, Papadimitropoulos A, et al. Engineering of a functional bone organ through endochondral ossification. Proceedings of the National Academy of Sciences. 2013;110(10):3997-4002.
86. Gerber H-P, Vu TH, Ryan AM, Kowalski J, Werb Z, Ferrara N. VEGF couples hypertrophic cartilage remodeling, ossification and angiogenesis during endochondral bone formation. Nature medicine. 1999;5(6):623.
87. Farrell E, van der Jagt OP, Koevoet W, Kops N, Van Manen CJ, Hellingman CA, et al. Chondrogenic priming of human bone marrow stromal cells: a better route to bone repair? Tissue Engineering Part C: Methods. 2008;15(2):285-95.
88. Knuth CA W-BJ, Ridwan Y, Wolvius EB, Farrell E. Mesenchymal stem cell-mediated endochondral ossification utilising micropellets and brief chondrogenic priming. Eur Cell Mater. 2017;34:142-61.
89. Thompson EM, Matsiko A, Kelly DJ, Gleeson JP, O'Brien FJ. An endochondral ossification-based approach to bone repair: chondrogenically primed mesenchymal stem cell-laden scaffolds support greater repair of critical-sized cranial defects than osteogenically stimulated constructs in vivo. Tissue Engineering Part A. 2016;22(5-6):556-67.
90. Pelttari K, Winter A, Steck E, Goetzke K, Hennig T, Ochs BG, et al. Premature induction of hypertrophy during in vitro chondrogenesis of human mesenchymal stem cells correlates with calcification and vascular invasion after ectopic transplantation in SCID mice. Arthritis & Rheumatology. 2006;54(10):3254-66.
91. Karsenty G, Wagner EF. Reaching a genetic and molecular understanding of skeletal development. Dev Cell. 2002;2(4):389-406.
92. Kronenberg HM. The role of the perichondrium in fetal bone development. Ann N Y Acad Sci. 2007;1116:59-64.
93. Maciulaitis R, D'Apote L, Buchanan A, Pioppo L, Schneider CK. Clinical development of advanced therapy medicinal products in Europe: evidence that regulators must be proactive. Molecular Therapy. 2012;20(3):479-82.

94. Herberts CA, Kwa MSG, Hermsen HPH. Risk factors in the development of stem cell therapy. Journal of translational medicine. 2011;9(1):29.
95. Lewis G. Injectable bone cements for use in vertebroplasty and kyphoplasty: State-of-the-art review. Journal of Biomedical Materials Research Part B: Applied Biomaterials. 2006;76(2):456-68.
96. Sculean A, Windisch P, Döri F, Keglevich T, Molnar B, Gera I. Emdogain in regenerative periodontal therapy. A review of the literature. Fogorvosi szemle. 2007;100(5):220-32, 11-9.
97. Esposito M, Grusovin MG, Papanikolaou N, Coulthard P, Worthington HV. Enamel matrix derivative (Emdogain) for periodontal tissue regeneration in intrabony defects. A Cochrane systematic review. European journal of oral implantology. 2009;2(3).

European cells and materials. 2019; 37. 277 - 291



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UNRAVELLING TISSUE ENGINEERED ENDOCHONDRAL OSSIFICATION; TOWARDS IMPROVED BONE REGENERATION

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Abstract

Endochondral ossification (EO) is the process by which the long bones of the body form developmentally and has proven a promising method in tissue engineering to achieve cell mediated bone formation. This review focuses on state of the art research pertaining to mesenchymal stem cell mediated endochondral bone formation, focusing on the role of donor cells, the extracellular matrix and host immune cells during tissue engineered bone formation. We highlight possible research avenues to improve graft outcome and bone output, as well as emerging research which, when applied to tissue engineered bone grafts offers new promise to improve the likelihood such grafts transition from bench side to bedside.

INTRODUCTION

Bone has an inherent ability to repair itself following small injuries (1), however when a critical size defect exists, or is created following surgery, the regenerative capacity of bone is exhausted making clinical intervention necessary. As a result bone is one of the most commonly transplanted tissues in the world (2). Autologous bone grafts are the current "gold standard" treatment option for such defects as they are a natural osteoinductive/osteoconductive material (3, 4) with low risk of immune rejection (5). Although roughly 90% of autologous grafts are considered successful (5, 6), their use is limited due to the availability of harvestable material, uncertain integration following implantation and risk of donor site morbidity (5). Although allogeneic and xenographic grafts are available they are associated with other risks, including disease transfer or immunological rejection (7). Common complications associated with bone grafts, regardless if they are autologous, allogeneic or xenogeneic, include insufficient vascularisation at the implant site leading to poor nutrient/oxygen delivery, cell death and core necrosis (3, 4). This highlights a clear and present need for new suitable graft alternatives.

Tissue engineering and regenerative medicine (8) based approaches to bone repair vary greatly. Bioactive or inert materials (table 1) are currently being developed, that should enhance bone regeneration by guided tissue regeneration. Although promising many of these materials and other TERM approaches also rely on the use of iliac crest bone, which then does not address the many issues surrounding the use of autologous bone. The use of various adult progenitor cells to create cell based alternatives recapitulating on one of the developmental pathways of bone formation to achieve bone regeneration and repair of critical sized bone defects has received much attention in recent decades. This review focuses on the state of the art strategies implemented in cell based TERM and focuses on considerations for improved bone regeneration and output.

CELL BASED STRATEGIES FOR BONE REPAIR; ENDOCHONDRAL VS INTRAMEMBRANOUS OSSIFICATION

Bone develops through either intramembranous (9) or endochondral ossification (EO) (10, 11). Although both processes vary greatly each results in bone formation. IMO involves the direct differentiation of mesenchymal cells to osteoblasts and is how most facial bones are formed developmentally (12). IMO can be achieved in TERM by either

direct differentiation or through the combination of MSCs with biomaterials (including but not limited to tricalcium phosphate or collagen sponges) (13). Although promising, this approach has not reached its full potential due to insufficient vascularisation of the implant, resulting in core necrosis (13, 14). This vascularisation is crucial for graft survival and is required for proper integration with patient's existing bone. With this in mind, EO is a more promising model for bone formation as it naturally induce vascularisation at the implant site (15-20).

Table 1: Bone graft related terminology and definition/examples

Term	Definition	Ref.
Osteoinductive	Material that is able to induce osteogenic differentiation of primitive cells; induces bone formation; process that is observed during bone repair (healing)	(1, 2)
Osteoconductive	Material that causes bone formation on the surface of the material; induces migration of bone forming cells to surface; observed regularly on bone implants; examples: hydroxyapatite, tricalcium phosphate	(1, 2)
Inert material	Not chemically active; material does not join/integrate directly with bone; example: titanium, steel	(3, 4)
Bioactive material	Cause a biological response allowing for tissue bonding to material; surface reactivity influences ability to bond to bone; example: bioactive glass and ceramics	(5)
Allogeneic graft	Tissue or cells obtained from donor material of same species as recipient; Osteoinductive and osteoconductive; can be fresh or frozen	(4)
Autologous graft	Tissue or cells obtained from patient receiving treatment; osteoinductive and osteoconductive	(4)
Xenogenic graft	Tissue or cells obtained from a non-human source; Example: bovine, porcine	(4)

1. *Lee JH, editor Development of osteoconductive and osteoinductive bone healing materials. 43rd Annual European Calcified Tissue Society Congress; 2016: BioScientifica.*
2. *Finkemeier CG. Bone-grafting and bone-graft substitutes. JBJS. 2002;84(3):454-64.*
3. *LeGeros RZ. Calcium phosphate-based osteoinductive materials. Chemical reviews. 2008;108(11):4742-53.*
4. *Roselló Llabrés X, Roselló Camps À, Jané Salas E, Alburquerque R, Velasco Ortega E, López López J. Graft materials in oral surgery: revision. Biomimetics, Biomaterials and Tissue Engineering, 2014, vol 19, num 1, p 1-7. 2014.*
5. *Ducheyne P, Qiu Q. Bioactive ceramics: the effect of surface reactivity on bone formation and bone cell function. Biomaterials. 1999;20(23-24):2287-303.*

Developmentally, EO relies on the establishment of a cartilage template which is achieved via condensation and differentiation of mesenchyme cells (17). Chondrocytes

within the template exhibit a zonal distribution, exhibiting clear divisions between the different stages of chondrocyte differentiation within the template. Resting chondrocytes display a seemingly sporadic distribution and are thought to maintain a population of cells which, when triggered, give rise to the more organized, disk like proliferating chondrocytes (21, 22). Proliferating chondrocytes contribute to longitudinal bone growth and are regulated by a complicated feedback loop which includes factors such as TGF-b, PTHrP, and Ihh (23, 24). These factors are also involved in initiating hypertrophic differentiation. When hypertrophic differentiation starts, chondrocytes secrete factors to recruit other cell types critical for successful EO (24, 25) (summarized figure 1). For example factors such as ANG-1, PDGFa, and VEGF will aid in the recruitment of the nearby vasculature to the cartilage template (26), which will ultimately result in the deliver pre-osteoblastic cells to the cartilage template (27). Factors released by the hypertrophic chondrocytes, including MMPs and other proteolytic enzymes, will contribute to early matrix remodelling (28) and release of RANKL and VEGF will recruit osteoclast cells which further contributes to proper matrix remodelling (29). Together osteoblastic cells delivered via the invaded vasculature, transdifferentiation of chondrocytes in the cartilage template and invading osteoblasts from the surrounding bone collar calcify the cartilage matrix and bone formation occurs (27, 30). The coordination of these events with cell/vascular recruitment ultimately controls effective bone formation in EO. This can be recapitulated in TERM by differentiating MSCs chondrogenically and implanted the cells subcutaneously either as pellets or seeded in scaffolds (31-34). This seems to mirror developmental EO and shows excellent integration with the host (35). Tissue engineered EO utilising mesenchymal stem cells has been proven a viable method to achieve bone formation (36-40). In 2006 Huang *et al*. showed the ability of chondrogenically primed MSCs loaded into a hyaluronan/gelatin scaffold to form bone (41) and in 2014 van der Stok and Bahney each independently demonstrated how these chondrogenic MSCs could also be used to partially repair a critical sized defect even without a biomaterial support (34, 42). Interestingly, this has been shown to be specific for chondrogenically differentiated MSC as chondrocytes following expansion and differentiation will not form bone or bone marrow *in vivo* despite similar culture characteristics. Whether this is to do with the developmental origin of these cells or their expression of specific proteins, such as Collagen type X (COLX), a hypertrophic associated collagen (which culture expanded chondrocytes do not express), is not known (43-45). It is also possible that chondrocytes do not interact with cells of the host in a similar fashion. In order to develop better TERM approaches to bone defect repair, recapitulating the EO process, we must understand how MSC mediated EO occurs and the kinetics of the process.

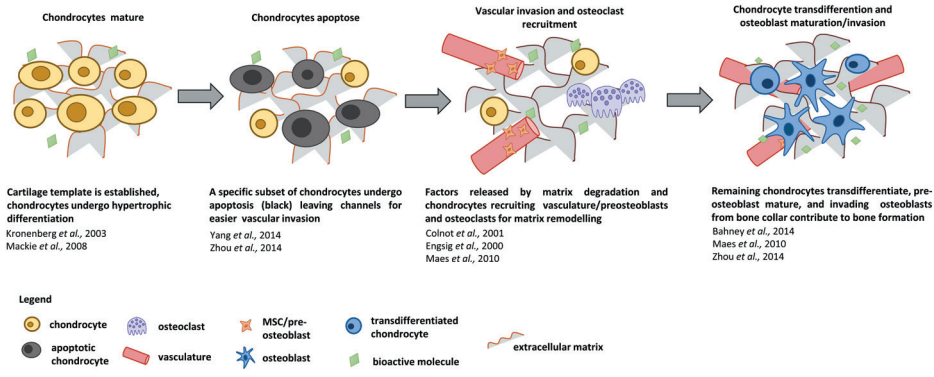

Figure 1: Snapshot of cellular invasion and behaviour during developmental endochondral ossification. Following the establishment of the cartilage template a specific subset of hypertrophic chondrocytes apoptose. This creates space for the nearby vasculature to invade and releases bioactive molecules within the matrix. At the same time pericytic like pre-osteoblasts invade via passive migration attached to the side of the vasculature. Factors released from the degraded extracellular matrix further aid in the recruitment of matrix remodeling osteoclasts. The non-apoptotic chondrocytes found within the matrix are capable of transdifferentiation into osteoblast like cells which in combination with mature osteoblasts contribute to bone formation.

THE DONOR'S ROLE: RECRUITMENT OF THE HOST AND LONG TERM INVOLVEMENT

The induction of vascular invasion, *de novo* formation of a marrow cavity and osteoclast activity observed in tissue engineered constructs demonstrates endogenous host cells have a role in the formation of new bone (46-49). Donor MSCs have been shown to directly contribute to the bone forming cell population in TERM EO. Using cell labelling methods implanted chondrogenically differentiated MSCs have been shown to persist within the bone matrix and contribute directly to bone formation (42, 46, 50). Prior research from our lab suggests that the initial bone formation is mediated by donor MSCs. Using immunocompetent transgenic rats overexpressing human placental alkaline phosphatase (hPLAP), donor cells were tracked following implantation into syngeneic wild type rats (46). A mixed population of both positive and negative hPLAP cells found embedded within the bone matrix demonstrated that cells were of both donor and host origin. Scotti *et al*. further suggested donor cells which persist in the newly formed bone may have undergone transdifferentiation to an osteoblastic like cell. They reported that donor and host bone had a zonal distribution. Host cells were found to contribute to bone formation at the outer periphery of the implant and donor cells in the central portion (50). Although Scotti *et al*. hypothesise over time these donor cells would be replaced with host cells, Bahney and colleagues suggest the majority of bone formation

is donor-derived (42). This research is in contrast to the developmental situation where it was believed that following hypertrophic differentiation of chondrocytes apoptosis was their only fate, as shown in earlier avian based research (51). This theory has been challenged as of late. Thanks to studies in development, fracture repair and TERM we know hypertrophic chondrocytes do not all apoptose. Rather, a subset of them are actually plastic and capable of transdifferentiating into osteoblasts, or osteoblast like cells, further aiding in the process of bone formation (30, 42, 52). Developmentally Yang *et al*. showed these transdifferentiated hypertrophic chondrocytes persisted throughout development being present not only in foetal bone but also in the bone of adult mice (30). These finds have changed how researchers view bone homeostasis in development and in TERM as it is clear chondrocytes do contribute to bone formation. In tissue engineering there is a trend towards development of acellular grafts which are indeed attractive from a clinical perspective. However, knowing that implanted cells play an important role in bone formation, it may be necessary to rethink such approaches in order to maximise bone output. Certainly in more challenging clinical situations.

THE ROLE OF THE EXTRACELLULAR MATRIX IN MSC MEDIATED ENDOCHONDRAL OSSIFICATION

During chondrogenic differentiation of MSCs a bioactive matrix is produced which can greatly influence EO *in vivo*. Studies suggest the quality of the matrix pre-implantation influences *in vivo* bone formation. Scotti et. al reported after longer priming, greater chondrogenic induction and glycosaminoglycan (GAG) production was achieved which resulted in better bone formation following implantation (32). We also reported how stronger chondrogenic induction can influence *in vivo* bone formation, however we hypothesised more GAG rich matrices had delayed marrow formation due to delayed remodelling (31). Perhaps this indicates that parameters can be set using extracellular matrix (ECM) components produced by chondrogenically differentiated MSCs by which to judge bone formation, but to assess this without destruction of the pellet itself would be difficult. Recently some have suggested the chondrogenic potential can be influenced through the addition of certain FGFs which modulate TGFβ receptors in turn altering the GAG concentration (53). If this is the case, researchers could utilise this to alter GAG production within the constructs pre-implantation, however research in this area yielded conflicting data and how TGFβ receptor modulation influence ECM production by MSCs is still an area of ongoing investigation (53, 54).

When trying to further understand how the ECM influences EO, we can also gain valuable insight from researchers that are using chondrogenically differentiated MSCs not to achieve EO but to use as a TE cartilage replacement. Chondrocytes formed via

differentiation of MSCs compared to "native" chondrocytes exhibit clear differences in structure, ECM deposition, cellular phenotypes, and mechanical properties, excellently reviewed by Somoza *et al*. (55, 56). Researchers are investigating how they can prevent TE MSC cartilage constructs from forming bone *in vivo*. For instance it has been shown how suppression of canonical WNT signalling during chondrogenic differentiation resulted in less hypertrophic constructs, containing less COLX in the ECM, which had a negative effect on bone formation *in vivo* (57). This may indicate that, for improved bone formation, the enhancement of the WNT signalling pathway during chondrogenic differentiation would have a beneficial effect on the ECM and cell behaviour for bone repair. Importantly this study also highlighted the importance of hypertrophic differentiation for the induction of bone formation with MSC based endochondral grafts.

Developmentally, hypertrophic differentiation precedes mineralisation and during this phase 45% of the collagens produced is COLX (58). COLX has been thought to add to the structural stability in the surrounding pericellular network of hypertrophic chondrocytes (59, 60), but from a bone formation stand point its role can be more clearly seen in previous transgenic (Tg) and knock-out (KO) studies. In such studies groups perinatal death has been reported in the absence of COLX (around 25% in Tg mice and 10% in KO mice) with the surviving mice exhibiting a range of phenotypes including dwarfism, skeletal abnormalities, defective haematopoiesis or even phenotypically normal mice (61-64). It is initially clear that the absence of COLX has an impact on the normal skeletal development in mice, but the exact mechanisms contributing to each of these abnormalities needs to be further explored to truly understand how COLX contributes to bone formation and the supportive role it plays during the process. Some scientists report in the absence of COLX abnormal GAG distribution and decreased heparin sulphate proteoglycan (HSPG) content around hypertrophic chondrocytes occurs (62). Proper proteoglycan distribution throughout the remodelled matrix is essential as it not only plays a role in stabilising the ECM, but also regulates the availability of growth factors trapped within the matrix which are crucial for EO, contributing to the induction of blood vessel invasion VEGF and the attraction of matrix remodelling cells such as osteoclasts in a timely manner(9, 65). Proper ECM arrangement is not only important with regard to the above mentioned aspects but also for proper placement of smaller structures like matrix vesicles.

Matrix vesicles are small structures which bud from the membrane of chondrocytes, osteoblasts, and other cells. These structures carry with them, among other things, a collection of bioactive enzymes, proteins and phospholipids, specific to the cell they are produced from, that are important in the initiation of calcification (66, 67). Matrix vesicles become entrapped in the ECM and help attract cells via their content (i.e. VEGF to attract blood vessels, BMPs to attract osteoblasts, etc.) making their point of anchoring and zonal distribution crucial for proper cell recruitment to the correct area (68, 69). There has

been research focusing on the interactions between COLX and annexin V binding which is found on matrix vesicles. Annexin V facilitates calcium influx into matrix vesicles which is important for the initiation of biomineralisation within the vesicles, in turn influences matrix mineralisation and bone formation. COLX is able to selectively bind to the annexin V, which is hypothesised to initiate this influx of calcium into matrix vesicles (70, 71). Others reported that when COLX is absent, vesicle distribution throughout the matrix is disrupted and subsequent bone formation is stunted (64, 71). This is alarming and shows proper placement of matrix vesicles is required for cell attraction to the proper site of bone formation. However this conclusion is challenged by others in the field who found that knocking out annexin V resulted in no change in mineralisation or bone formation (8). Although initially these results appear to be contradictory there could be a simple explanation. As we know, COLX plays a role in supporting and maintaining the proteoglycan and collagen organisation of the ECM. When it is absent these are no longer organised properly. Matrix vesicles have also been shown to associate with the hyaluronic acid binding region found in proteoglycans which can also result in calcium influx (71). If COLX is not present it is possible matrix vesicles associate more strongly with proteoglycans which would still allow them to be entrapped in the matrix, maybe no longer specifically at the border of the chondro-osseous junction, but still able to initiate mineralisation, thus allowing bone formation still takes place.

So far we have seen how COLX can influence bone formation during EO, however, there is another important area that is influenced by EO which is the proper development of the bone marrow niche, and the area crucial for proper haematopoiesis which studies have suggested is also regulated in part by COLX. It has been well established that important cytokines, chemokines, and growth factors bind and interact with HSPG which in part regulate or control an immune response (71, 72). Researchers have found when COLX is decreased there is also a decrease in HSPG and a dysregulation of the immune system of Tg mice. There is an increase in factors that play a role in regulating immune responses, including IL-4, IL-12, cutaneous T-cell attracting chemokine (CTACK) and leptin which have been shown to bind to HSPG, and major changes to the immune system itself. Often mice with defective or missing COLX have a severely decreased immune cell count. Although the immune cells that remain in the mouse often function properly the immune response they illicit cannot be controlled which ultimately has been found to lead to death in immune challenge studies (72). When mice with defective/missing COLX were challenged with an opportunistic parasite they were initially able to clear the parasitic infection but did not recover and ultimately died. Post-mortem investigation showed enlarged livers and increased parasite cysts in the brain, liver and lungs both indicative of a malfunctioned immune response (72). With a decreased HSPG count and an increased production of immune factors the body is unable to regulate the response properly. Again here researchers argue over the importance of COLX in regulating

the immune response as conflicting results have been shown (64, 73). However the differences observed between researchers may also come down to the genetic profile of the models they use.

OSTEOIMMUNOLOGY FROM A TISSUE ENGINEERING PERSPECTIVE

In large bone defects, the cells of the immune system play an important role. The complex interaction between cells of the skeletal system and the immune system is critical for successful bone repair and is initiated by an inflammatory response to the damaged tissue (74-77) (figure 2). This leads to the secretion of pro-inflammatory cytokines, including, TNFα, interleukin (IL)-6 and IL-1β (75, 78). These cytokines can induce angiogenesis and attract the first cells of the innate immune response (monocytes, macrophages, dendritic cells (DCs), neutrophils and natural killer (NK) cells). The innate immune cells subsequently release specific cytokines and growth factors which attract cells of the adaptive immune system (T and B cells) (79). Immune cells are not the only cells attracted during this inflammatory response. Bone-specific growth factors such as TGFβ and BMP-2 are also secreted leading to the recruitment of osteoprogenitor cells (including MSCs) to the site of inflammation (79). The combined expression of growth factors with secretion of inflammatory mediators induces the proliferation and differentiation of osteoprogenitor cells to osteoblasts (80-82). IMO and EO are the two processes by which osteoprogenitors can differentiate to osteoblasts. Unlike in IMO, during EO the secretion of TGFβ2 or 3, BMPs and other signalling molecules leads to the formation of a cartilage template that is replaced by woven bone, each of which can be influenced by immune cells (74, 78, 83-85). The majority of fractures heal via EO and previous studies have demonstrated the importance of the immune system during the repair process; lymphocytes, in particular, have been shown to be crucial for fracture healing (1). During bone remodelling, infiltrating T and B cells into the fracture callus have been shown to be negatively involved in the bone repair process (86, 87). During bone remodelling Th1, Th2 and Treg cells are thought to negatively influence osteoclast maturation, however Th17 cells show a positive effect on osteoclast formation (88-90). Mice lacking T and B cells appear to have accelerated fracture healing compared to those with a fully competent immune system (91). More specifically, CD8 T cells were demonstrated to inhibit fracture repair (92), however, other T cells on the other hand have varying effects on bone formation/regeneration depending of the subtype that was studied (87, 93, 94). Collectively, the complex interaction between the immune system and the cells of the skeletal system is critical for the outcome of the bone repair/regeneration as the manipulation of a specific subset of immune cells could greatly impact bone formation.

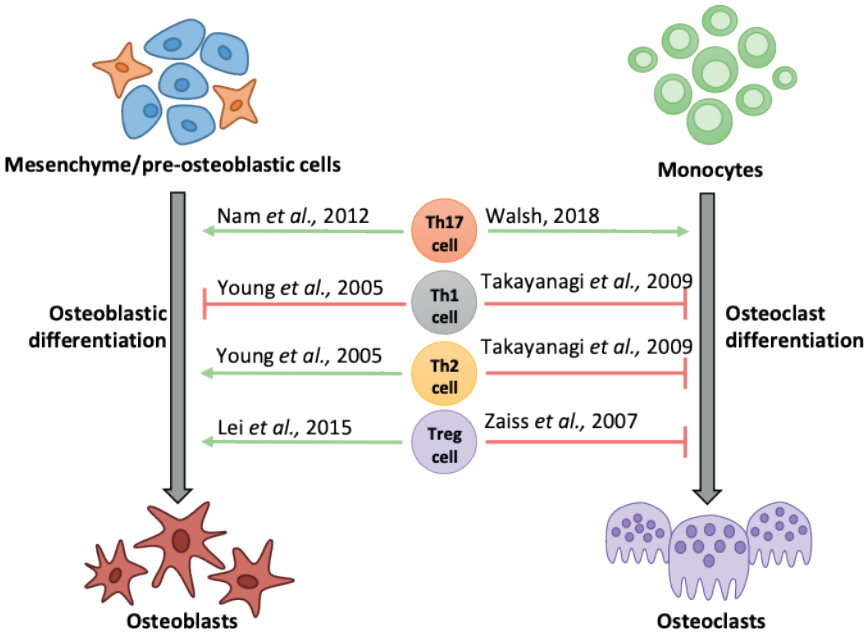

Figure 2: T cells can influence osteoblastic and osteclastic maturation. The release of cytokines and various growth factures during bone formation and fracture repair results in the recruitment of various immune cells which can influence bone formation and remodeling (green arrows-positive influence, red bar lines-negative influence).

The use of autologous cells for bone regeneration are ideal due to the lack of immune rejection upon implantation. However, autologous cells have drawbacks in the limited quantity of material that can be obtained. Moreover, the material that is obtained is usually of poor quality. This is due to the fact that autologous cells are generally obtained from elderly and diseased patients and therefore have poor proliferative and differentiation capacities compared to those that could be obtained from healthy individuals (95). Furthermore, treating a patient with their own cells can cause a major delay in treatment timetables due to the *in vitro* manipulations on the cells (e.g. expansion and quality control) before they can be administered back into the patient. Taking this into consideration, new and improved TERM-based approaches to bone repair need to be developed. The use of allogeneic cells would be preferable as there would be an immediate approved stock of cells ready to treat a patient. This advantage has led to an increased interest in the research of using allogeneic cells for TERM applications. There has been research already on allogeneic MSCs which demonstrate that they are somewhat “immunoevasive” due to low surface expression of costimulatory molecules (e.g. CD80 and CD86) and MHC class II (96-99). MSCs are known to be immunoevasive which is advantageous as MSCs will be implanted into an inflammatory environment during fracture repair (99-109). In normal

situations, implantation of allogeneic cells would lead to rejection of the cells by the adaptive immune system. However, allogeneic MSCs have be shown to evade the immune response and in some instances avoid rejection upon implantation. In studies focused on the use of allogeneic MSCs for bone repair, the immune response again played an important role in the process. Bone regeneration induced by allogeneic MSCs has been shown to be negatively impacted by Th1 T cells through the inhibition of osteogenesis-specific gene expression (osteocalcin, Runx2 and ALP) (110). On the other hand, osteogenesis was promoted by Th2, Th17 and regulatory T cells (79, 111, 112). While there have been numerous studies on allogeneic undifferentiated MSCs, there has been little to investigate how the immune system responds to allogeneic MSCs when they are pre-differentiated into another tissue type prior to implantation. Allogeneic undifferentiated MSCs have been shown to be non-immunogenic (96, 98, 113-115). Due to their immunoevasive nature, they can modify the immune system to their desired purpose. Few have investigated the effects of allogeneic chondrogenic MSCs on the immune system. Thus far results have been conflicting, with reports demonstrating allogeneic chondrogenic MSCs to be both immunogenic (116, 117) and non-immunogenic (109, 118-120).

The contradicting results were highly dependent on how the co-culturing work was performed during the experiments. Even in the *in vivo* setting, little is known about the effects of these pre-differentiated MSCs on the immune system. Our group has recently detailed the various studies that have focused the interactions between the immune system and allogeneic differentiated MSCs in the context of bone tissue engineering (119). More recently the “immune privileged” nature of allogeneic MSCs has been called into question. As excellently reviewed by Griffin and Lohan, it is well documented that host responses vary in response to the presence of allogeneic MSCs from minor inflammation to right out rejection (121, 122).

The idea that allogeneic MSCs could be recognized and targeted by the host is concerning for many in the field of tissue engineering. It is clear from these studies that there is more research that needs to be conducted to determine how pre-differentiated MSCs interact with the immune system in an allogeneic setting before these cells can be clinically applicable. It appears increasingly unlikely however that MSCs or differentiated MSCs are truly capable of completely evading the immune system. The question to be answered is whether or not this is an issue for concern.

FURTHER CONSIDERATIONS, TOWARDS IMPROVED BONE OUTPUT

MSC mediated endochondral bone formation has yielded some promising results in animal model defect repair, however treatment of large bone defects is still problematic. Although Harada *et al*. showed how chondrogenically primed rat MSCs could heal a

critical sized defect (123) since then no group has demonstrated such large bone defect repair. Although MSC mediated EO is capable of forming bone *in vivo*, the quantity usually formed, outside this study, is insufficient to treat large bone defects. From a translational perspective the volume of chondrogenic MSCs required to properly heal critical sized defects would require unmanageable cell numbers, incubator space, reagents and time to maintain which would make the cost of such constructs astronomical (34, 124). In order to treat large defects scale-up approaches are necessary to improve bone output.

When considering scaled up bone formation the need for successful vascularisation to maintain cell health during regeneration must be taken into account. As most cells of the body are rarely more than 100-200μm from a capillary due to diffusion limits which influence their behaviour (125, 126), meaning proper vascularisation in TERM constructs is critical. Although chondrocytes are thought to be well suited to survive in the initial defect site as their true environment is also hypoxic and avascular (127), remodelling, vessel invasion and bone formation introduces new cells with variable oxygen/nutrient requirements into the defect site (126) making vascularisation crucial to ensure these cells' survival. In small defect repair vascularisation occurs rapidly enough to allow graft survival and integration, however with a large defect natural vascularisation rates may not be sufficient meaning it must be induced or compensated for in the initial implanted construct to prevent cell death. Pre-vascularisation of chondrogenic grafts pre-implantation have shown more promising results (128, 129). Freeman *et al*. showed recently the pre-vascularisation of chondrogenic MSC can result in accelerated vascularisation, host cell survival and ossification versus non-vascularised counterparts (130). These constructs were implanted for only 4 weeks but it would be interesting to see how constructs perform following longer *in vivo* implantation or in an immunocompetent animals. These studies are promising but special care must be taken when selecting endothelial cell sources as the phenotype of the cell differs between tissue types they are isolated from (131, 132). Other groups have investigated how the addition of biologically relevant compounds which are known to influence endothelial cell behaviour such as VEGF could be utilised to improve graft vascularisation (133). However high doses of VEGF have been shown to result in uncontrollable bone formation indicating further research is required to make this a more viable option (134). By accelerating processes which are known to be important for *in vivo* bone formation, such as vascularisation, it could be possible to not only improve graft performance but also increase bone formation as developmental studies have shown that bone forming osteocytes invade the cartilage template via migration with the vasculature (134, 135). From a TERM approach prevascularising grafts or inducing faster vascularisation is advantageous as you not only tackle the issue of poor vascularisation but may also increase bone formation in the process.

With the complications associated with cell based approaches to tissue regeneration, there is a movement in the field to seek out possible cell free approaches which could

circumvent these limitations. MSCs used in endochondral TE bone grafts have been shown to directly contribute to the bone forming population. As previously discussed it has been shown how implanted chondrogenically differentiated MSCs persist within the bone matrix and contribute directly to bone formation, instructing host bone formation throughout the process (42, 46, 50, 136). These studies suggest implanted cells are essential for proper bone formation, however devitalised grafts derived from chondrogenically differentiated MSCs have also now been shown to form endochondral bone *in vivo* (137-140). Martin *et al*. have created decellularised grafts which maintain bone formation potential once implanted. This group utilises immortalised cell lines, eliminating many of the culture induced issues associated with MSCs, which are decellularised via activation of an engineered death inducible receptor within the cells (137-139). Once decellularised and implanted these constructs show promising bone formation. What is also interesting is the fact that these immortalised cells could be further manipulated to overexpress factors which are known to improve bone formation, such as BMP2, which would be incorporated in the ECM and could further improve bone output. Kelly *et al*. following this same line of research, showed matrices produces specifically by hypertrophic chondrogenically differentiated MSCs produced significantly more bone than non-hypertrophic matrices (140) indicating something produced specifically during hypertrophy could be key to improved bone formation. Although the bone formed by acellular grafts produced significantly less bone volume than cellularised counterparts, these cell free grafts were still able to recruit host vasculature and cells required for proper bone formation (137, 138). With further optimisation they could be a promising alternative to current autologous bone grafts. Although decellularised grafts and “off the shelf” treatment options are an ideal solution in tissue engineering, the fact remains that cell based approaches as of now yield better bone formation than acellular counterparts. As such a popular scale up approach is to use growth factors combined with novel biomaterials. Growth factors important for developmental induction of EO, such as BMP-2 (124, 141, 142), TGF-β (136, 142), VEGF (143, 144), PRP (145) as well as potentially novel factors are being characterised to determine if their use in combination with mesenchymal stem cells (MSCs) would improve bone output. These factors have shown variable results, sometimes performing as well as or better than iliac crest bone but sometimes less so (145). Two drawbacks associated with this approach are that these factors are extremely expensive and are used at supraphysiological levels which is associated with additional risk. For example high doses of BMP-2 have been shown to causes soft tissue swelling (146) abnormal excessive bone formation (147) and even an increased cancer risk (148) to name a few (149). As such researchers are also investigating other compounds which are known to be involved in EO which could possibly be used at more physiologically acceptable doses. This includes growth and differentiation factor 5 (GDF5). This protein is well known for its role in joint formation, chondrogenesis and hypertrophic differentiation and is also a

member of a subgroup of BMPs (150). Other proteins which are more recently identified as being important during EO, including connective tissue growth factor (also known as CCN2) and high mobility group box 1 have also been investigated as possible additions to improve bone formation as they have shown positive results *in vitro* for improved cell recruitment, vascularisation and osteogenesis (133, 151-153) but could perhaps be used at a more physiologically relevant dose compared to BMP2 (154). However even when supraphysiological doses are required researchers are looking for ways to possibly decrease the effective dosage required to prevent these unwanted side effects. By coupling or crosslinking factors to matrices the concentration of these compounds can be reduced to something more physiologically acceptable (155, 156). Further research into the identification of new biologically relevant compounds is also useful. Recent studies have identified new stem cell populations which activates in response to acute skeletal injury (157). By studying the secreted profile of these cells in comparison with controls perhaps new relevant targets could be identified which may not even require supraphysiological doses to be effective. Additionally the use of "organs-on-a-chip" and other computational models, which have been proven promising methods to identify/validate targets and for improved screening methods (158), could accelerate results and research.

CONCLUSIONS

Modelling grafts in TE after EO has been an active area of research in bone TE for years. The initial cartilage graft is well suited to survive in an avascular environment and it is capable of inducing the migration of all the previously mentioned cell types on its own. Although it has been shown to be a reproducible method of bone formation, progress to enhance the bone forming capacity of these constructs in order to properly fill large bone defects has been slow moving. However as our understanding of the interactions that take place improve, not only between donor and host cells but also those of a fully functioning immune system we will better understand how to improve our grafts. As research in the field continues we will be able to improve graft vascularisation, integration and bone output making these tissue-engineered endochondral grafts a viable alternative to autologous bone graft substitutes in the future.

References

1. Schindeler A, McDonald MM, Bokko P, Little DG, editors. Bone remodeling during fracture repair: the cellular picture. Seminars in cell & developmental biology; 2008: Elsevier.
2. Petite H, Viateau V, Bensaid W, Meunier A, de Pollak C, Bourguignon M, et al. Tissue-engineered bone regeneration. Nature biotechnology. 2000;18(9):959.
3. Lieberman JR, Friedlaender GE. Bone regeneration and repair: biology and clinical applications: Springer; 2005.
4. Frohlich M, Grayson WL, Wan LQ, Marolt D, Drobnic M, Vunjak-Novakovic G. Tissue engineered bone grafts: biological requirements, tissue culture and clinical relevance. Current stem cell research & therapy. 2008;3(4):254-64.
5. Bauer TW, Muschler GF. Bone graft materials: an overview of the basic science. Clinical Orthopaedics and Related Research®. 2000;371:10-27.
6. Hayden RE, Mullin DP, Patel AK. Reconstruction of the segmental mandibular defect: current state of the art. Current opinion in otolaryngology & head and neck surgery. 2012;20(4):231-6.
7. Gómez-Barrena E, Rosset P, Lozano D, Stanovici J, Ermthaller C, Gerbhard F. Bone fracture healing: Cell therapy in delayed unions and nonunions. Bone. 2015;70:93-101.
8. Grskovic I, Kutsch A, Frie C, Groma G, Stermann J, Schlötzer-Schrehardt U, et al. Depletion of annexin A5, annexin A6, and collagen X causes no gross changes in matrix vesicle–mediated mineralization, but lack of collagen X affects hematopoiesis and the Th1/Th2 response. Journal of Bone and Mineral Research. 2012;27(11):2399-412.
9. Kim S-H, Turnbull J, Guimond S. Extracellular matrix and cell signalling: the dynamic cooperation of integrin, proteoglycan and growth factor receptor. Journal of Endocrinology. 2011;209(2):139-51.
10. Shapiro F. Bone development and its relation to fracture repair. The role of mesenchymal osteoblasts and surface osteoblasts. Eur Cell Mater. 2008;15:53-76.
11. Yang Y. Skeletal morphogenesis during embryonic development. Crit Rev Eukaryot Gene Expr. 2009;19(3):197-218.
12. Thompson Z, Miclau T, Hu D, Helms JA. A model for intramembranous ossification during fracture healing. Journal of Orthopaedic Research. 2002;20(5):1091-8.
13. Meijer GJ, de Bruijn JD, Koole R, van Blitterswijk CA. Cell based bone tissue engineering in jaw defects. Biomaterials. 2008;29(21):3053-61.
14. Chatterjea A, Meijer G, van Blitterswijk C, de Boer J. Clinical application of human mesenchymal stromal cells for bone tissue engineering. Stem Cells Int. 2010;2010:215625.
15. Cervantes-Diaz F, Contreras P, Marcellini S. Evolutionary origin of endochondral ossification: the transdifferentiation hypothesis. Development genes and evolution. 2017;227(2):121-7.
16. Gawlitta D, Farrell E, Malda J, Creemers LB, Alblas J, Dhert WJ. Modulating endochondral ossification of multipotent stromal cells for bone regeneration. Tissue engineering Part B, Reviews. 2010;16(4):385-95.

17. Mackie EJ, Ahmed YA, Tatarczuch L, Chen KS, Mirams M. Endochondral ossification: how cartilage is converted into bone in the developing skeleton. Int J Biochem Cell Biol. 2008;40(1):46-62.
18. Medici D, Olsen BR. The role of endothelial-mesenchymal transition in heterotopic ossification. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research. 2012;27(8):1619-22.
19. Thompson EM, Matsiko A, Farrell E, Kelly DJ, O'Brien FJ. Recapitulating endochondral ossification: a promising route to in vivo bone regeneration. Journal of tissue engineering and regenerative medicine. 2015;9(8):889-902.
20. Yeung Tsang K, Wa Tsang S, Chan D, Cheah KS. The chondrocytic journey in endochondral bone growth and skeletal dysplasia. Birth Defects Res C Embryo Today. 2014;102(1):52-73.
21. Schrier L, Ferns SP, Barnes KM, Emons JA, Newman EI, Nilsson O, et al. Depletion of resting zone chondrocytes during growth plate senescence. Journal of Endocrinology. 2006;189(1):27-36.
22. Yang Y, Topol L, Lee H, Wu J. Wnt5a and Wnt5b exhibit distinct activities in coordinating chondrocyte proliferation and differentiation. Development. 2003;130(5):1003-15.
23. Chen J-h, Cao J-l, Chu Y-l, Wang Z-l, Yang Z-t, Wang H-l. T-2 toxin-induced apoptosis involving Fas, p53, Bcl-xL, Bcl-2, Bax and caspase-3 signaling pathways in human chondrocytes. Journal of Zhejiang University Science B. 2008;9(6):455-63.
24. Kronenberg HM. Developmental regulation of the growth plate. Nature. 2003;423(6937):332.
25. Ballock RT, O'KEEFE RJ. The biology of the growth plate. JBJS. 2003;85(4):715-26.
26. Colnot CI, Helms JA. A molecular analysis of matrix remodeling and angiogenesis during long bone development. Mechanisms of development. 2001;100(2):245-50.
27. Maes C, Kobayashi T, Selig MK, Torrekens S, Roth SI, Mackem S, et al. Osteoblast precursors, but not mature osteoblasts, move into developing and fractured bones along with invading blood vessels. Developmental cell. 2010;19(2):329-44.
28. Nishimura T. Role of extracellular matrix in development of skeletal muscle and postmortem aging of meat. Meat Science. 2015;109:48-55.
29. Engsig MT, Chen Q-J, Vu TH, Pedersen A-C, Therkidsen B, Lund LR, et al. Matrix metalloproteinase 9 and vascular endothelial growth factor are essential for osteoclast recruitment into developing long bones. The Journal of cell biology. 2000;151(4):879-90.
30. Yang L, Tsang KY, Tang HC, Chan D, Cheah KSE. Hypertrophic chondrocytes can become osteoblasts and osteocytes in endochondral bone formation. Proceedings of the National Academy of Sciences. 2014;111(33):12097-102.
31. Knuth C, Witte-Bouma J, Ridwan Y, Wolvius E, Farrell E. Mesenchymal stem cell-mediated endochondral ossification utilising micropellets and brief chondrogenic priming. European cells & materials. 2017;34:142-61.
32. Scotti C, Tonnarelli B, Papadimitropoulos A, Scherberich A, Schaeren S, Schauerte A, et al. Recapitulation of endochondral bone formation using human adult mesenchymal stem cells as a paradigm for developmental engineering. Proceedings of the National Academy of Sciences. 2010;107(16):7251-6.

33. Tonnarelli B, Centola M, Barbero A, Zeller R, Martin I. Re-engineering development to instruct tissue regeneration. Current topics in developmental biology. 108: Elsevier; 2014. p. 319-38.
34. van der Stok J, Koolen M, Jahr H, Kops N, Waarsing J, Weinans H, et al. Chondrogenically differentiated mesenchymal stromal cell pellets stimulate endochondral bone regeneration in critical-sized bone defects. Eur Cell Mater. 2014;27(137):e48.
35. Chan CK, Chen CC, Luppen CA, Kim JB, DeBoer AT, Wei K, et al. Endochondral ossification is required for haematopoietic stem-cell niche formation. Nature. 2009;457(7228):490-4.
36. Dickhut A, Pelttari K, Janicki P, Wagner W, Eckstein V, Egermann M, et al. Calcification or dedifferentiation: requirement to lock mesenchymal stem cells in a desired differentiation stage. Journal of cellular physiology. 2009;219(1):219-26.
37. Hennig T, Lorenz H, Thiel A, Goetzke K, Dickhut A, Geiger F, et al. Reduced chondrogenic potential of adipose tissue derived stromal cells correlates with an altered TGFbeta receptor and BMP profile and is overcome by BMP-6. Journal of cellular physiology. 2007;211(3):682-91.
38. Janicki P, Kasten P, Kleinschmidt K, Luginbuehl R, Richter W. Chondrogenic pre-induction of human mesenchymal stem cells on beta-TCP: enhanced bone quality by endochondral heterotopic bone formation. Acta biomaterialia. 2010;6(8):3292-301.
39. Jukes JM, Both SK, Leusink A, Sterk LM, van Blitterswijk CA, de Boer J. Endochondral bone tissue engineering using embryonic stem cells. Proc Natl Acad Sci U S A. 2008;105(19):6840-5.
40. Karoliina P, Eric S, Wiltrud R. The use of mesenchymal stem cells for chondrogenesis. Injury. 2008;39.
41. Huang JI, Durbhakula MM, Angele P, Johnstone B, Yoo JU. Lunate arthroplasty with autologous mesenchymal stem cells in a rabbit model. JBJS. 2006;88(4):744-52.
42. Bahney CS, Hu DP, Taylor AJ, Ferro F, Britz HM, Hallgrimsson B, et al. Stem cell-derived endochondral cartilage stimulates bone healing by tissue transformation. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research. 2014;29(5):1269-82.
43. Hellingman CA, Verwiel ET, Slagt I, Koevoet W, Poublon RM, Nolst-Trenite GJ, et al. Differences in cartilage-forming capacity of expanded human chondrocytes from ear and nose and their gene expression profiles. Cell Transplant. 2011;20(6):925-40.
44. Pelttari K, Winter A, Steck E, Goetzke K, Hennig T, Ochs BG, et al. Premature induction of hypertrophy during in vitro chondrogenesis of human mesenchymal stem cells correlates with calcification and vascular invasion after ectopic transplantation in SCID mice. Arthritis Rheum. 2006;54(10):3254-66.
45. Pleumeekers MM, Nimeskern L, Koevoet WL, Kops N, Poublon RM, Stok KS, et al. The in vitro and in vivo capacity of culture-expanded human cells from several sources encapsulated in alginate to form cartilage. Eur Cell Mater. 2014;27:264-80; discussion 78-80.
46. Farrell E, Both SK, Odörfer KI, Koevoet W, Kops N, O'Brien FJ, et al. In-vivo generation of bone via endochondral ossification by in-vitro chondrogenic priming of adult human and rat mesenchymal stem cells. BMC musculoskeletal disorders. 2011;12(1):31.

47. Tasso R, Augello A, Boccardo S, Salvi S, Carida M, Postiglione F, et al. Recruitment of a host's osteoprogenitor cells using exogenous mesenchymal stem cells seeded on porous ceramic. Tissue Eng Part A. 2009;15(8):2203-12.
48. Tasso R, Fais F, Reverberi D, Tortelli F, Cancedda R. The recruitment of two consecutive and different waves of host stem/progenitor cells during the development of tissue-engineered bone in a murine model. Biomaterials. 2010;31(8):2121-9.
49. Tortelli F, Tasso R, Loiacono F, Cancedda R. The development of tissue-engineered bone of different origin through endochondral and intramembranous ossification following the implantation of mesenchymal stem cells and osteoblasts in a murine model. Biomaterials. 2010;31(2):242-9.
50. Scotti C, Piccinini E, Takizawa H, Todorov A, Bourgine P, Papadimitropoulos A, et al. Engineering of a functional bone organ through endochondral ossification. Proceedings of the National Academy of Sciences. 2013;110(10):3997-4002.
51. Gibson GJ, Kohler WJ, Schaffler MB. Chondrocyte apoptosis in endochondral ossification of chick sterna. Developmental dynamics. 1995;203(4):468-76.
52. Zhou X, von der Mark K, Henry S, Norton W, Adams H, de Crombrugghe B. Chondrocytes transdifferentiate into osteoblasts in endochondral bone during development, postnatal growth and fracture healing in mice. PLoS genetics. 2014;10(12):e1004820.
53. Correa D, Somoza RA, Lin P, Greenberg S, Rom E, Duesler L, et al. Sequential exposure to fibroblast growth factors (FGF) 2, 9 and 18 enhances hMSC chondrogenic differentiation. Osteoarthritis and Cartilage. 2015;23(3):443-53.
54. de Kroon LM, Narcisi R, Davidson ENB, Cleary MA, van Beuningen HM, Koevoet WJ, et al. Activin receptor-like kinase receptors ALK5 and ALK1 are both required for TGFβ-induced chondrogenic differentiation of human bone marrow-derived mesenchymal stem cells. PloS one. 2015;10(12):e0146124.
55. Somoza RA, Correa D, Labat I, Sternberg H, Forrest ME, Khalil AM, et al. Transcriptome-wide analyses of human neonatal articular cartilage and human mesenchymal stem cell-derived cartilage provide a new molecular target for evaluating engineered cartilage. Tissue Engineering Part A. 2018;24(3-4):335-50.
56. Somoza RA, Welter JF, Correa D, Caplan AI. Chondrogenic differentiation of mesenchymal stem cells: challenges and unfulfilled expectations. Tissue Engineering Part B: Reviews. 2014;20(6):596-608.
57. Narcisi R, Cleary MA, Brama PA, Hoogduijn MJ, Tüysüz N, ten Berge D, et al. Long-term expansion, enhanced chondrogenic potential, and suppression of endochondral ossification of adult human MSCs via WNT signaling modulation. Stem cell reports. 2015;4(3):459-72.
58. Luvalle P, Daniels K, Hay ED, Olsen BR. Type X Collagen is Transcriptionally Activated and Specifically Localized During Sternal Cartilage Maturation. Matrix. 1992;12(5):404-13.
59. Schmid TM, Linsenmayer TF. Immunohistochemical localization of short chain cartilage collagen (type X) in avian tissues. The Journal of cell biology. 1985;100(2):598-605.

60. Shen G. The role of type X collagen in facilitating and regulating endochondral ossification of articular cartilage. Orthodontics & Craniofacial Research. 2005;8(1):11-7.
61. Campbell MR, Gress CJ, Appleman EH, Jacenko O. Chicken Collagen X Regulatory Sequences Restrict Transgene Expression to Hypertrophic Cartilage in Mice. The American Journal of Pathology. 2004;164(2):487-99.
62. Jacenko O, Chan D, Franklin A, Ito S, Underhill CB, Bateman JF, et al. A Dominant Interference Collagen X Mutation Disrupts Hypertrophic Chondrocyte Pericellular Matrix and Glycosaminoglycan and Proteoglycan Distribution in Transgenic Mice. The American Journal of Pathology. 2001;159(6):2257-69.
63. Jacenko O, Roberts DW, Campbell MR, McManus PM, Gress CJ, Tao Z. Linking Hematopoiesis to Endochondral Skeletogenesis through Analysis of Mice Transgenic for Collagen X. The American Journal of Pathology. 2002;160(6):2019-34.
64. Kwan KM, Pang MK, Zhou S, Cowan SK, Kong RY, Pfordte T, et al. Abnormal compartmentalization of cartilage matrix components in mice lacking collagen X: implications for function. J Cell Biol. 1997;136(2):459-71.
65. Yang Y-Q, Tan Y-Y, Wong R, Wenden A, Zhang L-K, Rabie ABM. The role of vascular endothelial growth factor in ossification. International journal of oral science. 2012;4(2):64-8.
66. Anderson HC, Garimella R, Tague SE. The role of matrix vesicles in growth plate development and biomineralization. Front Biosci. 2005;10(1):822-37.
67. Golub EE. Role of matrix vesicles in biomineralization. Biochimica et Biophysica Acta (BBA)-General Subjects. 2009;1790(12):1592-8.
68. Kirsch T, Harrison G, Golub EE, Nah H-D. The roles of annexins and types II and X collagen in matrix vesicle-mediated mineralization of growth plate cartilage. Journal of Biological Chemistry. 2000;275(45):35577-83.
69. Nahar NN, Missana LR, Garimella R, Tague SE, Anderson HC. Matrix vesicles are carriers of bone morphogenetic proteins (BMPs), vascular endothelial growth factor (VEGF), and noncollagenous matrix proteins. Journal of bone and mineral metabolism. 2008;26(5):514-9.
70. Kirsch T, Pfäffle M. Selective binding of anchorin CII (annexin V) to type II and X collagen and to chondrocalcin (C-propeptide of type II collagen) Implications for anchoring function between matrix vesicles and matrix proteins. FEBS letters. 1992;310(2):143-7.
71. Wu L, Genge B, Lloyd G, Wuthier R. Collagen-binding proteins in collagenase-released matrix vesicles from cartilage. Interaction between matrix vesicle proteins and different types of collagen. Journal of Biological Chemistry. 1991;266(2):1195-203.
72. Sweeney E, Campbell M, Watkins K, Hunter CA, Jacenko O. Altered endochondral ossification in collagen X mouse models leads to impaired immune responses. Developmental Dynamics. 2008;237(10):2693-704.
73. Rosatil R, Horanl GS, Piner03 GI, Garofalo S, Keene DR. Normal long bone growth and development in type X collagen. Nature genetics. 1994;8.

74. Kolar P, Schmidt-Bleek K, Schell H, Gaber T, Toben D, Schmidmaier G, et al. The early fracture hematoma and its potential role in fracture healing. Tissue engineering Part B, Reviews. 2010;16(4):427-34.
75. Mountziaris PM, Mikos AG. Modulation of the inflammatory response for enhanced bone tissue regeneration. Tissue engineering Part B, Reviews. 2008;14(2):179-86.
76. Pape HC, Marcucio R, Humphrey C, Colnot C, Knobe M, Harvey EJ. Trauma-induced inflammation and fracture healing. J Orthop Trauma. 2010;24(9):522-5.
77. Dar HY, Azam Z, Anupam R, Mondal RK, Srivastava RK. Osteoimmunology: The Nexus between bone and immune system. Front Biosci. 2018;23:464-92.
78. Gerstenfeld LC, Cullinane DM, Barnes GL, Graves DT, Einhorn TA. Fracture healing as a post-natal developmental process: molecular, spatial, and temporal aspects of its regulation. J Cell Biochem. 2003;88(5):873-84.
79. Kovach TK, Dighe AS, Lobo PI, Cui Q. Interactions between MSCs and immune cells: implications for bone healing. J Immunol Res. 2015;2015:752510.
80. Dimitriou R, Jones E, McGonagle D, Giannoudis PV. Bone regeneration: current concepts and future directions. BMC Med. 2011;9:66.
81. Loi F, Cordova LA, Pajarinen J, Lin TH, Yao Z, Goodman SB. Inflammation, fracture and bone repair. Bone. 2016;86:119-30.
82. Mizuno K, Mineo K, Tachibana T, Sumi M, Matsubara T, Hirohata K. The osteogenetic potential of fracture haematoma. Subperiosteal and intramuscular transplantation of the haematoma. J Bone Joint Surg Br. 1990;72(5):822-9.
83. Brighton CT. The biology of fracture repair. Instr Course Lect. 1984;33:60-82.
84. Einhorn TA, Gerstenfeld LC. Fracture healing: mechanisms and interventions. Nat Rev Rheumatol. 2015;11(1):45-54.
85. Kuntzman AJaT, G. J. The process of fracture repair. Anatomy and physiology for manual therapies: Wiley; 2010.
86. Konnecke I, Serra A, El Khassawna T, Schlundt C, Schell H, Hauser A, et al. T and B cells participate in bone repair by infiltrating the fracture callus in a two-wave fashion. Bone. 2014;64:155-65.
87. Young N, Mikhalkevich N, Yan Y, Chen D, Zheng W-p. Differential regulation of osteoblast activity by Th cell subsets mediated by parathyroid hormone and IFN-γ. The Journal of Immunology. 2005;175(12):8287-95.
88. Walsh MC, Takegahara N, Kim H, Choi Y. Updating osteoimmunology: regulation of bone cells by innate and adaptive immunity. Nature Reviews Rheumatology. 2018;14(3):146.
89. Takayanagi H. Osteoimmunology and the effects of the immune system on bone. Nature Reviews Rheumatology. 2009;5(12):667.
90. Zaiss MM, Axmann R, Zwerina J, Polzer K, Gückel E, Skapenko A, et al. Treg cells suppress osteoclast formation: a new link between the immune system and bone. Arthritis & Rheumatism: Official Journal of the American College of Rheumatology. 2007;56(12):4104-12.

91. Toben D, Schroeder I, El Khassawna T, Mehta M, Hoffmann JE, Frisch JT, et al. Fracture healing is accelerated in the absence of the adaptive immune system. J Bone Miner Res. 2011;26(1):113-24.
92. Reinke S, Geissler S, Taylor WR, Schmidt-Bleek K, Juelke K, Schwachmeyer V, et al. Terminally differentiated CD8(+) T cells negatively affect bone regeneration in humans. Sci Transl Med. 2013;5(177):177ra36.
93. Nam D, Mau E, Wang Y, Wright D, Silkstone D, Whetstone H, et al. T-lymphocytes enable osteoblast maturation via IL-17F during the early phase of fracture repair. PLoS One. 2012;7(6):e40044.
94. Lei H, Schmidt-Bleek K, Dienelt A, Reinke P, Volk H-D. Regulatory T cell-mediated anti-inflammatory effects promote successful tissue repair in both indirect and direct manners. Frontiers in pharmacology. 2015;6:184.
95. Mueller SM, Glowacki J. Age-related decline in the osteogenic potential of human bone marrow cells cultured in three-dimensional collagen sponges. J Cell Biochem. 2001;82(4):583-90.
96. Aggarwal S, Pittenger MF. Human mesenchymal stem cells modulate allogeneic immune cell responses. Blood. 2005;105(4):1815-22.
97. Le Blanc K, Ringden O. Immunomodulation by mesenchymal stem cells and clinical experience. J Intern Med. 2007;262(5):509-25.
98. Nauta AJ, Fibbe WE. Immunomodulatory properties of mesenchymal stromal cells. Blood. 2007;110(10):3499-506.
99. Ankrum JA, Ong JF, Karp JM. Mesenchymal stem cells: immune evasive, not immune privileged. Nature biotechnology. 2014;32(3):252.
100. Asari S, Itakura S, Ferreri K, Liu CP, Kuroda Y, Kandeel F, et al. Mesenchymal stem cells suppress B-cell terminal differentiation. Exp Hematol. 2009;37(5):604-15.
101. Corcione A, Benvenuto F, Ferretti E, Giunti D, Cappiello V, Cazzanti F, et al. Human mesenchymal stem cells modulate B-cell functions. Blood. 2006;107(1):367-72.
102. Djouad F, Charbonnier LM, Bouffi C, Louis-Plence P, Bony C, Apparailly F, et al. Mesenchymal stem cells inhibit the differentiation of dendritic cells through an interleukin-6-dependent mechanism. Stem cells. 2007;25(8):2025-32.
103. Jiang XX, Zhang Y, Liu B, Zhang SX, Wu Y, Yu XD, et al. Human mesenchymal stem cells inhibit differentiation and function of monocyte-derived dendritic cells. Blood. 2005;105(10):4120-6.
104. Liu WH, Liu JJ, Wu J, Zhang LL, Liu F, Yin L, et al. Novel mechanism of inhibition of dendritic cells maturation by mesenchymal stem cells via interleukin-10 and the JAK1/STAT3 signaling pathway. PLoS One. 2013;8(1):e55487.
105. Sotiropoulou PA, Perez SA, Gritzapis AD, Baxevanis CN, Papamichail M. Interactions between human mesenchymal stem cells and natural killer cells. Stem cells. 2006;24(1):74-85.
106. Spaggiari GM, Abdelrazik H, Becchetti F, Moretta L. MSCs inhibit monocyte-derived DC maturation and function by selectively interfering with the generation of immature DCs: central role of MSC-derived prostaglandin E2. Blood. 2009;113(26):6576-83.

107. Spaggiari GM, Capobianco A, Abdelrazik H, Becchetti F, Mingari MC, Moretta L. Mesenchymal stem cells inhibit natural killer-cell proliferation, cytotoxicity, and cytokine production: role of indoleamine 2,3-dioxygenase and prostaglandin E2. Blood. 2008;111(3):1327-33.
108. Zhao ZG, Xu W, Sun L, You Y, Li F, Li QB, et al. Immunomodulatory function of regulatory dendritic cells induced by mesenchymal stem cells. Immunol Invest. 2012;41(2):183-98.
109. Zheng ZH, Li XY, Ding J, Jia JF, Zhu P. Allogeneic mesenchymal stem cell and mesenchymal stem cell-differentiated chondrocyte suppress the responses of type II collagen-reactive T cells in rheumatoid arthritis. Rheumatology. 2008;47(1):22-30.
110. Dighe AS, Yang S, Madhu V, Balian G, Cui Q. Interferon gamma and T cells inhibit osteogenesis induced by allogeneic mesenchymal stromal cells. J Orthop Res. 2013;31(2):227-34.
111. Liu Y, Wang L, Kikuiri T, Akiyama K, Chen C, Xu X, et al. Mesenchymal stem cell-based tissue regeneration is governed by recipient T lymphocytes via IFN-gamma and TNF-alpha. Nature medicine. 2011;17(12):1594-601.
112. Omar OM, Graneli C, Ekstrom K, Karlsson C, Johansson A, Lausmaa J, et al. The stimulation of an osteogenic response by classical monocyte activation. Biomaterials. 2011;32(32):8190-204.
113. English K. Mechanisms of mesenchymal stromal cell immunomodulation. Immunol Cell Biol. 2013;91(1):19-26.
114. Gao F, Chiu SM, Motan DA, Zhang Z, Chen L, Ji HL, et al. Mesenchymal stem cells and immunomodulation: current status and future prospects. Cell Death Dis. 2016;7:e2062.
115. Hoogduijn MJ, Popp F, Verbeek R, Masoodi M, Nicolaou A, Baan C, et al. The immunomodulatory properties of mesenchymal stem cells and their use for immunotherapy. Int Immunopharmacol. 2010;10(12):1496-500.
116. Mukonoweshuro B, Brown CJ, Fisher J, Ingham E. Immunogenicity of undifferentiated and differentiated allogeneic mouse mesenchymal stem cells. J Tissue Eng. 2014;5:2041731414534255.
117. Ryan AE, Lohan P, O'Flynn L, Treacy O, Chen X, Coleman C, et al. Chondrogenic differentiation increases antidonor immune response to allogeneic mesenchymal stem cell transplantation. Molecular therapy : the journal of the American Society of Gene Therapy. 2014;22(3):655-67.
118. Adkisson HDt, Martin JA, Amendola RL, Milliman C, Mauch KA, Katwal AB, et al. The potential of human allogeneic juvenile chondrocytes for restoration of articular cartilage. Am J Sports Med. 2010;38(7):1324-33.
119. Kiernan CH, Hoogduijn MJ, Franquesa M, Wolvius EB, Brama PA, Farrell E. Allogeneic chondrogenically differentiated human mesenchymal stromal cells do not induce immunogenic responses from T lymphocytes in vitro. Cytotherapy. 2016;18(8):957-69.
120. Le Blanc K, Tammik C, Rosendahl K, Zetterberg E, Ringden O. HLA expression and immunologic properties of differentiated and undifferentiated mesenchymal stem cells. Exp Hematol. 2003;31(10):890-6.

121. Griffin MD, Ryan AE, Alagesan S, Lohan P, Treacy O, Ritter T. Anti-donor immune responses elicited by allogeneic mesenchymal stem cells: what have we learned so far? Immunology and Cell Biology. 2013;91(1):40-51.
122. Lohan P, Treacy O, Griffin MD, Ritter T, Ryan AE. Anti-donor immune responses elicited by allogeneic mesenchymal stem cells and their extracellular vesicles: are we still learning? Frontiers in immunology. 2017;8:1626.
123. Harada N, Watanabe Y, Sato K, Abe S, Yamanaka K, Sakai Y, et al. Bone regeneration in a massive rat femur defect through endochondral ossification achieved with chondrogenically differentiated MSCs in a degradable scaffold. Biomaterials. 2014;35(27):7800-10.
124. Penick KJ, Solchaga LA, Welter JF. High-throughput aggregate culture system to assess the chondrogenic potential of mesenchymal stem cells. Biotechniques. 2005;39(5):687.
125. Biederman-Thorson MA, Schmidt RF, Thews G. Human Physiology: Springer Science & Business Media; 2013.
126. Ko H, Milthorpe BK, McFarland CD. Engineering thick tissues-the vascularisation problem. European Cells and Materials. 2007.
127. Pfander D, Gelse K. Hypoxia and osteoarthritis: how chondrocytes survive hypoxic environments. Current opinion in rheumatology. 2007;19(5):457-62.
128. Ng J, Spiller K, Bernhard J, Vunjak-Novakovic G. Engineering Vascular Niche for Bone Tissue Regeneration. Biology and Engineering of Stem Cell Niches: Elsevier; 2017. p. 517-29.
129. Yousefi A-M, James PF, Akbarzadeh R, Subramanian A, Flavin C, Oudadesse H. Prospect of stem cells in bone tissue engineering: a review. Stem cells international. 2016;2016.
130. Freeman FE, Allen AB, Stevens HY, Guldberg RE, McNamara LM. Effects of in vitro endochondral priming and pre-vascularisation of human MSC cellular aggregates in vivo. Stem cell research & therapy. 2015;6(1):218.
131. Chi J-T, Chang HY, Haraldsen G, Jahnsen FL, Troyanskaya OG, Chang DS, et al. Endothelial cell diversity revealed by global expression profiling. Proceedings of the National Academy of Sciences. 2003;100(19):10623-8.
132. Garlanda C, Dejana E. Heterogeneity of endothelial cells: specific markers. Arteriosclerosis, thrombosis, and vascular biology. 1997;17(7):1193-202.
133. Takigawa M. CCN2: a master regulator of the genesis of bone and cartilage. Journal of cell communication and signaling. 2013;7(3):191-201.
134. Maes C, Goossens S, Bartunkova S, Drogat B, Coenegrachts L, Stockmans I, et al. Increased skeletal VEGF enhances β-catenin activity and results in excessively ossified bones. The EMBO Journal. 2010;29(2):424-41.
135. Maes C. Role and regulation of vascularization processes in endochondral bones. Calcified tissue international. 2013;92(4):307-23.

136. Mendes L, Katagiri H, Tam W, Chai Y, Geris L, Roberts S, et al. Advancing osteochondral tissue engineering: bone morphogenetic protein, transforming growth factor, and fibroblast growth factor signaling drive ordered differentiation of periosteal cells resulting in stable cartilage and bone formation in vivo. Stem cell research & therapy. 2018;9(1):42.
137. Bourgine P. Combination of cell immortalization and apoptosis induction to engineer decellularized matrices as bone graft materials: University_of_Basel; 2013.
138. Bourgine P, Le Magnen C, Pigeot S, Geurts J, Scherberich A, Martin I. Combination of immortalization and inducible death strategies to generate a human mesenchymal stromal cell line with controlled survival. Stem cell research. 2014;12(2):584-98.
139. Bourgine PE, Scotti C, Pigeot S, Tchang LA, Todorov A, Martin I. Osteoinductivity of engineered cartilaginous templates devitalized by inducible apoptosis. Proceedings of the National Academy of Sciences. 2014;111(49):17426-31.
140. Cunniffe GM, Vinardell T, Murphy JM, Thompson EM, Matsiko A, O'Brien FJ, et al. Porous decellularized tissue engineered hypertrophic cartilage as a scaffold for large bone defect healing. Acta biomaterialia. 2015;23:82-90.
141. Decambron A, Fournet A, Bensidhoum M, Manassero M, Sailhan F, Petite H, et al. Low-dose BMP-2 and MSC dual delivery onto coral scaffold for critical-size bone defect regeneration in sheep. Journal of Orthopaedic Research. 2017.
142. Stüdle C, Martin QV, Haumer A, Guerrero J, Centola M, Mehrkens A, et al. Spatially confined induction of endochondral ossification by functionalized hydrogels for ectopic engineering of osteochondral tissues. Biomaterials. 2018.
143. Carlevaro MF, Cermelli S, Cancedda R, Cancedda FD. Vascular endothelial growth factor (VEGF) in cartilage neovascularization and chondrocyte differentiation: auto-paracrine role during endochondral bone formation. J Cell Sci. 2000;113(1):59-69.
144. Gerber H-P, Vu TH, Ryan AM, Kowalski J, Werb Z, Ferrara N. VEGF couples hypertrophic cartilage remodeling, ossification and angiogenesis during endochondral bone formation. Nature medicine. 1999;5(6):623.
145. Janssen NG, Weijs WL, Koole R, Rosenberg AJ, Meijer GJ. Tissue engineering strategies for alveolar cleft reconstruction: a systematic review of the literature. Clinical oral investigations. 2013.
146. Shahlaie K, Kim KD. Occipitocervical fusion using recombinant human bone morphogenetic protein-2: adverse effects due to tissue swelling and seroma. Spine. 2008;33(21):2361-6.
147. Zara JN, Siu RK, Zhang X, Shen J, Ngo R, Lee M, et al. High doses of bone morphogenetic protein 2 induce structurally abnormal bone and inflammation in vivo. Tissue Engineering Part A. 2011;17(9-10):1389-99.
148. Carragee EJ, Chu G, Rohatgi R, Hurwitz EL, Weiner BK, Yoon ST, et al. Cancer risk after use of recombinant bone morphogenetic protein-2 for spinal arthrodesis. JBJS. 2013;95(17):1537-45.

149. Carreira A, Lojudice F, Halcsik E, Navarro R, Sogayar M, Granjeiro J. Bone morphogenetic proteins: facts, challenges, and future perspectives. Journal of dental research. 2014;93(4):335-45.
150. Coleman CM, Vaughan EE, Browe DC, Mooney E, Howard L, Barry F. Growth differentiation factor-5 enhances in vitro mesenchymal stromal cell chondrogenesis and hypertrophy. Stem cells and development. 2013;22(13):1968-76.
151. Khattab HM, Aoyama E, Kubota S, Takigawa M. Physical interaction of CCN2 with diverse growth factors involved in chondrocyte differentiation during endochondral ossification. Journal of cell communication and signaling. 2015;9(3):247-54.
152. Kubota S, Takigawa M. The role of CCN2 in cartilage and bone development. Journal of cell communication and signaling. 2011;5(3):209-17.
153. Taniguchi N, Yoshida K, Ito T, Tsuda M, Mishima Y, Furumatsu T, et al. Stage-Specific Secretion of HMGB1 in Cartilage Regulates Endochondral Ossification. Molecular and Cellular Biology. 2007;27.
154. Fahmy-Garcia S, van Driel M, Witte-Buoma J, Walles H, van Leeuwen J, van Osch G, et al. NELL-1, HMGB1, and CCN2 Enhance Migration and Vasculogenesis, But Not Osteogenic Differentiation Compared to BMP2. Tissue Eng Part A. 2017.
155. Mumcuoglu D, Fahmy-Garcia S, Ridwan Y, Nicke J, Farrell E, Kluijtmans SG, et al. Injectable BMP-2 delivery system based on collagen-derived microspheres and alginate induced bone formation in a time- and dose-dependent manner. Eur Cell Mater. 2018;35:242-54.
156. Quinlan E, Thompson EM, Matsiko A, O'brien FJ, López-Noriega A. Long-term controlled delivery of rhBMP-2 from collagen–hydroxyapatite scaffolds for superior bone tissue regeneration. Journal of Controlled Release. 2015;207:112-9.
157. Chan CK, Gulati GS, Sinha R, Tompkins JV, Lopez M, Carter AC, et al. Identification of the Human Skeletal Stem Cell. Cell. 2018;175(1):43-56. e21.
158. Esch EW, Bahinski A, Huh D. Organs-on-chips at the frontiers of drug discovery. Nature reviews Drug discovery. 2015;14(4):248.

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3

ISOLATING PAEDIATRIC MESENCHYMAL STEM CELLS WITH ENHANCED EXPANSION AND DIFFERENTIATION CAPABILITIES

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Abstract

Mesenchymal stem cells/marrow stromal cells (MSCs) are attractive for applications ranging from research and development to use in clinical therapeutics. However, the most commonly studied MSCs, adult bone marrow MSCs (A-MSCs), are limited by significant donor variation resulting in inconsistent expansion rates and multilineage differentiation capabilities. We have recently obtained permission to isolate paediatric MSCs (P-MSCs) from surplus iliac crest bone chips. Here, we developed a simple and easily replicable isolation protocol yielding P-MSCs which adhere to MSC defining guidelines. After confirming immunophenotypic marker expression we compared expansion rates, senescence, morphology and trilineage differentiation of P-MSCs to A-MSCs for multiple donors. We found P-MSCs have faster *in vitro* replication, consistently show significantly lower senescence and are capable of more reproducible multilineage differentiation than A-MSCs. We therefore believe P-MSCs are a promising candidate for use in research applications and potentially as part of an allogeneic therapeutic treatment.

INTRODUCTION

Over the last decades interest in mesenchymal stem cells/marrow stromal cells (MSCs) has grown; many have recognised their potential to advance scientific discovery and improve clinical treatment options (1-3). MSCs unlike other lineage-committed progenitors or terminally differentiated cells are capable of multilineage differentiation which is desirable for a number of applications ranging from developmental research to use in advanced therapeutic medicinal products (ATMPs) (4-7). MSCs are attractive for these applications as they can be easily isolated, cultured and expanded *in vitro* (2, 8). They have been found in a variety of tissues, blood and even urine (9-11). Regardless of their point of isolation, MSCs must adhere to criteria determined by the International Society for Cellular Therapy (ISCT) as outlined by M. Dominici et al. (12). Briefly, cells must (i) be plastic adherent, (ii) retain their multipotent differentiation capacity and (iii) express a panel of surface antigens. Although a diverse variety of MSCs meet these criteria, there are still numerous differences between populations depending on the method and tissue they are isolated from including variability in *in vitro* expansion, differentiation capability and cell surface marker expression (8, 13-16). These differences and the inherent donor variation observed between MSCs makes clinical translation and their use in ATMPs challenging.

In order for MSCs to be used as part of AMTPs, a sufficient quantity of cells must be obtained which are capable of producing consistent outcomes that satisfy the regulatory requirements set by the European Medicines Agency (EMA) and the Food and Drug Administration (FDA) (17-19). When removed from the environment of their *in vivo* niche and expanded *in vitro*, MSCs rapidly lose their ability to replicate and differentiate, meaning their characteristics change unpredictably over time in culture (16, 20-22). This variation is often observed in bone marrow (BM) MSC populations which as of now is still considered the gold standard when it comes to MSCs (23, 24). Other MSC sources such as umbilical cord and adipose tissue are being actively characterised with promising but conflicting results (11, 25-27). An ideal MSC source would allow isolation with minimal patient discomfort and yield cells capable of reproducibly meeting EMA/FDA regulatory requirements (28). Although BM MSCs are currently the gold standard for MSCs their isolation is associated with a painful procedure and harvesting of such material results in substantial patient discomfort and recovery time when used (27, 29). Many researchers, us among them, use surplus clinical material obtained from patients undergoing surgical procedures (total hip or knee replacement for example). However the age and disease

status of the donors often negatively influences MSC performance (20). Kretlow et al. found cell attachment, proliferation and differentiation were all affected as donor age increased (30, 31). MSCs from aged donors were less capable of secreting and maintaining a chondrogenic matrix (32), and had a decreased bone forming potential *in vivo* (33). It has also been reported cells from the elderly often exhibit cellular dysregulation Which negatively impacts stem cell populations (31, 34, 35). Additionally MSCs from elderly patients have been shown to have age-induced gene expression changes and earlier replicative senescence which further negatively effects MSC performance (31, 36). Cellular dysregulation in aging populations has also been hypothesised to add to the pathogenesis of these diseases which results in a damaged stem cell population (31, 34). As neither cell dysregulation or senescence is useful for research or the clinics, MSCs isolated from adult or geriatric populations are not an ideal cell source. MSCs isolated from younger patients have shown promise (11).

Recently we have gained access to small quantities of surplus bone from paediatric patients undergoing craniofacial reconstruction surgery from which we can easily isolate paediatric MSCs (P-MSCs). The resulting MSCs are plastic adherent, maintain MSC related immunophenotype and are capable of consistent differentiation. Here we outline how these cells are obtained, isolated and cultured as well as describe the morphological and phenotypic characteristics of these novel MSCs to allow others in the scientific community to utilise them for their own applications. We compare P-MSCs to adult MSCs (A-MSCs) isolated from BM and find P-MSCs to be capable of more consistent multilineage differentiation. We believe P-MSCs to be a promising candidate for use in both research as well as clinical applications.

MATERIALS AND METHODS

Mesenchymal stem cell isolation and expansion

All samples were harvested with the approval of the medical ethics committee at Erasmus Medical Centre (ErasmusMC, Netherlands). Adult-MSCs were isolated and expanded as previously described (MEC-2004-142 & MEC-2015-644) (13). Paediatric mesenchymal stem cells (P-MSC) were isolated from leftover iliac crest bone chip material obtained from patients undergoing cleft palate reconstructive surgery (MEC-2014-16; 9-13 years). P-MSCs were isolated by gently swirling 10mL of expansion medium (αMEM containing 10 % serum (lot # 41Q204K, Gibco), 50 mg/mL gentamycin, 1.5 mg/mL fungizone, 25 µg/mL L-ascorbic acid 2-phosphate and 1 ng/mL fibroblast growth factor-2 (Instruchemie)) with iliac crest bone chips. Medium was removed and the process was repeated with an additional 10 mL expansion medium. The cell suspension from the combined medium of both washes was plated in a T75 flask and iliac crest chips were processed for histology.

Flasks were washed 24 hours after plating with PBS to remove non- adherent cells and debris. Cells were cultured at 37°C and 5% carbon dioxide (CO_2). Expansion medium was refreshed twice a week. P-MSCs were passed at approximately 80-90% confluency using 0.05% trypsin and replated at approximately 2,300 cells/cm^2. After passages 2-4 A-MSCs were used for trilineage differentiation and after passage 5 for FACs analysis, immunocharacterisation and b-galactosidase stainings. P-MSCs were always used after passage 5 unless otherwise noted (b-galactosidase staining, passage 8).

FACS analysis

A-MSCs and P-MSCs were trypsinised at passage 5 and rinsed in FACS flow. Cells were incubated for 30 minutes in 100 µL FACS buffer (BD Biosciences) containing antibodies against CD90 (APC), CD105 (FITC), CD73 (PE), CD271 (APC), CD166 (PE), HLA-DR (PerCP), HLA-ABC (FITC) or CD45 (PerCP). MSCs were washed with FACS flow, centrifuged at 689 g for 5 minutes, resuspended in 200 µL of FACS flow and analysed on a FACS Jazz flow cytometer (all antibodies BD Biosciences). Post-analysis was completed using FlowJo software version 10.0.7 (Treestar Inc.).

Senescence staining and quantification

The percentage of senescent cells was determined by staining for senescence-associated lysosomal β-galactosidase using a modification of Debacq-Chainiaux et al's protocol (37). A-MSCs and P-MSCs were seeded at 2,300 cells/cm^2 in complete expansion medium (as described above). On day 3 cells were refreshed and after 6 days cells were fixed in 1% [v/v] formaldehyde (Sigma) and 0.5% glutaraldehyde [v/v] (Sigma) in PBS for 15 minutes at 4°C. After washing with distilled water, cells were incubated for 24 hours at 37°C with 250 µL/cm^2 staining solution (1 mg X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Roche Diagnostics), 1.64 mg potassium hexacyanoferrate (III) (Sigma), 2.1 mg potassium hexacyanoferrate (II) trihydrate (Sigma), 2 µmol magnesium chloride hexahydrate (Sigma), 150 µmol sodium chloride, 7.3 µmol monohydrous citric acid (Sigma), 25.3 µmol disbasic dodium phosphate dihydrate (Sigma)) per mL distilled water; pH 6.0). After rinsing in distilled water, cells were counterstained with 1 g/L neutral red (Sigma) in a solution of 0.2% acetic acid. The number of positive cells was quantified and plotted relative to total cell number.

Chondrogenic differentiation

2x10^5 A-MSCs or P-MSCs were suspended in 500 mL of chondrogenic medium (high-glucose DMEM supplemented with 50 µg/mL gentamycin (Invitrogen), 1.5 µg/mL fungizone (Invitrogen), 1 mM sodium pyruvate (Invitrogen), 40 µg/mL proline (Sigma), 1:100 v/v insulin-transferrin-selenium (ITS+; BD Biosciences), 10 ng/mL transforming growth factor β3 (Peprotech), 25 µg/mL L-ascorbic acid 2-phosphate (Sigma), and 100

nM dexamethasone (Sigma)) in 15 mL polypropylene tubes. Samples were centrifuged at 200 g for 8 minutes. Medium was replaced twice weekly for 21 days (P-MSCs) or for 28-35 days (A-MSCs). Samples were formalin fixed for histology (4% (w/v) formaldehyde in PBS for 2 hrs).

Osteogenic differentiation

A-MSCs or P-MSCs were plated at a density of $3x10^3$ cells/cm^2 in expansion medium (previously described). 24 hours following seeding medium was replaced with osteogenic induction medium (high-glucose DMEM supplemented with 10% serum, 50 mg/mL gentamycin, 1.5 mg/mL fungizone, 10 mM glycerol phosphate (Sigma), 0.1 mM dexamethasone (Sigma), and 0.1 mM L-ascorbic acid 2-phosphate (Sigma)). Medium was refreshed as previously described for 14-21 days, depending on when cell sheets began to pull away from the outer perimeter of the well or when calcium deposition was observed macroscopically, at which point the culture was ended. Cells were cultured at 37°C and 5% CO_2. Samples were prepared for histology (fixed in 70% EtOH at 4°C) following the end of culture.

Adipogenic differentiation

A-MSCs or P-MSCs were plated at a density of $2.1x10^4$ cells/cm^2 in adipogenic induction medium (high-glucose DMEM supplemented with 10 % serum, 50 mg/mL gentamycin, 1.5 mg/mL fungizone, 0.2 mM indomethacin (Sigma), 0.01 mg/mL insulin (Sigma), 0.5 mM 3 iso-butyl-1-methyl-xanthine (Sigma)). Medium was refreshed as previously described and cells were maintained at 37°C and 5% CO_2. Samples were prepared for histology (fixed in 4% (w/v) formalin) following harvest.

Haematoxylin-eosin staining

Paediatric bone chips were fixed for 24 hours in 4% (w/v) formalin, decalcified in 10% EDTA (w/v) for 30 days and paraffin embedded. Chondrogenic MSC pellets were fixed in 4% (w/v) formalin for 2 hours and paraffin embedded. 6 mm-thick sections were cut, deparaffinised and stained with haematoxylin-eosin (H&E). H&E staining was performed by incubating deparrafinised samples in Gill's haematoxylin (Sigma) for 5 minutes, washed in none distilled water for 5 minutes, washed in distilled water, and counterstained for 45 seconds with 2% Eosin (Merck; in 50% ethanol, 0.5% acetic acid). Samples were fixed in 70% EtOH for 10 seconds and rehydrated (sequentially in 96% EtOH, 100% EtOH, and xylene for 1 minute). Samples were mounted in Entellan (Depex).

Thionine staining

Deparaffinised samples were incubated in 0.04% thionine (prepared in 0.01M sodium acetate, pH 4.5) for 5 minutes, differentiated in 70% EtOH for 10 seconds and then rehydrated as previously described. Samples were mounted in Entellan (Depex).

von Kossa staining

Osteogenically differentiated MSCs were fixed in 4% (w/v) formalin for 1 hour. Following a rinse with ultrapure water, samples were incubated in 5% w/v silver nitrate (Sigma) for approximately 30 minutes under direct light provided by a light box. Following incubation samples were washed in ultrapure water and counterstained with nuclear fast red (Merck) for five minutes. Samples were dehydrated in 70% EtOH for 10 seconds followed by 96% EtOH and 100% EtOH for one minute. Samples were imaged in 100% EtOH directly following staining.

Oil red staining

Following a 15 minute fixation in 4% (w/v) formalin samples were rinsed in distilled water for 10 minutes. 0.5% w/v Oil-red O (in 2-propanol; Sigma) was added to samples for 10 minutes followed by further rinsing with distilled water. Samples were imaged in distilled water.

P-MSC-PBMCs co-culture

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats from healthy male donors (Sanquin, Rotterdam) by a Ficoll-Paque PLUS gradient separation as previously described (GE Healthcare) (38). PBMCs were resuspended in human serum conditioned medium (HCM) (RPMI-1640 medium, 1% GlutaMAX (Life Technologies), 50 µg/mL gentamycin, 1.5 µg/mL fungizone, 10% human serum (Sigma-Aldrich)) and stored at -80^{o}C until use. P-MSCs were trypsinised as previously described and seeded in low-evaporation round bottom 96 well plates. 24 hours following seeding, 1x10^{7} PBMCs were labelled with 20 µL of CFSE. T cells were stimulated by adding anti-CD3/CD28 antibodies (1 µL/10^{6} cells) to PBMC suspension with an anti- goat linker antibody (2 µl/106 cells). Stimulated 100,000 PBMCs were co-cultured with P-MSCs at a P-MSC:PBMC ratios of 1:2.5, 1:5, 1:10 or 1:20., PBMCs were harvested 5 days later and stained with CD3-PerCP (clone SK7), CD8- PE-Cy7 and CD4-APC (BD Biosciences). Samples were fixed in 3.6% paraformaldehyde and analysed using a FACS Jazz flow cytometer (BD Biosciences) and post-analysis was completed using FlowJo software version 10.0.7 (Treestar Inc.). Data is represented as reciprocal of the mean fluorescence intensity (MFI).

Statistical analysis

Mann Whitney U analysis was performed using SPSS (Ver 21. SPSS Inc, Chicago, USA) on data used in figures 1, 2, and 3. Kruskal-Wallis analysis with Dunn's multiple comparison was performed on figure 4. Data are shown as mean ± SD, P-values under 0.05 were considered significant.

RESULTS

Paediatric MSCs express a panel of established MSC cell surface markers

P-MSCs were isolated from small quantities of surplus bone biopsies from patients undergoing cleft pallet reconstruction surgery. The environment from which the P-MSCs are isolated contains both bone and bone marrow elements (figure 1).

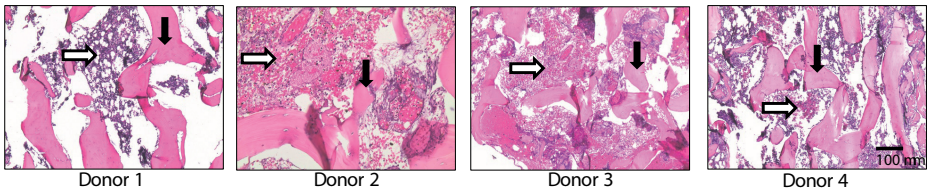

Figure 1: P-MSCs are isolated from illiac crest rest material containing both bone and bone marrow. Hematoxylin and Eosin stained sections of the illiac crest chips from which P-MSCs are isolated. The cell source environment is rich in both bone marrow elements and bone (black arrows indicate bone, white arrow bone marrow).

In order to prove isolated cells were indeed true MSCs we characterised the immunophenotypic expression of common MSC markers. These markers included a panel which are known to be expressed on MSCs including CD105, CD90, CD73, CD271, CD166 and HLA-ABC as well as a commonly used negative marker, lymphocyte associated CD45 (12, 39). Both A-MSCs and P-MSCs were analysed following 5 passages. MSC markers were expressed at a similar level in P-MSCs and A-MSCs (figure 2).

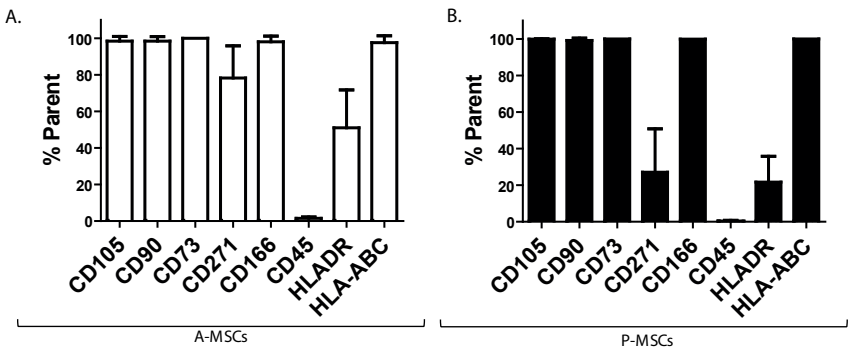

*Figure 2: P-MSCs and A-MSCs express similar levels of general stem cell markers. General MSC related markers which are commonly expressed on A-MSCs **(A)** are also expressed at a similar level in P-MSCs **(B)** (N.S. differences between A-MSCs and P-MSCs; Mann Whitney U test) Both populations are negative for haematopoietic marker CD45 however are positive for a panel of other immune related markers.*

Both populations were negative for CD45, and positive HLA-ABC. About half the P-MSCs and A-MSCs population were positive for HLA-DR which was not surprising as HLA-DR expression can increase with *in vitro* culture of MSCs (40). We found no significant difference in HLA-DR expression between P-MSCs and A-MSCs (figure 2).

Paediatric MSCs have enhanced expansion properties compared to adult MSCs

During expansion P-MSCs exhibit a typical MSC morphology similar to that observed in A-MSCs (figure 3a). Although the total number of days A-MSCs and P-MSCs took to establish the initial culture (figure 3b) and reach passage 3 (figure 3c) did not change, P-MSCs expanded significantly faster than A-MSCs, yielding more cells after the same time in culture (figure 3d; $p<0.02$). This difference in cell number could be attributed to cell size. A-MSCs enlarged the longer they were in culture, whereas P-MSCs remained small (figure 3a, 4a).

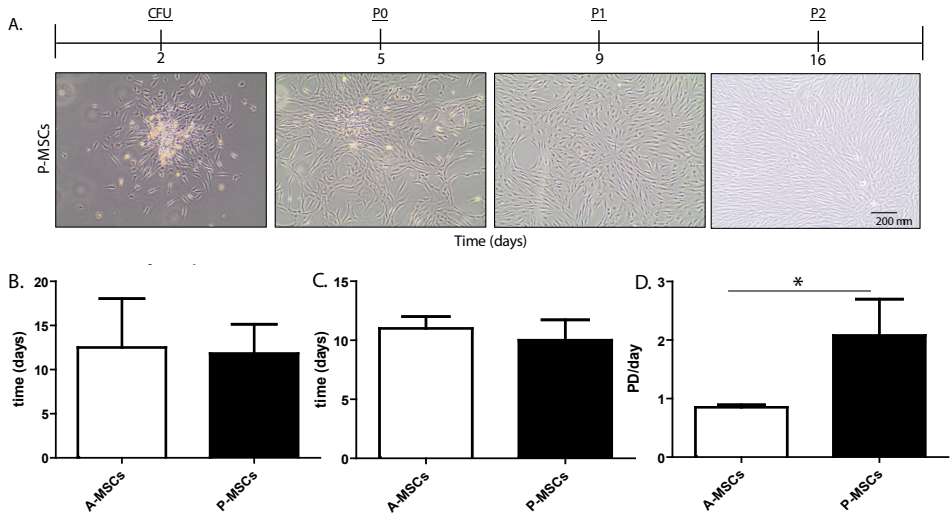

*Figure 3: P-MSCs and A-MSCs have a similar rate of expansion but P-MSCs undergo more population doublings. **A)** Cell morphology typically observed during expansion of P-MSCs (representative donor). **B)** Total days taken to establish culture from plating initial cell suspension to passage 0 does not differ between P-MSCs and A-MSCs. **C)** Expansion time from passage 0 to passage 3 does not differ between A-MSCs and P-MSCs. **D)** Total number of population doublings between passage 1 and passage 3 is greater in P-MSCs compared to A-MSCs ($p<0.0238$; Mann Whitney U test). (Abbreviations: CFU-colony forming units; P0: passage 0;P1: passage 1; P2: passage 2; P3: passage 3; PD: population doublings)*

P-MSCs are a less senescent cell source compared to A-MSCs

As increased cell size is a hallmark of senescence, a permanent cell cycle arrest that A-MSCs have been shown to undergo *in vitro*, we compared senescence between A-MSCs

and P-MSCs (41, 42) We observed cell enlargement of A-MSCs compared to P-MSCs (figure 4a). Senescence-associated lysosomal β-galactosidase staining showed P-MSCs, even after extensive passage (p8; figure 4b, c), contained significantly less senescent cells than A-MSCs at an earlier passage (p5; figure 4b, c).

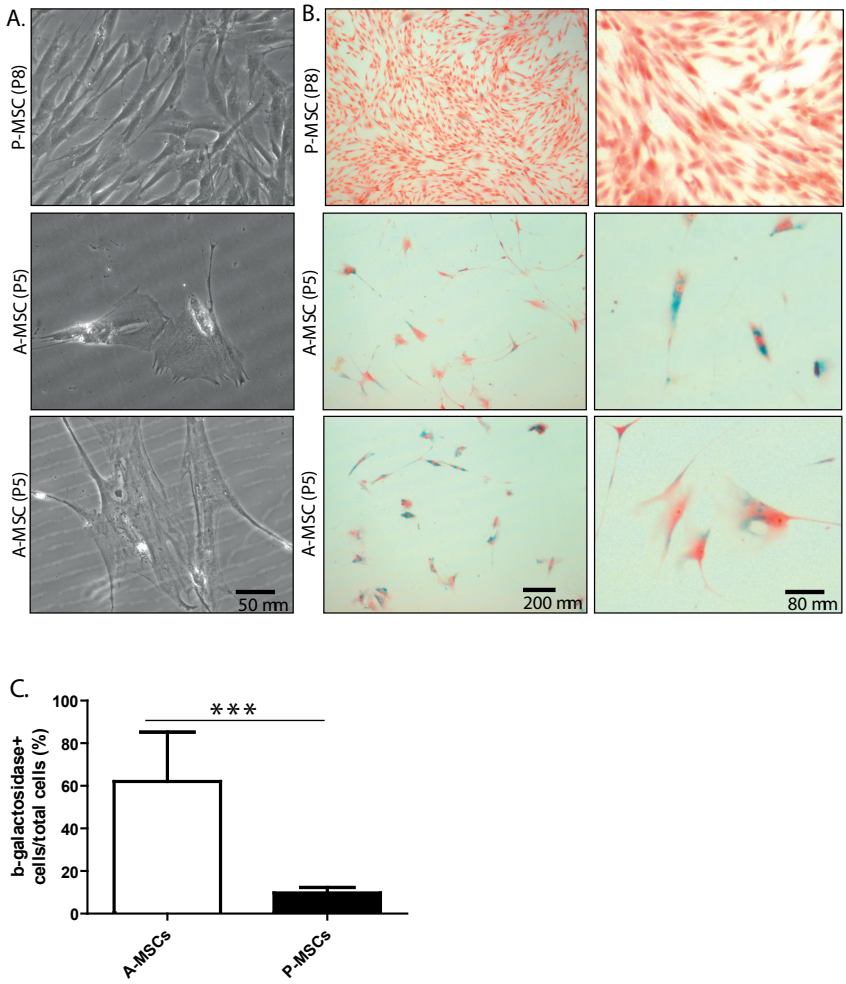

Figure 4: P-MSCs contain significantly less senescent cells than A-MSCs. A) Cell morphology observed during expansion of P-MSCs (p8) and of A-MSCs (p5). A-MSCs display a larger cell morphology compared to P-MSCs even though they are an earlier passage. Both A-MSCs and P-MSCs were seeded at the 2,300 cells/cm2 and expanded under normal conditions for 6 days. B) b-galactosidase staining of both P-MSCs (p8) and A-MSCs (p5). P-MSCs contain far less positively b-galactosidase stained cells than A-MSCs. C) Percentage of senescent cells counted in P-MSCs is significantly lower than that of A-MSCs (n=4; p<0.000; Mann Whitney test). (Abbreviations: P5: passage 5, P8: passage 8)

In monolayer P-MSCs reduce T cell proliferation at a similar level as A-MSCs

Our lab has previously shown that A-MSCs repress the proliferation of allogeneic T cells, a feature essential for many anti-inflammatory MSC-based therapeutics (38). To examine if P-MSCs repress T cell proliferation, allogeneic T cells from PBMC fractions were CD3/CD28 stimulated and added in suspension to P-MSC monolayers. In both CD4+ and CD8+ T cell subsets, P-MSCs inhibited T cell proliferation in a dose-dependent manner and to similar extend as we have previously reported for A-MSCs (figure 5). Here we found P-MSCs exhibited a similar level of inhibition to what was reported by A-MSCs (38).

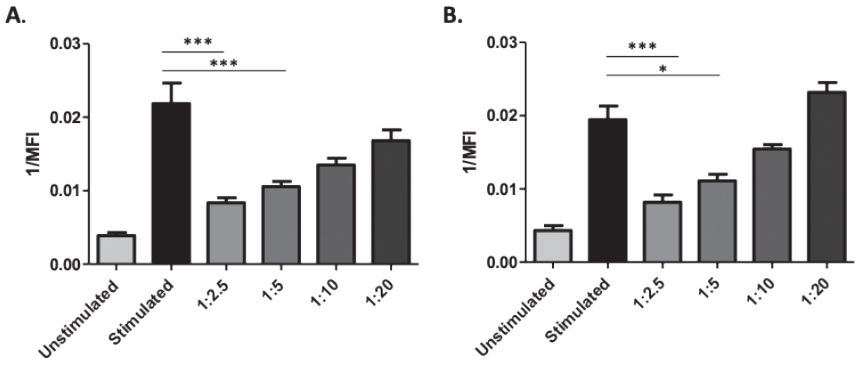

Figure 5: P-MSCs reduce allogeneic CD4+ and CD8+ T cell proliferation in monolayer. Stimulated (+CD3/CD28) PBMCs co-cultured with P-MSCs at different MSC:PBMC ratios (1:2.5, 1:5, 1:10, 1:20). CD4+ ***(A)*** *and CD8+* ***(B)*** *proliferation rates were found to decrease in a dose dependent manner following 5 days in culture. (N=3 P-MSC donors N=2 PBMC donors) Abbreviation: MFI-mean fluorescent intensity. (Kruskal-Wallis with Dunn's post hoc correction; ***P>0.001, *P>0.05)*

Paediatric MSCs exhibit more consistent multilineage differentiation capacity compared to adult donors

A-MSCs are known to exhibit inconsistent differentiation capabilities which varies greatly between donors. This severely limits their use in applications where consistency is essential (43). Compared to A-MSCs donors (passages 2-5; figure 6a) the trilineage differentiation potential of P-MSCs (passage 5; figure 6b) was found to be more consistent. Even though P-MSCs were used several passages beyond that used for the A-MSCs, P-MSCs more consistently underwent adipogenesis, osteogenesis and chondrogenesis with only 1 out of 12 donors not being able to make bone or cartilage following treatment (figure 6b). A-MSCs showed much more variability in their differentiation potential. Out of the 14 A-MSC donors tested, 3 donors failed to undergo adipogenesis, 5 donors failed to osteogenically differentiate and 5 donors were unable to chondrogenically differentiate (figure 6a).

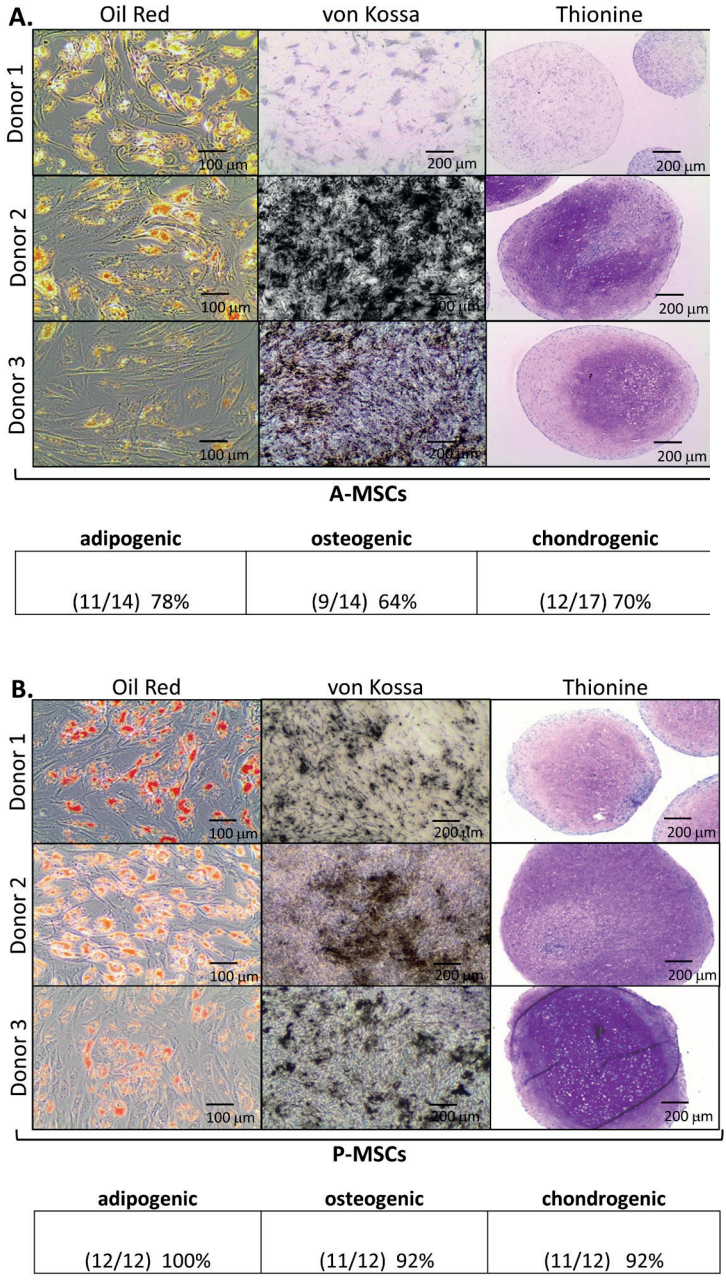

Figure 6: P-MSCs show more consistent capacity for multilineage differentiation. A) A-MSCs (passage 2-4) show great variation in differentiation capacity. A-MSCs are known to exhibit far greater variation in differentiation capacity (Chamberlain, Fox et al. 2007; Noort, Scherjon et al. 2003). B) P-MSCs (passage 5-6) are capable of tri-lineage differentiation with all donors but one being capable of osteogenic and chondrogenic differentiation. All P-MSCs tested could adipogenically differentiation.

DISCUSSION

Identifying cell sources with enhanced and reliable differentiation capabilities and expansion properties is necessary in order for MSCs to be more readily utilised both in research and in ATMPs. Here we have established a simple isolation protocol to obtain P-MSCs from surplus iliac crest bone chip material (figure 1). We confirmed P-MSCs expressed cell surface markers typically used to properly identify MSC populations. P-MSCs showed similar expression levels of general stem cell markers CD105, CD90, CD73 and CD166 compared to A-MSCs, as well as the absence of the hematopoietic marker CD45 (figure 2). As these markers are conventionally used to identify MSCs (44, 45) we are confident that this marker expression in combination with other results presented here we have proved P-MSCs are indeed MSCs. In this study A-MSCs displayed a higher expression of CD271 and HLA-DR compared to P-MSCs. It has been previously reported that in MSCs CD271 as well as HLA-DR expression decrease over time in culture (46, 47). As P-MSCs undergo more population doublings compared to A-MSCs at the same passage (figure 3d) this increased cellular division could have contributed to the decreased expression of both CD271 and HLA-DR we observe in P-MSCs.

P-MSCs expanded more rapidly than A-MSC donors, which might be attributed to the relatively low senescence in P-MSCs (figure 4). Senescent cells are much larger than non-senescent cells (48). A-MSCs have more enlarged senescent cells which do not divide (49, 50) making it easy to understand why the population doublings in A-MSCs are effected. Additionally, by preventing proliferation, senescence can also blocks differentiation pathways requiring proliferation, such as chondrogenic differentiation (51). Being able to obtain cells with higher proliferation and differentiation capacity in a shorter time than is possible with A-MSCs makes P-MSCs an attractive cell source.

If P-MSCs are to be utilised in an allogeneic setting it is important to show P-MSCs maintain immunomodulatory capabilities typically observed in A-MSCs (38, 52). MSCs are known to be able to manipulate T-cell proliferation and phenotypic behaviour and their immunosuppressive nature makes them an interesting candidate from a clinical perspective (52-54). In this study we found P-MSCs were capable of inhibiting T-cell proliferation at a similar level to what we previously reported with A-MSCs using a 1:5 ratio (MSC:PBMCs) (38). For use in an allogenic model it is advantageous for P-MSCs to inhibit T-cell proliferation to prevent an unwanted immune reaction following transplantation. However how P-MSCs interact with other immune cell types including antigen presenting cells needs to be determined in order to further understand how they would respond to a fully functional immune system.

A-MSCs have been reported to have inconsistent multilineage differentiation capabilities (15, 20, 55). P-MSCs were capable of more consistent multilineage differentiation compared to the A-MSCs in this study. Senescence could have contributed in part to the difference

we observed here as senescent MSC cell populations undergo phenotypic changes (56, 57) and exhibit chromosomal abnormalities (58, 59) which ultimately could influence their differentiation capacity (51). In this study it is plausible that a combination of factors influenced the differentiation capacity of these cell populations. It is logical cells from a younger, healthier patient which contain less senescent cells would be capable of better multilineage differentiation than senescent cells obtained from elderly patients. Having a cell source with more consistent differentiation capacities is ideal as it allows for more reproducible results.

Here we have described an easy isolation protocol which allows access to a P-MSC population with enhanced expansive and differentiation potential compared to A-MSCs. P-MSCs with their rapid expansion, low senescence and consistent multilineage differentiation are therefore prime candidates for applications from drug screening and development to use in ATMPs.

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Disclosure Statement

Authors declare there is no conflict of interest.

References

1. Deans, R.J., andMoseley, A.B. Mesenchymal stem cells: biology and potential clinical uses. Experimental hematology 28, 875, 2000.
2. Barry, F.P., andMurphy, J.M. Mesenchymal stem cells: clinical applications and biological characterization. The international journal of biochemistry & cell biology 36, 568, 2004.
3. Caplan, A.I., andBruder, S.P. Mesenchymal stem cells: building blocks for molecular medicine in the 21st century. Trends in molecular medicine 7, 259, 2001.
4. Caplan, A.I. Mesenchymal stem cells. Journal of orthopaedic research 9, 641, 1991.
5. García-Gómez, I., Elvira, G., Zapata, A.G., Lamana, M.L., Ramírez, M., García Castro, J., García Arranz, M., Vicente, A., Bueren, J., andGarcía-Olmo, D. Mesenchymal stem cells: biological properties and clinical applications. Expert opinion on biological therapy 10, 1453, 2010.
6. Goldring, C.E.P., Duffy, P.A., Benvenisty, N., Andrews, P.W., Ben-David, U., Eakins, R., French, N., Hanley, N.A., Kelly, L., andKitteringham, N.R. Assessing the safety of stem cell therapeutics. Cell stem cell 8, 618, 2011.
7. Mikkelsen, T.S., Ku, M., Jaffe, D.B., Issac, B., Lieberman, E., Giannoukos, G., Alvarez, P., Brockman, W., Kim, T.-K., andKoche, R.P. Genome-wide maps of chromatin state in pluripotent and lineage-committed cells. Nature 448, 553, 2007.
8. Hass, R., Kasper, C., Böhm, S., andJacobs, R. Different populations and sources of human mesenchymal stem cells (MSC): a comparison of adult and neonatal tissue-derived MSC. Cell Communication and Signaling 9, 12, 2011.
9. Pittenger, M.F., Mackay, A.M., Beck, S.C., Jaiswal, R.K., Douglas, R., Mosca, J.D., Moorman, M.A., Simonetti, D.W., Craig, S., andMarshak, D.R. Multilineage Potential of Adult Human Mesenchymal Stem Cells. Science 284, 143, 1999.
10. Schosserer, M., Reynoso, R., Wally, V., Jug, B., Kantner, V., Weilner, S., Buric, I., Grillari, J., Bauer, J.W., andGrillari-Voglauer, R. Urine is a novel source of autologous mesenchymal stem cells for patients with epidermolysis bullosa. BMC Research Notes 8, 767, 2015.
11. Trivanović, D., Kocić, J., Mojsilović, S., Krstić, A., Ilić, V., Okić-Đorđević, I., Santibanez, J.F., Jovčić, G., Terzić, M., andBugarski, D. Mesenchymal stem cells isolated from peripheral blood and umbilical cord Wharton's jelly. Srpski arhiv za celokupno lekarstvo 141, 178, 2013.
12. Dominici, M., Le Blanc, K., Mueller, I., Slaper-Cortenbach, I., Marini, F.C., Krause, D.S., Deans, R.J., Keating, A., Prockop, D.J., andHorwitz, E.M. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 8, 315, 2006.
13. Narcisi, R., Cleary, M.A., Brama, P.A.J., Hoogduijn, M.J., Tüysüz, N., ten Berge, D., andvan Osch, G.J.V.M. Long-term expansion, enhanced chondrogenic potential, and suppression of endochondral ossification of adult human MSCs via WNT signaling modulation. Stem cell reports 4, 459, 2015.

14. Cleary, M.A., Narcisi, R., Focke, K., van der Linden, R., Brama, P.A.J., andvan Osch, G. Expression of CD105 on expanded mesenchymal stem cells does not predict their chondrogenic potential. Osteoarthritis and cartilage 24, 868, 2016.
15. Sivasubramaniyan, K., Lehnen, D., Ghazanfari, R., Sobiesiak, M., Harichandan, A., Mortha, E., Petkova, N., Grimm, S., Cerabona, F., andde Zwart, P. Phenotypic and functional heterogeneity of human bone marrow–and amnion-derived MSC subsets. Annals of the New York Academy of Sciences 1266, 94, 2012.
16. Baksh, D., Song, L., andTuan, R.S. Adult mesenchymal stem cells: characterization, differentiation, and application in cell and gene therapy. Journal of cellular and molecular medicine 8, 301, 2004.
17. Bianco, P., Barker, R., Brüstle, O., Cattaneo, E., Clevers, H., Daley, G.Q., De Luca, M., Goldstein, L., Lindvall, O., andMummery, C. Regulation of stem cell therapies under attack in Europe: for whom the bell tolls. The EMBO journal 32, 1489, 2013.
18. Cyranoski, D. FDA challenges stem-cell clinic. Nature Publishing Group; 2010.
19. Knoepfler, P.S. From bench to FDA to bedside: US regulatory trends for new stem cell therapies. Advanced drug delivery reviews 82, 192, 2015.
20. Li, Z., Liu, C., Xie, Z., Song, P., Zhao, R.C.H., Guo, L., Liu, Z., andWu, Y. Epigenetic dysregulation in mesenchymal stem cell aging and spontaneous differentiation. PloS one 6, e20526, 2011.
21. Bonab, M.M., Alimoghaddam, K., Talebian, F., Ghaffari, S.H., Ghavamzadeh, A., andNikbin, B. Aging of mesenchymal stem cell in vitro. BMC cell biology 7, 14, 2006.
22. Banfi, A., Muraglia, A., Dozin, B., Mastrogiacomo, M., Cancedda, R., andQuarto, R. Proliferation kinetics and differentiation potential of ex vivo expanded human bone marrow stromal cells: implications for their use in cell therapy. Experimental hematology 28, 707, 2000.
23. Maciulaitis, R., D'Apote, L., Buchanan, A., Pioppo, L., andSchneider, C.K. Clinical development of advanced therapy medicinal products in Europe: evidence that regulators must be proactive. Molecular Therapy 20, 479, 2012.
24. Herberts, C.A., Kwa, M.S.G., andHermsen, H.P.H. Risk factors in the development of stem cell therapy. Journal of translational medicine 9, 29, 2011.
25. Mareschi, K., Biasin, E., Piacibello, W., Aglietta, M., Madon, E., andFagioli, F. Isolation of human mesenchymal stem cells: bone marrow versus umbilical cord blood. haematologica 86, 1099, 2001.
26. Muraglia, A., Cancedda, R., andQuarto, R. Clonal mesenchymal progenitors from human bone marrow differentiate in vitro according to a hierarchical model. J Cell Sci 113, 1161, 2000.
27. Barlow, S., Brooke, G., Chatterjee, K., Price, G., Pelekanos, R., Rossetti, T., Doody, M., Venter, D., Pain, S., andGilshenan, K. Comparison of human placenta-and bone marrow–derived multipotent mesenchymal stem cells. Stem cells and development 17, 1095, 2008.
28. Torre, M.L., Lucarelli, E., Guidi, S., Ferrari, M., Alessandri, G., De Girolamo, L., Pessina, A., Ferrero, I., andGruppo Italiano Staminali, M. Ex vivo expanded mesenchymal stromal cell minimal quality requirements for clinical application. Stem cells and development 24, 677, 2014.

29. Kristjánsson, B., andHonsawek, S. Current perspectives in mesenchymal stem cell therapies for osteoarthritis. Stem cells international 20142014.
30. Kretlow, J.D., Jin, Y.-Q., Liu, W., Zhang, W.J., Hong, T.-H., Zhou, G., Baggett, L.S., Mikos, A.G., andCao, Y. Donor age and cell passage affects differentiation potential of murine bone marrow-derived stem cells. BMC cell biology 9, 60, 2008.
31. Wagner, W., Bork, S., Horn, P., Krunic, D., Walenda, T., Diehlmann, A., Benes, V., Blake, J., Huber, F.-X., andEckstein, V. Aging and replicative senescence have related effects on human stem and progenitor cells. PloS one 4, e5846, 2009.
32. Zheng, H., Martin, J.A., Duwayri, Y., Falcon, G., andBuckwalter, J.A. Impact of aging on rat bone marrow-derived stem cell chondrogenesis. The Journals of Gerontology Series A: Biological Sciences and Medical Sciences 62, 136, 2007.
33. Sethe, S., Scutt, A., andStolzing, A. Aging of mesenchymal stem cells. Ageing research reviews 5, 91, 2006.
34. Carrington, J.L. Aging bone and cartilage: cross-cutting issues. Biochemical and biophysical research communications 328, 700, 2005.
35. Chambers, S.M., Shaw, C.A., Gatza, C., Fisk, C.J., Donehower, L.A., andGoodell, M.A. Aging hematopoietic stem cells decline in function and exhibit epigenetic dysregulation. PLoS biology 5, e201, 2007.
36. Rando, T.A. Stem cells, ageing and the quest for immortality. Nature 441, 1080, 2006.
37. Debacq-Chainiaux, F., Erusalimsky, J.D., Campisi, J., andToussaint, O. Protocols to detect senescence-associated beta-galactosidase (SA-βgal) activity, a biomarker of senescent cells in culture and in vivo. 4, 1798, 2009.
38. Kiernan, C.H., Hoogduijn, M.J., Franquesa, M., Wolvius, E.B., Brama, P.A.J., andFarrell, E. Allogeneic chondrogenically differentiated human mesenchymal stromal cells do not induce immunogenic responses from T lymphocytes in vitro. Cytotherapy 18, 957, 2016.
39. Lv, F.-J., Tuan, R.S., Cheung, K., andLeung, V.Y.L. Concise review: the surface markers and identity of human mesenchymal stem cells. Stem cells 32, 1408, 2014.
40. Sotiropoulou, P.A., Perez, S.A., Salagianni, M., Baxevanis, C.N., andPapamichail, M. Characterization of the optimal culture conditions for clinical scale production of human mesenchymal stem cells. Stem cells 24, 462, 2006.
41. Greenberg, S.B., Grove, G.L., andCristofalo, V.J. Cell size in aging monolayer cultures. In Vitro Cellular & Developmental Biology-Plant 13, 297, 1977.
42. Tsai, C.-C., Chen, Y.-J., Yew, T.-L., Chen, L.-L., Wang, J.-Y., Chiu, C.-H., andHung, S.-C. Hypoxia inhibits senescence and maintains mesenchymal stem cell properties through down-regulation of E2A-p21 by HIF-TWIST. Blood 117, 459, 2011.
43. Narcisi, R., Arikan, O.H., Lehmann, J., ten Berge, D., andvan Osch, G.J.V.M. Differential effects of small molecule WNT agonists on the multilineage differentiation capacity of human mesenchymal stem cells. Tissue Engineering Part A 22, 1264, 2016.

44. Beane, O.S., Fonseca, V.C., Cooper, L.L., Koren, G., andDarling, E.M. Impact of aging on the regenerative properties of bone marrow-, muscle-, and adipose-derived mesenchymal stem/stromal cells. PloS one 9, e115963, 2014.
45. Bruna, F., Contador, D., Conget, P., Erranz, B., Sossa, C.L., andArango-Rodríguez, M.L. Regenerative potential of mesenchymal stromal cells: age-related changes. Stem cells international 20162016.
46. Alegre-Aguarón, E., Desportes, P., García-Álvarez, F., Castiella, T., Larrad, L., andMartínez-Lorenzo, M.J. Differences in surface marker expression and chondrogenic potential among various tissue-derived mesenchymal cells from elderly patients with osteoarthritis. Cells Tissues Organs 196, 231, 2012.
47. Mareddy, S., Crawford, R., Brooke, G., andXiao, Y. Clonal isolation and characterization of bone marrow stromal cells from patients with osteoarthritis. Tissue engineering 13, 819, 2007.
48. Nelson, G., Wordsworth, J., Wang, C., Jurk, D., Lawless, C., Martin-Ruiz, C., andvon Zglinicki, T. A senescent cell bystander effect: senescence-induced senescence. Aging cell 11, 345, 2012.
49. van Deursen, J.M. The role of senescent cells in ageing. Nature 509, 439, 2014.
50. Wagner, W., Horn, P., Castoldi, M., Diehlmann, A., Bork, S., Saffrich, R., Benes, V., Blake, J., Pfister, S., andEckstein, V. Replicative senescence of mesenchymal stem cells: a continuous and organized process. PloS one 3, e2213, 2008.
51. Dexheimer, V., Frank, S., andRichter, W. Proliferation as a requirement for in vitro chondrogenesis of human mesenchymal stem cells. Stem cells and development 21, 2160, 2012.
52. Aggarwal, S., andPittenger, M.F. Human mesenchymal stem cells modulate allogeneic immune cell responses. Blood 105, 1815, 2005.
53. Di Nicola, M., Carlo-Stella, C., Magni, M., Milanesi, M., Longoni, P.D., Matteucci, P., Grisanti, S., andGianni, A.M. Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli. Blood 99, 3838, 2002.
54. Roemeling-van Rhijn, M., Khairoun, M., Korevaar, S.S., Lievers, E., Leuning, D.G., Ijzermans, J.N.M., Betjes, M.G.H., Genever, P.G., van Kooten, C., andde Fijter, H.J.W. Human bone marrow-and adipose tissue-derived mesenchymal stromal cells are immunosuppressive in vitro and in a humanized allograft rejection model. Journal of stem cell research & therapy 2013.
55. Choudhery, M.S., Badowski, M., Muise, A., Pierce, J., andHarris, D.T. Donor age negatively impacts adipose tissue-derived mesenchymal stem cell expansion and differentiation. Journal of translational medicine 12, 8, 2014.
56. Vacanti, V., Kong, E., Suzuki, G., Sato, K., Canty, J.M., andLee, T. Phenotypic changes of adult porcine mesenchymal stem cells induced by prolonged passaging in culture. Journal of cellular physiology 205, 194, 2005.
57. Coppé, J.-P., Desprez, P.-Y., Krtolica, A., andCampisi, J. The senescence-associated secretory phenotype: the dark side of tumor suppression. Annual Review of Pathological Mechanical Disease 5, 99, 2010.

58. Wagner, W., Horn, P., Castoldi, M., Diehlmann, A., Bork, S., Saffrich, R., Benes, V., Blake, J., Pfister, S., Eckstein, V., andHo, A.D. Replicative Senescence of Mesenchymal Stem Cells: A Continuous and Organized Process. PLOS ONE 3, e2213, 2008.
59. Turinetto, V., Vitale, E., andGiachino, C. Senescence in Human Mesenchymal Stem Cells: Functional Changes and Implications in Stem Cell-Based Therapy. International Journal of Molecular Sciences 17, 1164, 2016.

Submitted: European cells and materials

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COLLAGEN TYPE X IS ESSENTIAL FOR SUCCESSFUL MESENCHYMAL STEM CELL MEDIATED CARTILAGE FORMATION AND SUBSEQUENT ENDOCHONDRAL OSSIFICATION

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Abstract

In tissue engineering, endochondral ossification (EO) is often replicated by chondrogenically differentiating mesenchymal stromal cells (MSCs) *in vitro* and achieving bone formation via *in vivo* implantation. The resulting marrow containing bone construct is promising as a treatment for bone defects, however limited bone formation capacity has prevented them from reaching their full potential. This is further complicated as it is not fully understood how this bone formation is achieved. It has recently been shown that acellular grafts derived from chondrogenically differentiated MSCs can initiate this bone formation, however which component(s) within these decellularised matrices that contribute to this bone formation has yet to be determined. Collagen type X (COLX), a hypertrophy associated collagen found within these constructs, is involved in matrix organisation, calcium binding and matrix vesicle compartmentalisation. However the importance of COLX during tissue engineered chondrogenesis and subsequent bone formation is unknown. Here we investigate the importance of COLX via shRNA mediated gene silencing in primary MSCs. We found a significant knock-down of COLX disrupted the production of key components of the extracellular matrix and the secreted profile by chondrogenically differentiated MSCs. Following *in vivo* implantation we observed disrupted bone formation in knock-down constructs. We confirm the importance of COLX during both chondrogenic differentiation and subsequent EO in this tissue engineered setting.

INTRODUCTION

Endochondral ossification (EO) is the process by which the long bones of the body are formed developmentally. It relies on mesenchyme cells which condense and undergo chondrogenic differentiation resulting in the formation of a cartilage anlagen (1) which is subsequently remodelled by osteoclasts (2), invaded by nearby vasculature (3) and ultimately serves as the template for bone formation by osteoblasts (4). It has been shown that EO can be mimicked in a tissue engineering setting by chondrogenically differentiating mesenchymal stem cells (MSCs) pre implantation (5-9). Although such grafts could one day offer an attractive alternative to currently available treatment options, these constructs exhibit variable degrees of bone formation and often are unable to bridge large bone defects (5, 10). Their clinical translation is further complicated due to the fact that despite years of research it has not been fully understood how exactly these constructs achieve bone formation. Recently it has been shown that devitalised grafts derived from chondrogenic MSCs formed endochondral bone following *in vivo* implantation (11, 12), indicating that components within the chondrogenic matrix itself are capable of inducing bone formation independent of the presence of MSCs. Kelly et al. further showed that biomaterials containing decellularised matrices specifically from hypertrophic chondrogenically differentiated MSCs were far better at inducing this bone formation than non-hypertrophic counterparts (12), however what exactly within these hypertrophic matrices is responsible for this difference remains to be determined.

During chondrogenic differentiation, collagen type X (COLX), the major marker of hypertrophic chondrocytes, can be detected within the first few days (10, 13). Developmentally, following hypertrophic differentiation the extracellular matrix (ECM) is remodeled to allow for invasion of the template by nearby vasculature (14, 15), mineralization of the cartilage template is initiated, osteoclastic remodeling occurs (15, 16) and ultimately endochondral bone formation takes place (15, 17). COLX is produced specifically by hypertrophic chondrocytes (HCs) and comprises more than 45% of the total collagens produced (18). It is thought to play a number of important roles during EO even contributing to the structural support of the pericellular network (19, 20), support that is essential during matrix remodeling (19). Additionally COLX may play a role in initiating biomineralisation by binding to annexin V (21, 22). Annexin V facilitates the influx of Ca^{2+} into matrix vesicles, initiating mineralisation (23). Others hypothesise that COLX is also essential for the proper distribution of collagens and proteoglycans throughout the ECM (24, 25). However these hypotheses have been hard to verify as previous research

utilising transgenic (Tg) mouse models in which COLX has been removed, truncated or disrupted via other methods produce conflicting data (25-29). Some groups show early developmental irregularities attributed to a lack of COLX including perinatal death, dwarfism and various skeletal abnormalities (25, 27); one group reports a complete absence of phenotypic changes (29). Studies have also reported, convincingly, that a lack of COLX results in a compromised immune system with an inability to illicit and control a proper immune reaction (30-32) while others find no such deficiency (29). These different findings could come down to the genetic variation between different strains of mice used between labs, variation in the methodology used to achieve knock-down/interference of COLX, or even differences in sample processing, analysis or staining techniques but obviously this make understanding the role COLX increasingly challenging. Furthermore, COLX has only been studied from a developmental stand point. How COLX contributes both to chondrogenic differentiation of MSCs and subsequent endochondral bone formation has not been studied. The goal of this research was to determine if COLX is essential for MSC mediated chondrogenic differentiation and subsequent endochondral bone formation. *COL10A1* expression was knocked-down via short hairpin RNA (shRNA) directed against the *COL10A1* mRNA sequence delivered by lentivirus. In this way we could specifically knock-down the production of COLX in MSCs and determine its contribution to tissue engineered chondrogenesis and endochondral bone formation. *COL10A1* knock-down resulted in disrupted chondrogenic differentiation. The production of key ECM components was severely decreased when the knock-down was highly efficient. We also observed several secreted factors linked to the recruitment of key cell types involved in EO were also down-regulated. Following *in vivo* implantation we observed EO was likewise affected. This however was only observed when knock-down efficiency was greater than 80%, indicating a minimal production of *COL10A1* is sufficient to prevent these adverse effects. Here we show the importance of COLX in MSC mediated chondrogenesis and EO, furthering our understanding of tissue engineered endochondral bone formation.

MATERIALS AND METHODS

Mesenchymal stem cell culture

Mesenchymal stem cells (MSCs) were isolated from leftover material from patients undergoing alveolar bone graft surgery (Medical Ethical Testing Commission approval code 2014-106); following informed or parental consent as previously described in accordance with relevant guidelines and regulations (33). Cells were plated at approximately 2,300 cells/cm^2 in complete expansion medium (αMEM containing 10% fetal bovine serum (lot# 41Q204K, Gibco, Bleiswijk, Netherlands), 50 µg/mL gentamycin

and 1.5 µg/mL fungizone; supplemented with 25 µg/mL L-ascorbic acid 2-phosphate and 1 ng/ml fibroblast growth factor-2 (Instruchemie, Delfzijl, Netherlands)) at 37°C and 5% carbon dioxide (CO_2) in a humidified atmosphere. Medium was replenished every 3-4 d until cells reached approximately 90% confluency at which time they were passaged using 0.05% trypsin.

Lentiviral generation

MISSION® TRC1.5 shRNA targeting collagen type X (TRCN0000082798) and control plasmids (SHC001 & SHC002) were purchased (Sigma-Aldrich, Zwijndrecht, Netherlands) and transformed into DH5-α E.coli (Invitrogen, Landsmeer, Netherlands). Plasmids were isolated using an endofree maxiprep kit (Qiagen, Venlo, Netherlands) as per manufacturer's instructions. HEK293FT cells were expanded in complete HEK medium (high glucose DMEM, 10% fetal bovine serum, 50 µg/mL gentamycin, 1.5 µg/mL fungizone) supplemented with 50 µg/mL geneticin (Thermoscientific; Bleiswijk, Netherlands) and maintained at 37°C and 5% CO_2 in a humidified atmosphere. 24 h prior to transfection $3x10^5$ cells/cm^2 were plated in tissue culture petri dishes (Corning, Amsterdam, Netherlands) in high glucose DMEM containing 10% fetal bovine serum. Cells were co-transfected with plasmids via calcium phosphate precipitation (3 µg pLP1 plasmid, 3 µg pLP2 plasmid, 3 µg pLP/VSVG plasmid; Virapower packaging mix), 7.5 µg shRNA plasmid). Medium was refreshed 24 h post transfection with HEK medium containing 20mM HEPES. Viral supernatant was harvested and filtered (0.45 µm filter) 48 h following transfection and stored at -80°C until used. P24 concentration was determined using INNOTEST® HIV Antigen mAb enzyme immunoassay (Fujirebio, Gent, Belgium) performed as per manufacture's instruction.

Lentiviral transduction

MSCs were transduced at 30% confluency. Cells were incubated with 40 or 60 ng/ml p24 particles (depending on the batch used and the optimal dose determined by titration experiments) and 20 µg/mL protamine sulphate (Sigma-Aldrich) in complete expansion medium for 48 h (figure 1-3). Following incubation cells were washed, expanded and selected after reaching 80% confluency. Selection was achieved in 72 h using 5 µg/mL puromycin in complete expansion medium. Post selection, cells were washed and expanded until 90% confluency at which point they either harvested for RNA (homogenized in 350 µl trizol (Thermoscientific) and stored at -80°C until isolated) or used for differentiation studies. P24 particle, protamine sulphate and puromycin concentrations were determined prior to experimental start via titration experiments (figure 1-3). Knock-down percentage was calculated by subtracting the average COLX gene expression from COLX knock-down pellets from the average of the non-treated pellets' COLX gene expression. This was divided by the average of the non-treated COLX gene expression and then multiplied by 100 to obtain a percentage.

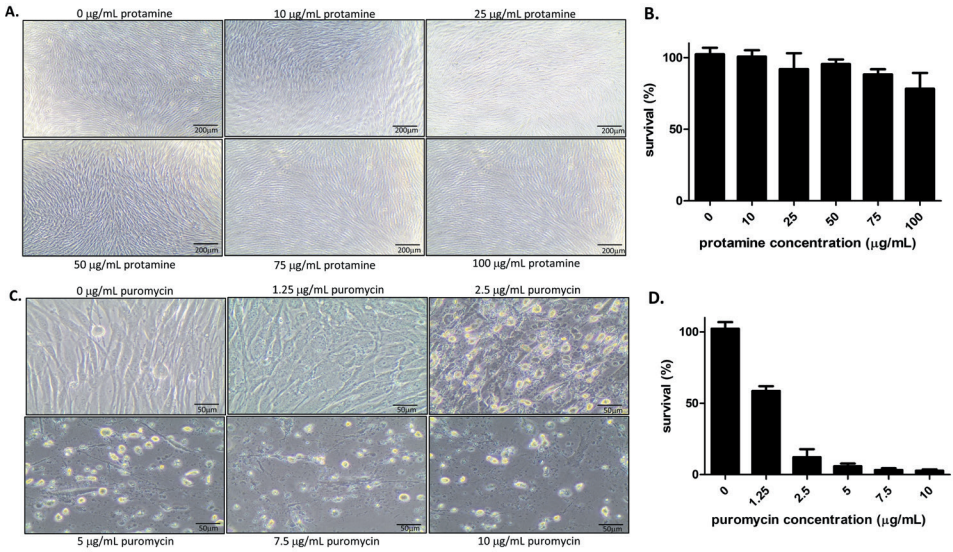

Figure 1: Titration of protamine and puromycin for use in lentiviral transduction. A, B) Addition of protamine sulfate did not significantly affect cell survival. 10, 25, 50, 75 or 100 mg/mL protamine sulfate was added to MSCs. After 48 hours cultures were refreshed for 24 hours after which time morphology unaffected ***(A)****. Cell survival was assessed via MTT assay* ***(B)****. At highest concentration protamine sulfate did not reduce cell survival.* ***C, D)*** *5 mg/mL puromycin concentration is effective for induction of MSC death. 1, 2.5, 5, 7.5 or 10 mg/mL puromycin was added to MSCs. After 48 hours significant cell death was observed in concentrations above 2.5 mg/mL puromycin* ***(A)****. Cell survival was assessed via MTT assay* ***(B).***

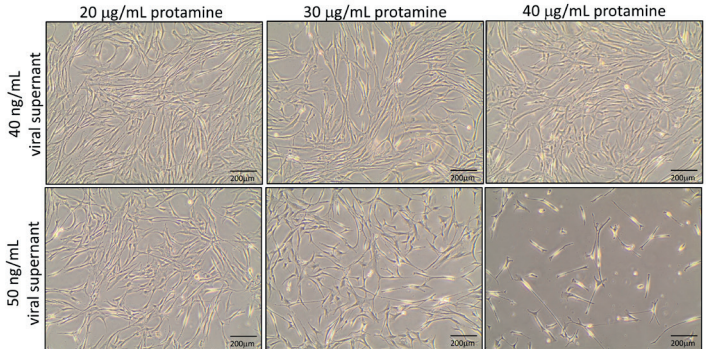

Figure 2: Determining lowest effective concentration of protamine and viral supernatant for lentiviral batch #1 (used on donors 1 &2). Multiple concentrations (based off P24 values) of viral supernatant were tested in combination with several concentration of protamine. Cells were transduced, selected and expanded. Following expansion, cells treated with viral doses less than 40ng/mL or over 50ng/mL failed to expand. With viral supernatant batch #1, 40ng/mL viral supernatant and 20mg/mL protamine was found to be the lowest effective dose and was used for main experiments.

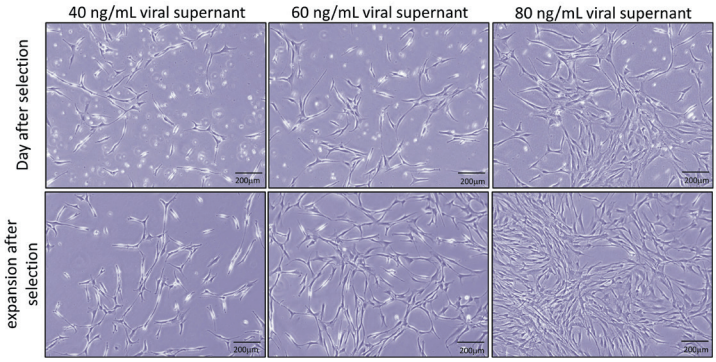

Figure 3: Determining lowest effective concentration of viral supernant for lentiviral batch #2 (used on donors 3). Multiple concentrations (based off P24 values) of viral supernatant were tested in combination with 20 mg/mL protamine. Cells were transduced, selected and expanded. Following expansion, cells treated with viral doses less than 60ng/mL failed to expand. With viral supernatant batch #2 higher concentrations of viral supernatant were required than what was needed with batch #1 to allow for get proper cell expansion following puromycin selection.

Chondrogenic differentiation

MSCs were chondrogenically differentiated in complete chondrogenic medium (high-glucose DMEM supplemented with 50 µg/mL gentamycin (Thermoscientific), 1.5 µg/mL fungizone (Thermoscientific), 1mM sodium pyruvate (Thermoscientific), 40 µg/mL proline (Sigma; Zwijndrecht, Netherlands), 1:100 v/v insulin-transferrin-selenium (ITS+; BD Biosciences; Temse, Beligum), 10 ng/mL transforming growth factor β3 (Peprotech; London, UK), 25 µg/mL L-ascorbic acid 2-phosphate (Sigma-Aldrich), and 100nM dexamethasone (Sigma-Aldrich)) using $2x10^5$ MSCs suspended in 15 mL polypropylene tubes, centrifuged at 300 g for 8 min. Cultures were maintained in a humidified atmosphere at 37°C and 5% CO_2 and medium was replenished every 3-4 d for 21 d. Samples were harvested following 7, 14 and 21 d in culture for RNA isolation (as previously described), GAG/DNA analysis (stored at -80°C), western blot (snap frozen), histology (4% formalin fixed for 2 h), ELISA (supernatant) or migration (conditioned medium; described below).

Osteogenic differentiation

MSCs were plated at a density of $3x10^3$ cells/cm^2 in expansion medium. 24 h after plating the medium was changed to osteogenic induction medium (high-glucose DMEM supplemented with 10 % v/v fetal bovine serum, 50 µg/mL gentamycin, 1.5 µg/mL fungizone, 10 mM glycerol -3-phosphate (Sigma), 0.1 µM dexamethasone (Sigma), and 0.1 mM L-ascorbic acid 2-phosphate (Sigma)). Cultures were maintained at 37°C and 5% CO_2 in a humidified atmosphere. Medium was refreshed every 3-4 d for 13-21 d. Once cell sheets began to detach from outer periphery of well, culture was ended

and samples were fixed in 4% (w/v) formalin. Von Kossa staining was performed as previously described (33).

Adipogenic differentiation

MSCs were plated at a density of 2.1x10^4 cells/cm^2 in adipogenic induction medium (high-glucose DMEM supplemented with 10% serum, 50 µg/mL gentamycin, 1.5 µg/mL fungizone, 0.2 mM indomethacin (Sigma-Aldrich), 0.01 mg/mL insulin (Sigma-Aldrich), 0.5 mM 3 iso-butyl-1-methyl-xanthine (Sigma-Aldrich) and 1 µM dexamethasone). Cultures were maintained at 37°C and 5% CO_2 in a humidified atmosphere. Medium was refreshed as every 3-4 d for 19-21 d after which samples were prepared for histology (4% (w/v) formalin fixed). Oil red O staining was performed as previously described (33).

Cell viability testing

MSCs were plated at a density of 2.1x10^4 cells/cm^2 and treated with either protamine sulphate (10, 25, 50, 75, or 100 µg/mL) or puromycin (1.25, 2.5, 5, 7.5 or 10 µg/mL) in MSC expansion medium for 48 h at 37°C, 5% CO_2 in a humidified atmosphere. Then the cells were washed with PBS and cells were incubated in 0.5 mg/mL MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; Sigma-Aldrich) in MSC expansion medium in the dark at 37°C and 5% CO_2 for 3 h in a humidified atmosphere. Then, the medium was aspirated, and the cells were washed with PBS. The MTT-tetrazolium crystals formed by healthy cells were dissolved in 100% Ethanol. Absorbance was measured at 570 nm and 670 nm, the A570:A670 ratio was compared to none treated control MSCs.

Western blot

Pellets were snap frozen in liquid nitrogen and homogenised in 100 µl M-PER (Mammalian Protein Extraction Reagent, Thermoscientific) with 1x halt protease inhibitor cocktail (Thermoscientific) followed by water bath sonication (10 cycles of 30s and 30s rest). Total protein was measured by BCA assay (Thermoscientific). 10 µg total protein was incubated at 95°C for 10 min and separated on a gradient gel (Bolt 4-12% Bis-Tris Plus, Thermoscientific) under non-reducing conditions. Proteins were transferred to a PVDF membrane and blocked with 5% w/v fat-free milk in 0.1% v/v TBS-Tween. The membrane was cut at 70 kDa and each piece incubated overnight with its respective antibody: mouse monoclonal anti-ColX, clone X53 (Quartett) at 1:1000 dilution and rabbit monoclonal anti α-tubulin (Cell Signaling) at 1:2000 dilution. This was followed by incubation for 1 h at room temperature with the respective secondary antibody: HRP conjugated goat anti-mouse at 1:2000 dilution (Thermoscientific) and HRP conjugated goat anti-rabbit at 1:2000 dilution (Thermoscientific). Protein bands

were visualised using the Super Signal West detection kit (Thermoscientific) on the digital camera Alliance 2.7 (Uvitec, Cambridge, UK) with an exposure time of 15 (ColX) or 17 min (α-tubulin; raw images supplementary figure 4).

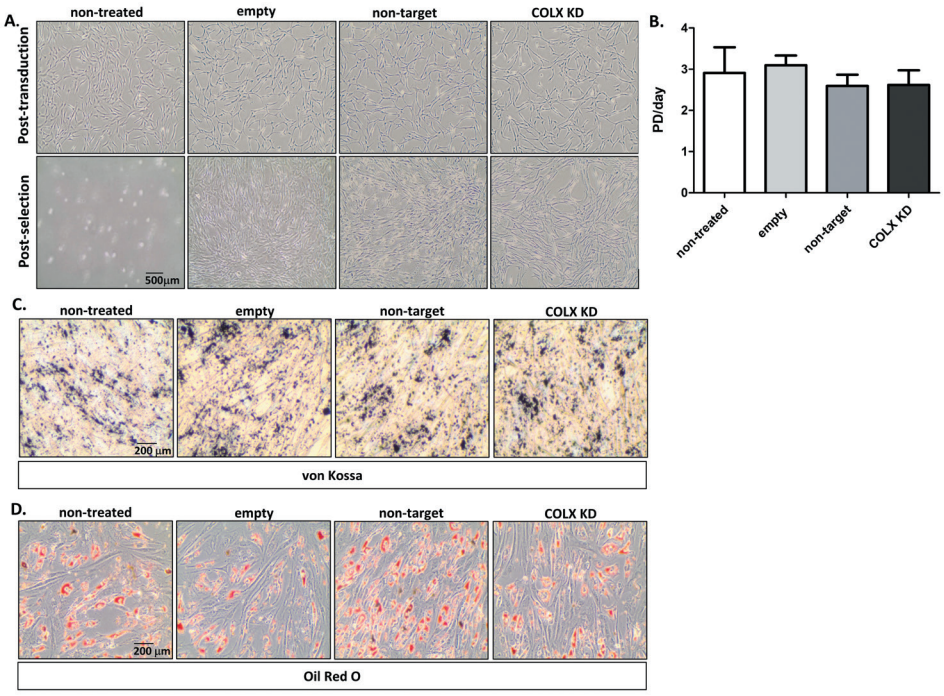

Figure 4: Lentiviral infection does not hinder MSC expansion or differentiation capabilities. A) MSCs were transduced with lentivirus containing an empty vector, non-target shRNA or COL10A1 shRNA. MSCs were then selected based on puromycin resistance Following transduction cells maintain MSC like morphology and post-selection positively infected cells maintain both morphology and proliferative capacity. B) Transduced MSCs post-selection expand at a similar rate to non-treated MSCs, undergoing a similar number of population doublings (n=4; Linear mixed model with Bonferroni post-hoc comparison: non-significant; PD/day: population doublings per day). Transduced MSCs maintain capacity to differentiate osteogenically (C) and adipogenically (D). (abbreviations: PD, population doublings).

RNA isolation and gene expression

Samples were homogenised in 350 µl trizol to which 70 µl 100% chloroform was added and thoroughly mixed. Following a 10 min incubation at room temperature and phase separation at 10,000x g for 15 min, the aqueous phase was mixed with an equal volume of 70% ethanol and transferred to RNeasy® kit columns. RNA was isolation and purified using the RNeasy® microkit (Qiagen) following manufacturer's instructions. cDNA was reverse transcribed as per manufacturer's instruction using a

First Strand cDNA Synthesis Kit (Thermoscientific) and PCR was performed as previously described (34). For gene expression of monolayer, day 7 and day 14 of chondrogenic differentiation 3 different MSC donors were analysed. For gene expression analysed at day 21, one MSC donor was analysed (3 pellets/donor/condition). Gene expression is shown as delta delta CT.

Biochemical assay

Chondrogenically differentiated samples stored at -80°C after which they were digested overnight at 60°C in buffer containing papain ((0.2M NaH_2PO_4, 0.01M EDTA, 0.01M cysteine HCl, 250 µg/mL papain (Sigma-Aldrich), pH 6). GAGs were then quantified by dimethylmethylene blue assay (pH 3) with a chondroitin sulphate C (Sigma-Aldrich) standard utilizing an A530:590 ratio. Ethidium bromide was used to determine the DNA content of these samples. Calf thymus DNA (Sigma-Aldrich) was used as a standard.

Cytokine quantification

Culture medium was refreshed 24 h before harvest from pellets on the day of implantation. Supernatant was applied to a human cytokine angiogenic multiplex chemiluminescent ELISA (Cat. No. 150251HU, Quansys Biosciences, Logan UT) as per manufacturer's instructions. This angiogenic multiplex included the following targets: angiotensin II (Ang-2), fibroblast growth factor-2 (FGF-2), hepatocyte growth factor (HGF), interleukin-8 (IL-8), platelet derived growth factor-BB (PDGF-BB), tissue inhibitor of metalloproteinase 1 and 2 (TIMP-1 and TIMP-2), tumor necrosis factor (TNF). ELISA plate was imaged on the Amersham imager 600 (GE) and images were quantified using the Q-view software® provided by the ELISA manufacturer (Quansys Bioscience).

Histology

In vitro samples were fixed in 4% formalin for 2 h prior to paraffin embedding. *In vivo* samples were formalin fixed for 24 h and decalcified in 10% EDTA w/v in H_2O for 10 d refreshing the EDTA every other day. Following paraffin embedding 6 µm-thick sections were cut from all samples. Sections were deparaffinised and stained with haematoxylin-eosin (H&E), safranin O, tartrate-resistant acid phosphatase (TRAP), collagen type II or collagen type X as previously described (34).

Subcutaneous implantation

Experiments were conducted with approval by the Animal Experiments Committee at the Erasmus Medical Centre, Rotterdam (EU license number 15-114-02) adhering to all relevant guidelines and regulations. All work protocols used were reviewed and

approved by the Erasmus Medical Centre Animal Experiments Committee. 8 week-old athymic nude mice (Balb/c nudes, Charles River; Sulzfeld, Germany) were housed under standard light dark cycles with ad libitum food and water access (12 mice in total; housed in groups of 2). Pain medication (buprenorphine 0.05mg/kg bodyweight) was administered pre-operatively under anesthesia (1-3% isoflurane). Dorsal incisions were created in which a subcutaneous pocket was made. Per pocket 3 pellets of control or virally transduced pellets were implanted subcutaneously (conditions were randomised between mice and physical positions). 3 implantations were performed per experimental condition (3 pellets or 600,000 cells/implantation). Incisions were closed using staples. 8 or 10 weeks post implantation animals were euthanized by CO_2 asphyxiation.

Micro CT longitudinal imaging

µCT scans were performed at the Applied Molecular Imaging Erasmsus MC facility using the Quantum FX (Perkin-Elmer; Groningen, Netherlands). Under anesthesia (1-2.5% isoflurane) scans were acquired using a field of view of 60mm (90 kV/160 mA, 4.5 min) bi-weekly. Using the Analyze 11.0 software (AnalyzeDirect, Netherlands) scans were quantified by converting original linear attenuation coefficient measurements by linear transformation to Hounsfield units (HU). Median spatial filters were applied with a 3x3x3 kernel size. Values over corresponding to a density of 0.133 g/cm^3 (determined by phantom scans) were segmented out and quantified as calcified tissue.

Figures and statistical analysis

Graph figures were created using GraphPad Software (La Jolla California USA). Graphs depict delta CT values (35), setting non-treated value to one. Linear mixed model with Bonferroni post-hoc comparison was performed using SPSS on expansion data in figure 1 (Ver 21. SPSS Inc, Chicago, USA).

RESULTS

Lentivirally transduced MSCs maintain proliferative and differentiation capacity following puromycin selection

We first optimised concentrations of (protamine sulphate 20ng/mL) and puromycin (5 µg/mL) via titration experiments (figure 1). Minimal doses of both were chosen to avoid unnecessary cell stress during the transduction process. For each viral batch the optimal concentration (as determined by P24 viral antigen concentration) was determined based on minimum dose at which cells maintained proliferative capacity

(compared to non-transduced controls from the same donor) following selection (figure 2-3).

Following transduction with optimal lentiviral and protamine sulphate concentrations, MSCs maintained a healthy spindle like morphology which was retained after puromycin selection (figure 4a). MSCs recovered quickly from selection stress and all grew at a similar rate, undergoing comparable population doublings with non-treated controls (figure 4a, b). Furthermore, transduced cells maintained their differentiation potential upon osteogenic (figure 4c) and adipogenic (figure 4d) induction.

COL10A1 can be efficiently knocked-down via lentivirally delivered shRNA

MSCs from each condition were chondrogenically differentiated for 21 days via pellet culture. Following differentiation, *COL10A1* expression was effectively knocked-down with an efficiency ranging between 80 to 99% depending by the MSC donor (figure 5a; donor 1: >99%; donor 2: 99%; donor 3: 80% knock-down). Gene and protein expression of COLX was found to be significantly downregulated compared to non-treated, empty vector and non-target controls which exhibited normal expression levels for all donors (figure 5b, c). We confirmed COLX protein downregulation with immunohistochemistry and western blot (figure 5b, c). *COL10A1* knock-down pellets from donor 1 were too small to be processed for immunohistochemistry. We observed a complete absence of positive staining in *COL10A1* knock-down pellets from donor 2 and a slight positivity in the pellets from donor 3, however the quantity was far less than that observed in controls (figure 5c). Western blot analysis also showed *COL10A1* shRNA could successfully inhibit COLX protein production (figure 5b), showing the validity of our silencing approach at both transcript and protein level.

Absence of _COL10A1_ during chondrogenic differentiation of MSCs effects the ECM

After confirming the efficiency of the *COL10A1* knock-down we wanted to investigate how chondrogenic differentiation of MSCs was affected in its absence. For all donors non-treated, empty vector and non-target controls maintained their chondrogenic potential indicating lentiviral transduction and RNA-induced silencing complex (RISC) activation did not negatively affect chondrogenic differentiation of MSCs from each donor (figure 5-7). *COL10A1* knock-down pellets were significantly smaller than all controls for all donors which lead us to investigate if the absence of COLX affected other matrix components.

When COLX production was mostly absent as in donors 1 and 2, a significant decrease in GAGs (figure 6a, b) and ACAN gene expression (figure 3c) was observed. However when a less effective knock-down of *COL10A1* was achieved, as was the case in donor 3, matrix production was less affected (figure 6). We observed a similar trend with regard

to collagen type II production at both protein (figure 7a) and gene expression (figure 7b) level. We also show this to be true for collagen type I on gene expression (figure 7c). Overall, *COL10A1* knock-down affected production of crucial chondrogenic ECM components following differentiation.

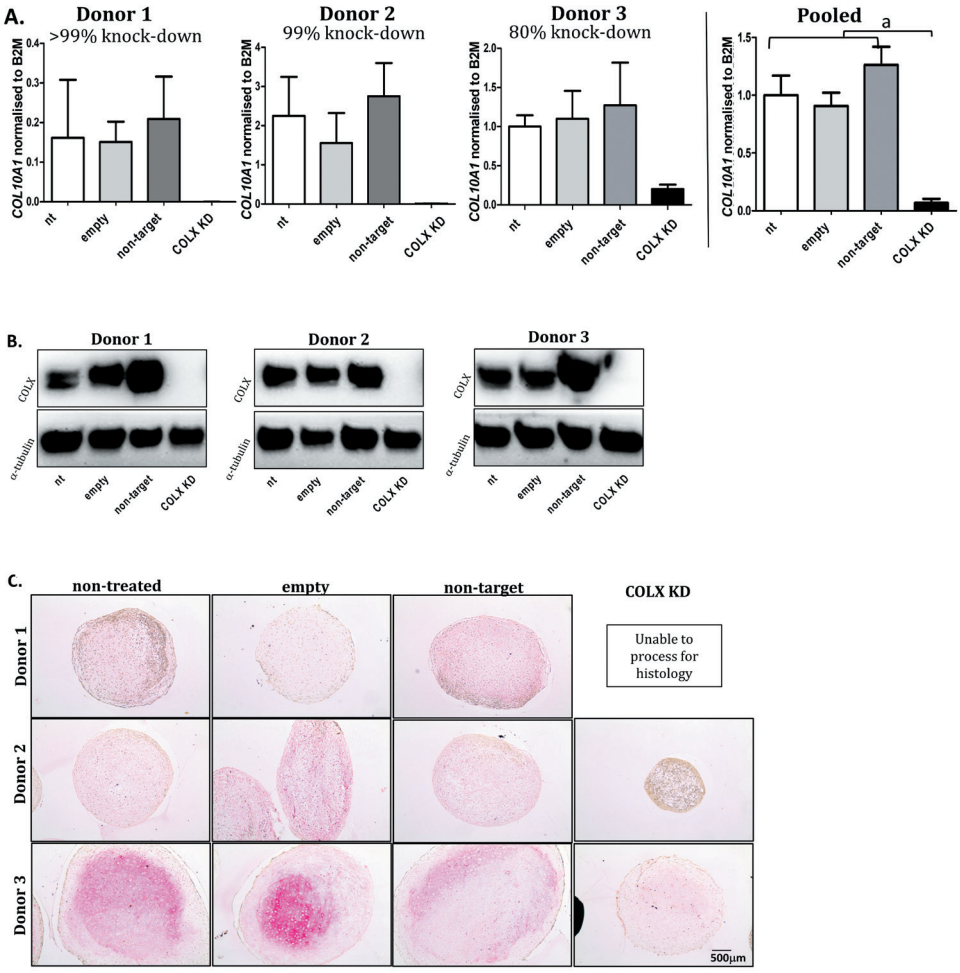

*Figure 5: COL10A1 can be efficiently knocked-down using lentivirally delivered shRNA. MSCs were transduced with lentivirus containing an empty vector, non-target shRNA or COL10A1 shRNA. MSCs were then selected based on puromycin resistance, expanded and chondrogenically differentiated for 21 days at which time they were harvested for analysis. **A)** Compared to control conditions MSCs transduced with shRNA directed against COL10A1 show significant down regulation of gene expression, the level of knock-down varying between donors (% NT vs COLX KD; Pooled data: Linear mixed model with bonferonni correction: a=p<0.05). **B)** This down regulation is also observed at the protein level via western blot and (C) COLX immunohistochemistry.*

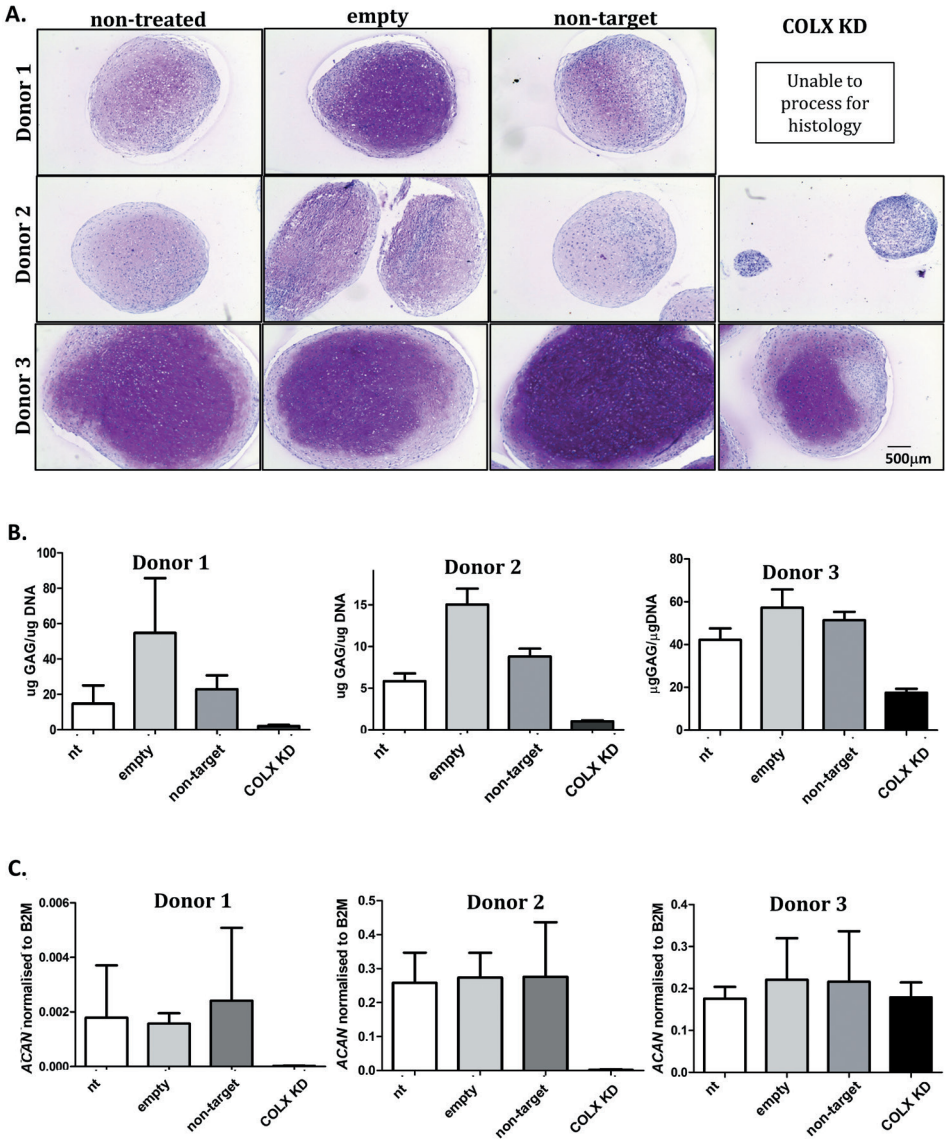

Figure 6: A small quantity of COL10A1 is required for the production of glycoaminoglycans during chondrogenic differentiation of MSCs . A) Thionine staining and (B) GAG quantification show when COL10A1 knock-down is over 99% the production of glycoaminoglcans (GAGs) is decreased (donors 1 &2). Production of 20% of the original COL10A1 quantity is enough to trigger production of GAGs (donor 3). C) A similar pattern is also observed in ACAN gene expression. Staining and expression of genes did not vary greatly between non-treated, empty and non-target conditions.

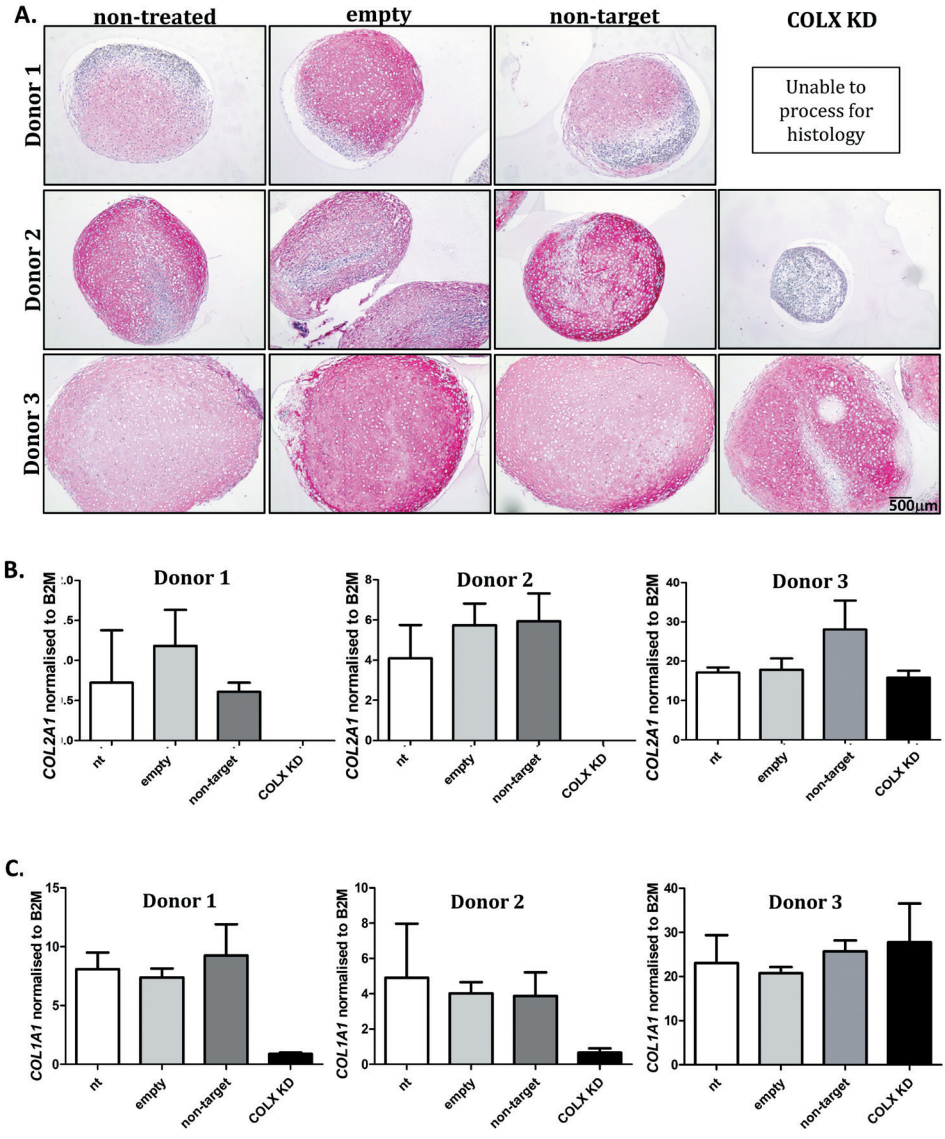

Figure 7: A small quantity of COL10A1 is required for proper collagen type II producion during chondrogenic differentiation of MSCs. A) Collagen type II (COLII) immunohistochemistry and **(B)** *gene expression show that when COL10A1 is knocked down with a greater efficiency (over 99%) production of COL2A1 is effected (donors 1 & 2). When the knock down is less efficient (donor 3) COL2A1 production is not affected. This same trend was observed in COL1A1 gene expression.* **(C)**. *Staining and expression of genes did not vary greatly between non-treated, empty and non-target conditions.*

A complete cartilage template is essential for tissue engineered MSC mediated EO

After observing that a knock-down of *COL10A1* in MSCs affected tissue engineered cartilage formation and confirming our control conditions for each donor were unaffected, we wanted to determine how a lack of *COL10A1* affected endochondral bone formation following subcutaneous implantation in nude mice, a model we have previously shown results in endochondral bone formation (6, 10). Chondrogenically differentiated MSC pellets were cultured for 21 d *in vitro* and implanted subcutaneously in nude mice. All control conditions from these donors underwent mineralisation (figure 8a, b).

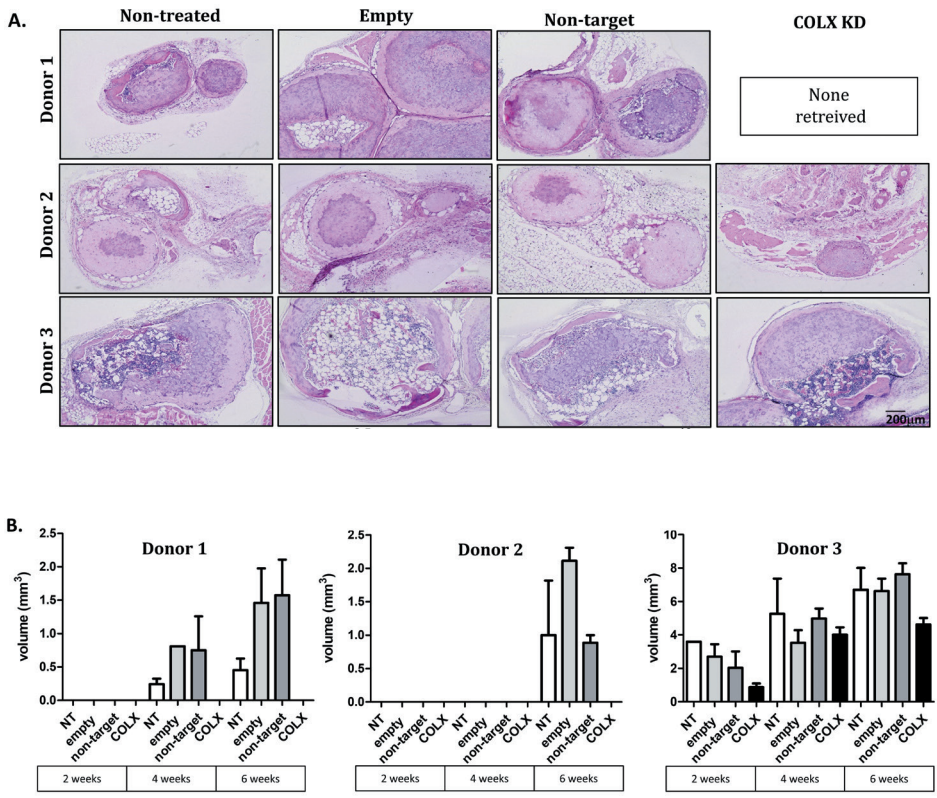

Figure 8: When COL10A1 production is absent in chondrogenically differentiated MSCs bone formation is hindered however a less significant knock-down results in normal bone formation. A) Following 8 (donors 1&2) or 10 weeks (donor 3) of in vivo implantation control conditions show the production of bone, bone marrow and calcified cartilage. Donor 1 COL10A1 knock-down pellets could not be retrieved following implantation however one pellet from Donor 2 showed signs of mineralisation. Pellets from Donor 3 appeared similar to control conditions however (B) mineralisation was initially delayed in these COL10A1 knock-down pellets as observed in longitudinal mCT.

COL10A1 knock-down pellets from donors 1 and 2 never showed *in vivo* mineralisation via µCT (figure 8b). No pellets which contained *COL10A1* shRNA were retrieved from donor 1 and only 1 of the 9 implanted pellets was retrieved from donor 2. This pellet showed calcification around the outer periphery but no marrow formation and was substantially smaller than control pellets. All pellets from donor 3 containing shRNA against *COL10A1* not only showed evidence of calcification on µCT (figure 8b), albeit less than that observed in other conditions, but also after 10 weeks *in vivo* showed the presence of mature bone, bone marrow and calcified cartilage. Here we show how an almost complete down-regulation of *COL10A1* in MSCs pre-implantation will hinder *in vivo* bone formation, however if down-regulation is not as efficient, bone formation will proceed normally.

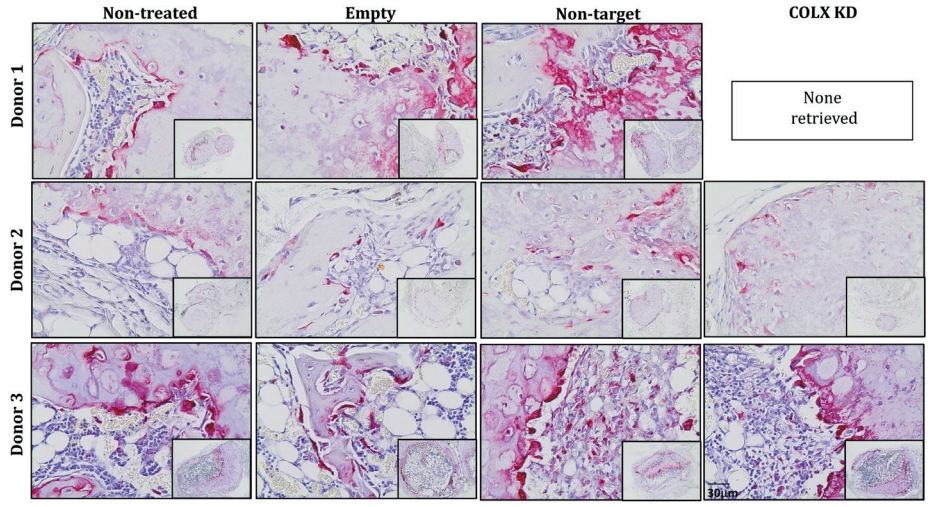

Figure 9: Osteoclast activity in response to COL10A1 knock-down of chondrogenically differentiated MSCs following in vivo implantation is affected in relation to the efficiency of the knock-down. TRAP staining of implanted pellets following 8 (donors 1&2) or 10 (donor 3) weeks of in vivo implantation. Pellets containing COL10A1 shRNA could not be recovered following implantation from donor 1. 1 of the 9 COL10A1 shRNA containing pellets from donor 2 was recovered but only exhibited TRAP positive staining on the outer periphary. All implanted pellets from donor 3 were recovered and displayed TRAP positivity throughout the construct. Staining did not vary greatly between non-treated, empty and non-target conditions.

Chondrogenic matrices with decreased *COL10A1* exhibit decreased osteoclastic remodelling

As *in vivo* endochondral bone formation was differentially affected depending on the efficiency of the *COL10A1* knock-down we wanted to determine if the presence of

osteoclasts was also affected in a similar manner, as they are known to be important for matrix remodelling, an important process for successful endochondral bone formation (2). Tartrate-resistant acid phosphatase (TRAP) was performed (figure 9) and in the pellet containing shRNA against *COL10A1* from donor 2 a different pattern of staining was observed versus controls. Non-treated, empty vector and scrambled pellets all showed positive staining in the central marrow cavity whereas *COL10A1* shRNA containing pellets only showed staining around the outer periphery. This was starkly different from *COL10A1* shRNA containing pellets from donor 3 which exhibited a similar staining pattern to what was observed in all the different control condition pellets (figure 9).

When *COL10A1* expression is significantly knocked-down less osteoclasts were observed *in vivo*, however, why this happens could be an indirect effect from the *COL10A1* knock-down instead of direct, further research is required to determine this.

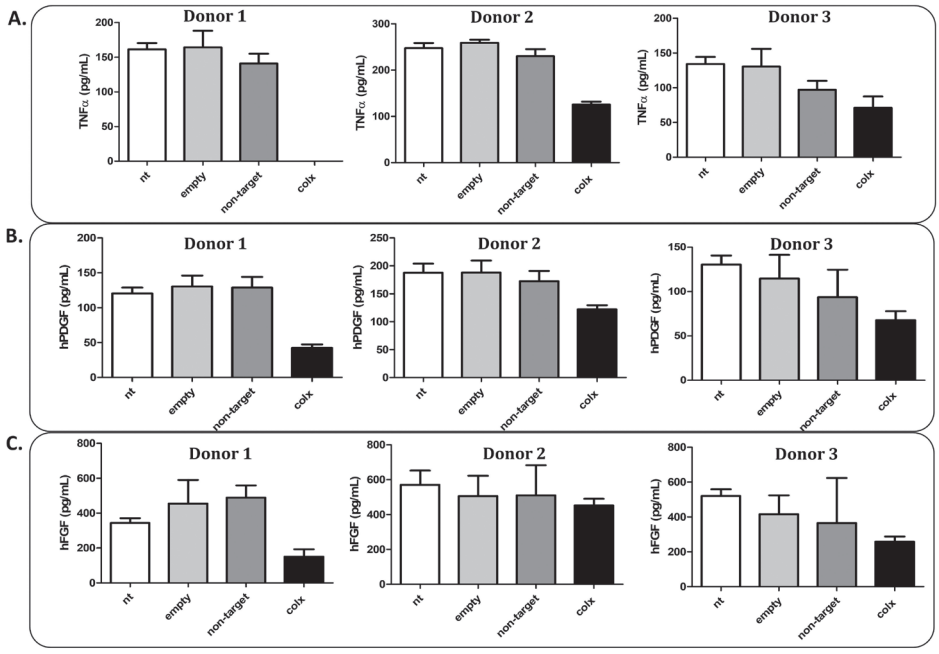

Figure 10: When COL10A1 production is absent the production of factors related to osteoclastic and osteoblastic migration and maturation are decreased but a small quantity rescues this effect. A) Production of TNF-a is down regulated when COL10A1 is downregulated in chondrogenic MSCs. B) A significant knock down of COL10A1 is required for other factors related to osteoblastic migration and maturation, PDGF and FGF to be downregulated (donor 1). A small quantity of COL10A1 remaining in knock-down conditions shows no inhibition of this production (donors 2 and 3). Production did not vary greatly between non-treated, empty and non-target conditions.

Down-regulation of *COL10A1* affects the secretion profile of chondrogenic MSCs

Following implantation we observed some calcification and altered cell recruitment on *COL10A1* knock-down pellet from donor 2 but less extensive differences on COLX knock-down pellets from donor 3 (figure 8). In order to further understand why these differences occurred, we investigated the secreted profile of known factors which are secreted from chondrogenic MSCs which influence EO. We found in donor 1 two factors involved in endothelial migration and osteoblastic migration/maturation, platelet derived growth factor (PDGF) and fibroblast growth factor 2 (FGF) (1, 36, 37), were lowly expressed (figure 10).

These factors were both detected at a higher level in donors 2 and 3. A similar trend was observed in TNF-α, a factor linked to inflammation and osteoclastic maturation (figure 10). This shows that the secreted profile of chondrogenically differentiated MSCs, at least in part, is influenced based on the production of *COL10A1*.

DISCUSSION

The role of COLX in EO is often debated. Where some developmental studies have found significant perinatal death, delayed bone formation and a compromised immune system, others report no major difference between knock out and wild type controls (25, 30, 38, 39). However, how COLX contributes to tissue engineered cartilage formation and subsequent endochondral bone formation has yet to be determined. Here we investigated the role of COLX from a tissue engineering perspective using culture expanded MSCs. As chondrogenically differentiated MSCs express COLX early during differentiation and have previously been shown to form bone following *in vivo* implantation (5-7) this system was a suitable model for our study. We included a number of lentiviral controls to ensure the act of transfecting primary MSCs with a lentivirus (empty vector control (SHC001)) and the activation of RISC (via a non-target control shRNA (SHC002)) was not having detrimental effects on cell behavior. We ensured the proper concentration of protamine sulphate, puromycin and lentiviral supernatant were used by completing extensive testing prior to experimental start. This allowed us to minimise unnecessary stress to MSCs throughout the study.

In these studies matrix production was significantly altered, resulting in the distribution of matrix components around hypertrophic cells and throughout the growth plate (25, 30). COLX is also known to play a role in the movement and accumulation of proteoglycans and has been shown to associate with other collagens within the matrix (40-42). Using chondrogenically differentiated MSCs which are prone to hypertrophic differentiation (13, 43, 44) we found GAG quantity was significantly decreased and other major ECM components including COLII and aggrecan were significantly downregulated

or almost absent when COLX knock-down was greater than 80% (gene expression). In previous *COL10A1* knock-downdown and Tg mouse model studies, adjacent non-hypertrophic chondrocytes which were unaffected by the knock-down were still able to produce normal cartilage matrix components (29). As these non-affected cells were not present in our model it could explain why matrix production was more severely affected. We and others have previously reported *COL10A1* expression occurs early in chondrogenic differentiation of MSCs, being observed within the first few day (10, 13). In tissue engineering it appears a certain threshold of *COL10A1* production is crucial for proper cartilage matrix formation during chondrogenic differentiation. When *COL10A1* is efficiently down-regulated proper matrix formation is hindered. Not only are crucial ECM components not produced, but the secreted profile of the constructs is affected. Here we shown a small quantity of *COL10A1* (roughly 20% of the original; donor 3) is sufficient to allow the MSC construct to undergo ossification and marrow formation *in vivo*, albeit less than non-knockdown conditions, indicating perhaps a minimum threshold of COLX production exists which allows for these processes to still occur. A greater number of donors would be helpful to determine exactly what this threshold is. It would be of great interest to see if the overexpression of *COL10A1* in this model would improve chondrogenic differentiation or increase the secretion of factors which influence *in vivo* matrix remodeling and bone formation.

Schmid type metaphyseal chondrodysplasia, a disease resulting in dwarfism, *coxa vara* and other skeletomuscular defects, is the result of mutated or deficient COLX production (45-47). As we observed a significant effect when COLX production was decreased during chondrogenesis we wanted to investigate how this affected bone formation following implantation. Here we report a severe decrease in COLX production has not only major consequences on MSC chondrogenic differentiation but also affects endochondral bone formation. When COLX was significantly down regulated, as in donor 1, bone formation was not observed. However, in the second donor which also had a highly efficient knock-down, only one pellet from one of the implant pockets were retrievable. Here we observed calcification of the construct but no marrow formation. This donor overall appeared to be slightly delayed in bone formation, as controls had very little marrow formation following implantation. It is possible had the *in vivo* time frame been increased, marrow formation would have occurred but this would require further investigation to verify. With donor 3, where knock-down efficiency was under 90%, matrix remodeling and bone formation appeared relatively unaffected but was still less than untreated controls regarding levels of mineralisation. To further understand these differences, we investigated the secretion of some relevant molecules by these constructs.

During EO the recruitment and invasion of the nearby vasculature, the initiation of osteoclast migration and remodeling of the cartilaginous matrix, and osteoblastic

maturation and bone deposition are all essential. Much of this can be initiated by different secreted factors which are released or found within the matrix of our implanted chondrogenic pellets (8, 9, 48, 49). In this study the production of TNFα was downregulated when COLX expression was decreased. TNFα is produced at high levels by hypertrophic chondrocytes (50). TNFα is not only a major inflammatory factor involved in bone fracture healing (51) but also an important factor in bone formation and metabolism. It is involved in osteoclast differentiation and has been shown to mediate bone turnover (52, 53). In fracture repair studies there is a decreased recruitment of osteoclasts as well as delayed endochondral bone formation in TNFα null mice (54). Other factors including PDGF and FGF, which are expressed consistently during all stages of fracture repair (55), were also found to be affected by a lack of COLX. PDGF has been shown to be important for osteoblastic differentiation (56, 57) and is also thought to contribute to the stabilisation of blood vessels by orchestrating migration and assembly of pericytes, which are known to contribute to proper vasculature formation (37, 58). FGFs plays a role in EO and are also important for the proliferation and differentiation of chondrocytes (59, 60). Additionally FGFs have been shown to play a role in osteoclast formation and maturation (61-63). In this study we found a significant decrease in TNFα, PDGF and FGF when COLX expression was knocked down over 99% compared to controls which were did not show a difference in the production of this or other reported factors (donor 1). In COLX knock-down constructs from this donor constructs were never retrieved following implantation. These constructs were most likely resorbed or degraded by the host. However, donors which had a decreased COLX expression but still maintained expression of TNFα, PDGF and FGF were retrieved and formed bone following implantation (donors 2 and 3). In retrieved pellets from these donors we observed bone and signs of osteoclastic remodeling, as evident by TRAP positive staining. In these donors it could be the production of these secreted factors was high enough to initiate the migration of osteoclasts and osteoblasts required to initiate bone formation. A full secreted profile analysis would be helpful to further elucidate the effects of a *COL10A1* knock-down on the chondrogenic secretome of differentiated MSCs.

Here we observed significant effects of COLX during both cartilage and bone formation, however, Rosati et al found no major defects or skeletal abnormalities when COLX production was inhibited (39). This could simply be explained by the species differences, as we did not look at mouse cells in this study nor did we have a fully immune competent environment. Additionally, in the Rosati et al study it is possible that other collagens and components in the ECM were able to compensate for in the absence of COLX by surrounding cells in the growth plate. As our model was a single cell culture followed by subcutaneous implantation in a normally non-bony environment there were no such cells close to the construct to compensate for the lack of COLX. As is often the case with MSC studies we do observe a certain amount of inter and

intra donor variation. Although inter donor variation is well documented in MSCs, the intra donor variation we observed could be attributed to the random integration nature of lentiviral delivery. Perhaps this could have been controlled partially for via single cell expansion, which is extremely difficult to achieve with a primary MSC cell source. This variation has also been observed by other researchers utilising primary MSCs (64). However, it is clear from a tissue engineering perspective that COLX is essential for proper cartilage and bone formation. Its presence is required for proper cartilage differentiation pre-implantation and necessary for subsequent endochondral bone formation. With this in mind it could be possible to further improve future graft performance with chondrogenically differentiated MSCs by over expressing or increasing hypertrophic differentiation preimplantation, increasing the production of important factors which are influenced by the presence of COLX as reported here, although further studies are required to prove this. By improving our understanding of how these constructs achieve endochondral bone formation we will no doubt improve graft performance and the clinical relevance of such constructs.

CONCLUSION

In this study we show how the absence of COLX in MSCs not only hinders the ability of MSCs to undergo chondrogenesis but also effects subsequent endochondral ossification following *in vivo* implantation. From a tissue engineering perspective this study improves our understanding of how MSC mediated endochondral ossification is achieved. Understanding COLX plays a crucial role in this method of bone formation can further improve graft construction in the future.

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Conflict of interest
Authors declare no conflict of interest.

References

1. Ornitz DM. FGF signaling in the developing endochondral skeleton. Cytokine & growth factor reviews. 2005;16(2):205-13.
2. Ortega N, Behonick DJ, Werb Z. Matrix remodeling during endochondral ossification. Trends in cell biology. 2004;14(2):86-93.
3. Maes C, Kobayashi T, Selig MK, Torrekens S, Roth SI, Mackem S, et al. Osteoblast precursors, but not mature osteoblasts, move into developing and fractured bones along with invading blood vessels. Developmental cell. 2010;19(2):329-44.
4. Mackie E. Osteoblasts: novel roles in orchestration of skeletal architecture. The international journal of biochemistry & cell biology. 2003;35(9):1301-5.
5. van der Stok J, Koolen MKE, Jahr H, Kops N, Waarsing JH, Weinans H, et al. Chondrogenically differentiated mesenchymal stromal cell pellets stimulate endochondral bone regeneration in critical-sized bone defects. Eur Cell Mater [Internet]. 2014 2014; 27:[137-48; discussion 48 pp.]. Available from: http://europepmc.org/abstract/MED/24554271.
6. Farrell E, Both SK, Odorfer KI, Koevoet W, Kops N, O'Brien FJ, et al. In-vivo generation of bone via endochondral ossification by in-vitro chondrogenic priming of adult human and rat mesenchymal stem cells. BMC Musculoskelet Disord. 2011;12:31.
7. Farrell E, van der Jagt OP, Koevoet W, Kops N, Van Manen CJ, Hellingman CA, et al. Chondrogenic priming of human bone marrow stromal cells: a better route to bone repair? Tissue Engineering Part C: Methods. 2008;15(2):285-95.
8. Scotti C, Tonnarelli B, Papadimitropoulos A, Scherberich A, Schaeren S, Schauerte A, et al. Recapitulation of endochondral bone formation using human adult mesenchymal stem cells as a paradigm for developmental engineering. Proceedings of the National Academy of Sciences. 2010;107(16):7251-6.
9. Scotti C, Piccinini E, Takizawa H, Todorov A, Bourgine P, Papadimitropoulos A, et al. Engineering of a functional bone organ through endochondral ossification. Proceedings of the National Academy of Sciences. 2013;110(10):3997-4002.
10. Knuth CA W-BJ, Ridwan Y, Wolvius EB, Farrell E. Mesenchymal stem cell-mediated endochondral ossification utilising micropellets and brief chondrogenic priming. Eur Cell Mater. 2017;34:142-61.
11. Bourgine PE, Scotti C, Pigeot S, Tchang LA, Todorov A, Martin I. Osteoinductivity of engineered cartilaginous templates devitalized by inducible apoptosis. Proceedings of the National Academy of Sciences. 2014;111(49):17426-31.
12. Cunniffe GM, Vinardell T, Murphy JM, Thompson EM, Matsiko A, O'Brien FJ, et al. Porous decellularized tissue engineered hypertrophic cartilage as a scaffold for large bone defect healing. Acta biomaterialia. 2015;23:82-90.

13. Pelttari K, Winter A, Steck E, Goetzke K, Hennig T, Ochs BG, et al. Premature induction of hypertrophy during in vitro chondrogenesis of human mesenchymal stem cells correlates with calcification and vascular invasion after ectopic transplantation in SCID mice. Arthritis & Rheumatology. 2006;54(10):3254-66.
14. Bahney CS, Hu DP, Theodore Miclau Iii RSM. The multifaceted role of the vasculature in endochondral fracture repair. Frontiers in endocrinology. 2015;6.
15. Colnot CI, Helms JA. A molecular analysis of matrix remodeling and angiogenesis during long bone development. Mechanisms of development. 2001;100(2):245-50.
16. Touaitahuata H, Cres G, de Rossi S, Vives V, Blangy A. The mineral dissolution function of osteoclasts is dispensable for hypertrophic cartilage degradation during long bone development and growth. Developmental biology. 2014;393(1):57-70.
17. Deckers MML, Van Bezooijen RL, Van Der Horst G, Hoogendam J, van der Bent C, Papapoulos SE, et al. Bone morphogenetic proteins stimulate angiogenesis through osteoblast-derived vascular endothelial growth factor A. Endocrinology. 2002;143(4):1545-53.
18. Luvalle P, Daniels K, Hay ED, Olsen BR. Type X Collagen is Transcriptionally Activated and Specifically Localized During Sternal Cartilage Maturation. Matrix. 1992;12(5):404-13.
19. Shen G. The role of type X collagen in facilitating and regulating endochondral ossification of articular cartilage. Orthodontics & Craniofacial Research. 2005;8(1):11-7.
20. Schmid TM, Linsenmayer TF. Immunohistochemical localization of short chain cartilage collagen (type X) in avian tissues. The Journal of cell biology. 1985;100(2):598-605.
21. Kirsch T, Pfäffle M. Selective binding of anchorin CII (annexin V) to type II and X collagen and to chondrocalcin (C-propeptide of type II collagen) Implications for anchoring function between matrix vesicles and matrix proteins. FEBS letters. 1992;310(2):143-7.
22. Wu LN, Genge BR, Lloyd GC, Wuthier RE. Collagen-binding proteins in collagenase-released matrix vesicles from cartilage. Interaction between matrix vesicle proteins and different types of collagen. Journal of Biological Chemistry. 1991;266(2):1195-203.
23. Anderson HC, Garimella R, Tague SE. The role of matrix vesicles in growth plate development and biomineralization. Front Biosci. 2005;10(1):822-37.
24. Chan D, Jacenko O. Phenotypic and biochemical consequences of collagen X mutations in mice and humans. Matrix Biology. 1998;17(3):169-84.
25. Jacenko O, Chan D, Franklin A, Ito S, Underhill CB, Bateman JF, et al. A Dominant Interference Collagen X Mutation Disrupts Hypertrophic Chondrocyte Pericellular Matrix and Glycosaminoglycan and Proteoglycan Distribution in Transgenic Mice. The American Journal of Pathology. 2001;159(6):2257-69.
26. Kim TK, Sharma B, Williams CG, Ruffner MA, Malik A, McFarland EG, et al. Experimental model for cartilage tissue engineering to regenerate the zonal organization of articular cartilage. Osteoarthritis and cartilage. 2003;11(9):653-64.

27. Campbell MR, Gress CJ, Appleman EH, Jacenko O. Chicken Collagen X Regulatory Sequences Restrict Transgene Expression to Hypertrophic Cartilage in Mice. The American Journal of Pathology. 2004;164(2):487-99.
28. Kwan KM, Pang MKM, Zhou S, Cowan SK, Kong RYC, Pfordte T, et al. Abnormal compartmentalization of cartilage matrix components in mice lacking collagen X: implications for function. The Journal of cell biology. 1997;136(2):459-71.
29. Rosati R, Horan GSB, Pinero GJ, Garofalo S, Keene DR, Horton WA, et al. Normal long bone growth and development in type X collagen-null mice. Nature genetics. 1994;8(2):129-35.
30. Jacenko O, Roberts DW, Campbell MR, McManus PM, Gress CJ, Tao Z. Linking Hematopoiesis to Endochondral Skeletogenesis through Analysis of Mice Transgenic for Collagen X. The American Journal of Pathology. 2002;160(6):2019-34.
31. Sweeney E, Roberts D, Corbo T, Jacenko O. Congenic mice confirm that collagen X is required for proper hematopoietic development. PloS one. 2010;5(3):e9518.
32. Sweeney E, Campbell M, Watkins K, Hunter CA, Jacenko O. Altered endochondral ossification in collagen X mouse models leads to impaired immune responses. Developmental Dynamics. 2008;237(10):2693-704.
33. Knuth CA, Kiernan CH, Palomares Cabeza V, Lehmann J, Witte-Buoma J, ten Berge D, et al. Isolating paediatric mesenchymal stem cells with enhanced expansion and differentiation capabilities. Tissue Engineering. 2018(ja).
34. Knuth C, Witte-Bouma J, Ridwan RY, Wolvius E, Farrell E. Mesenchymal stem cell-mediated endochondral ossification utilising micropellets and brief chondrogenic priming, towards scale-up. 2015.
35. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method. methods. 2001;25(4):402-8.
36. Kim SJ, Kim SY, Kwon CH, Kim YK. Differential effect of FGF and PDGF on cell proliferation and migration in osteoblastic cells. Growth Factors. 2007;25(2):77-86.
37. Xie H, Cui Z, Wang L, Xia Z, Hu Y, Xian L, et al. PDGF-BB secreted by preosteoclasts induces angiogenesis during coupling with osteogenesis. Nature medicine. 2014;20(11):1270.
38. Kwan KM, Pang MK, Zhou S, Cowan SK, Kong RY, Pfordte T, et al. Abnormal compartmentalization of cartilage matrix components in mice lacking collagen X: implications for function. J Cell Biol. 1997;136(2):459-71.
39. Rosatil R, Horanl GS, Piner03 GI, Garofalo S, Keene DR. Normal long bone growth and development in type X collagen. Nature genetics. 1994;8.
40. Chen Q, Linsenmayer C, Gu H, Schmid TM, Linsenmayer TF. Domains of type X collagen: alteration of cartilage matrix by fibril association and proteoglycan accumulation. The Journal of cell biology. 1992;117(3):687-94.
41. Chen Q, Gibney E, Fitch JM, Linsenmayer C, Schmid TM, Linsenmayer T. Long-range movement and fibril association of type X collagen within embryonic cartilage matrix. Proceedings of the National Academy of Sciences. 1990;87(20):8046-50.

42. Poole AR, Pidoux I. Immunoelectron microscopic studies of type X collagen in endochondral ossification. The Journal of cell biology. 1989;109(5):2547-54.
43. Mueller MB, Tuan RS. Functional characterization of hypertrophy in chondrogenesis of human mesenchymal stem cells. Arthritis & Rheumatology. 2008;58(5):1377-88.
44. Narcisi R, Cleary MA, Brama PA, Hoogduijn MJ, Tüysüz N, ten Berge D, et al. Long-term expansion, enhanced chondrogenic potential, and suppression of endochondral ossification of adult human MSCs via WNT signaling modulation. Stem cell reports. 2015;4(3):459-72.
45. Warman ML, Abbott M, Apte SS, Hefferon T, McIntosh I, Cohn DH, et al. A type X collagen mutation causes Schmid metaphyseal chondrodysplasia. Nature genetics. 1993;5(1):79.
46. Mcintosh I, Abbott MH, Warman ML, Olsen BR, Francomano CA. Additional mutations of type X collagen confirm COL10A1 as the Schmid metaphyseal chondrodysplasia locus. Human molecular genetics. 1994;3(2):303-7.
47. Chan D, Weng YM, Graham HK, Sillence DO, Bateman JF. A nonsense mutation in the carboxyl-terminal domain of type X collagen causes haploinsufficiency in schmid metaphyseal chondrodysplasia. The Journal of clinical investigation. 1998;101(7):1490-9.
48. Boeuf S, Richter W. Chondrogenesis of mesenchymal stem cells: role of tissue source and inducing factors. Stem cell research & therapy. 2010;1(4):31.
49. Tonnarelli B, Centola M, Barbero A, Zeller R, Martin I. Re-engineering development to instruct tissue regeneration. Current topics in developmental biology. 108: Elsevier; 2014. p. 319-38.
50. Kon T, Cho TJ, Aizawa T, Yamazaki M, Nooh N, Graves D, et al. Expression of Osteoprotegerin, Receptor Activator of NF-κB Ligand (Osteoprotegerin Ligand) and Related Proinflammatory Cytokines During Fracture Healing. Journal of Bone and Mineral Research. 2001;16(6):1004-14.
51. Gerstenfeld LC, Cullinane DM, Barnes GL, Graves DT, Einhorn TA. Fracture healing as a post-natal developmental process: Molecular, spatial, and temporal aspects of its regulation. Journal of cellular biochemistry. 2003;88(5):873-84.
52. Bertolini DR, Nedwin GE, Bringman TS, Smith DD, Mundy GR. Stimulation of bone resorption and inhibition of bone formation in vitro by human tumour necrosis factors. Nature. 1986;319(6053):516.
53. Kimble RB, Bain S, Pacifici R. The functional block of TNF but not of IL-6 prevents bone loss in ovariectomized mice. Journal of Bone and Mineral Research. 1997;12(6):935-41.
54. Council Directive 93/42/EEC of 14 June 1993 concerning medical devices. 1993:1-43.
55. Bolander ME. Regulation of fracture repair by growth factors. Proceedings of the Society for Experimental Biology and Medicine. 1992;200(2):165-70.
56. Li A, Xia X, Yeh J, Kua H, Liu H, Mishina Y, et al. PDGF-AA promotes osteogenic differentiation and migration of mesenchymal stem cell by down-regulating PDGFRα and derepressing BMP-Smad1/5/8 signaling. PLoS One. 2014;9(12):e113785.
57. Yu X, Hsieh SC, Bao W, Graves DT. Temporal expression of PDGF receptors and PDGF regulatory effects on osteoblastic cells in mineralizing cultures. American Journal of Physiology-Cell Physiology. 1997;272(5):C1709-C16.

58. Caplan AI, Correa D. PDGF in bone formation and regeneration: new insights into a novel mechanism involving MSCs. Journal of Orthopaedic Research. 2011;29(12):1795-803.
59. Kronenberg HM. Developmental regulation of the growth plate. Nature. 2003;423(6937):332.
60. Ornitz DM, Marie PJ. FGF signaling pathways in endochondral and intramembranous bone development and human genetic disease. Genes & development. 2002;16(12):1446-65.
61. Nakagawa N, Yasuda H, Yano K, Mochizuki S-i, Kobayashi N, Fujimoto H, et al. Basic fibroblast growth factor induces osteoclast formation by reciprocally regulating the production of osteoclast differentiation factor and osteoclastogenesis inhibitory factor in mouse osteoblastic cells. Biochemical and biophysical research communications. 1999;265(1):158-63.
62. Hurley M, Lee S, Raisz L, Bernecker P, Lorenzo J. Basic fibroblast growth factor induces osteoclast formation in murine bone marrow cultures. Bone. 1998;22(4):309-16.
63. Kawaguchi H, Chikazu D, Nakamura K, Kumegawa M, Hakeda Y. Direct and indirect actions of fibroblast growth factor 2 on osteoclastic bone resorption in cultures. Journal of bone and mineral research. 2000;15(3):466-73.
64. Lin P, Lin Y, Lennon DP, Correa D, Schluchter M, Caplan AI. Efficient lentiviral transduction of human mesenchymal stem cells that preserves proliferation and differentiation capabilities. Stem cells translational medicine. 2012;1(12):886-97.

European cells and materials. 2017; 34. 142-161

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MESENCHYMAL STEM CELL-MEDIATED ENDOCHONDRAL OSSIFICATION UTILISING MICROPELLETS AND BRIEF CHONDROGENIC PRIMING

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Abstract

With limited autologous and donor bone graft availability, there is an increasing need for alternative graft substitutes. We have previously shown that chondrogenically priming mesenchymal stem cell pellets for 28 days *in vitro* will reproducibly result in endochondral bone formation after *in vivo* implantation. However, pellet priming time is quite extensive for clinical applications. A micropellet (µpellet)-fibrin construct was developed and coupled with a decreased priming period determined by an *in vitro* time course experiment. *In vitro* data showed expression of chondrogenic genes and matrix production after 7 days of chondrogenic priming, indicating briefer priming could possibly be used to induce bone formation *in vivo*. Both 7 and 28 day primed pellet, pellet-fibrin and µpellet-fibrin constructs were cultured for *in vitro* analysis and implanted subcutaneously into nude mice for 8 weeks. µpellet-fibrin constructs cultured *in vitro* for 7 or 28 days showed comparable bone to standard pellets *in vivo*. MSC mediated bone formation was achieved following only 7 days *in vitro* priming. Bone formation *in vivo* appeared to be influenced by overall matrix production pre-implantation. Given this short priming time and the injectable nature of the µpellet-fibrin constructs this approach might be further developed as an injectable bone substitute leading to a minimally invasive treatment option which would allow for tailored filling of bone defects.

INTRODUCTION

Autologous graft material for reconstruction of large bone defects is limited and as harvesting is associated with additional patient risk and discomfort there is an increasing need for suitable graft alternatives (1-3).

"Developmental engineering," mimicking the processes of tissue development and healing to create biologically relevant replacements for congenitally missing, worn out or damaged tissues, is commonly implemented in bone tissue engineering to develop replacements for autologous grafts (4, 5). Bone formation predominantly proceeds via two mechanisms, intramembranous (IO) and endochondral ossification (EO), both of which are exploited for bone graft development using stem cells and other cell types with varying degrees of success (6-9). Although the IO route of bone formation is often used in tissue engineering (TE), grafts often exhibit poor survival due to insufficient vascularisation and perfusion following implantation (10). For these reasons this work focused on achieving tissue engineered bone via the EO pathway.

Endochondral bone formation relies on a cartilage intermediate (11, 12). Mesenchymal cells condense, undergo chondrogenic differentiation, leading to the production of a cartilage template rich in collagens, glycosaminoglycans (GAGs), and bioactive signalling molecules (13, 14). As the process continues the chondrogenic matrix is calcified and degraded which results in the release of these molecules (14-16), triggering cell migration, blood vessel invasion, and ultimately bone formation (3, 17-19). It has previously been shown that EO can be mimicked to achieve bone formation using chondrogenically primed MSC pellets (20, 21). Chondrogenically differentiated MSC pellets *in vitro* for 28 days results in vascularised, endochondral bone when subcutaneously implanted *in vivo* for 8 weeks. This approach to TE bone formation is advantageous as cartilage is better suited to survive in the initial avascular environment and naturally promotes the revascularisation of the graft material when implanted into defect sites (22). Whilst a promising approach to bone regeneration, hurdles remain when trying to implement such techniques clinically. A major obstacle is the lengthy *in vitro* priming time required to differentiate pellets to stimulate endochondral ossification *in vivo*, a process which is essential as undifferentiated MSC pellets typically fail to form bone or form an insufficient quantity to heal defects (21). It has recently been shown *in vitro* priming can be reduced to 14 days, however, it has not been investigated whether further reduction is possible to allow for swifter patient treatment (23). *In vitro* priming time is further compounded by the fact that inherent differences between MSC donors results in variable degrees of

chondrogenic differentiation which can affect the quantity and quality of bone produced *in vivo* (21, 24, 25). In order for these constructs to be clinically relevant criteria for *in vitro* differentiation which is shown to lead to reproducible bone formation must be identified. However *in vitro* priming and donor variation are not the only obstacle between these constructs and clinical translation. Isolation of MSCs from bone marrow requires a painful surgical procedure and there would always be a secondary surgical procedure following the construction of MSC pellets required for graft implantation to treat the defect. The development of an injectable bone substitute would allow for a less invasive treatment option and possibly decreased recovery.

To address these issues we have created chondrogenically primed constructs comprised of micropellets (μpellets) suspended within a fibrin hydrogel. This approach exploits the reproducible and swift bone forming capacity of MSC pellets coupled with a shorter *in vitro* priming time to decrease the time required pre-implantation. μpellet constructs are promising in that their small size allows them to pass easily through a needle making them ideal for an injectable therapy. This would allow clinicians to properly fill a defect site without the need of a tailor printed/designed implant to fit specific void shapes and would result in decreased patient discomfort and possibly recovery time.

In this study we aimed to examine the bone forming potential of these μpellet constructs. We investigated decreased *in vitro* priming with the goal of achieving bone formation in a shorter period of time than has been reported. Additionally we observe how differences in chondrogenic potential pre-implantation influence endochondral bone formed *in vivo*.

METHODS AND MATERIALS

MSC isolation

Human bone marrow MSCs were isolated from 3 separate consenting patients undergoing total hip arthroplasty (Medical Ethical Testing Commission (METC) approval code 2004-142; donor 1: female, 60; donor 2: male, 34; donor 3: male, 58) and expanded in αMEM containing 10 % FBS serum (lot # 41Q204K, Gibco; Bleiswijk, Netherlands; 50 μg/mL gentamycin, 1.5 μg/mL fungizone, 25 μg/mL L-ascorbic acid 2-phosphate and 1 ng/ml fibroblast growth factor-2 (all from Instruchemie, Delfzijl, Netherlands). Heparinised bone marrow aspirates were taken from the greater trochanter and plated at 30-100 $x10^6$ nucleated cells per T175 flask (Corning; Amsterdam, Netherlands). 24h after plating, flasks were washed and refreshed to remove non adherent cells and debris. Cells were cultured at 37°C and 5% carbon dioxide (CO_2) and medium was refreshed twice a week. MSCs were passed at 85-90% using 0.05% trypsin (Gibco; Bleiswijk, Netherlands) and replated at approximately 2,800 cells/cm^2, cells were expanded to passage 4 at which time they were chondrogenically differentiated.

Chondrogenic differentiation

2x10^5 MSCs were suspended in complete chondrogenic medium (high-glucose DMEM supplemented with 50 µg/mL gentamycin (Invitrogen; Landsmeer, Netherlands), 1.5 µg/mL fungizone (Invitrogen; Landsmeer, Netherlands), 1mM sodium pyruvate (Invitrogen; Landsmeer, Netherlands), 40 µg/mL proline (Sigma; Zwijndrecht, Netherlands), 1:100 v/v insulin-transferrin-selenium (ITS+; BD Biosciences; Temse, Beligum), 10ng/mL transforming growth factor β3 (Peprotech; London, UK), 25 µg/mL L-ascorbic acid 2-phosphate (Sigma; Zwijndrecht, Netherlands), and 100 nM dexamethasone (Sigma; Zwijndrecht, Netherlands)) in 15 mL polypropylene tubes and centrifuged at 200g for 8min to create standard pellets. To generate µpellets, 3x10^6 MSCs per well were seeded in Aggrewell™800 plates (StemCell Technologies; Evergem, Belgium) in 1 mL chondrogenic medium and centrifuged at 300g for 10 min and incubated overnight creating 300 µpellets (10,000 cells per pellet) per well. Following 24h of incubation µpellets were transferred to 24 well plates (Corning; Zwijndrecht, Netherlands) or fibrin encapsulated. Cells undergoing chondrogenic differentiation were cultured at a ratio of 4x10^5 cells/mL of complete chondrogenic medium. All conditions were cultured at 37°C in 5% CO_2.

Chondrogenic time course

MSCs (donor information above) were chondrogenically differentiated via standard pellet culture conditions as described. Samples were harvested for histology following 2, 5, 7, 10, 14, and 28 days of differentiation. Samples were fixed in 4% (w/v) formaldehyde in PBS for 2h. Samples for biochemical assays were harvested after 7, 14, 21, and 28 days. Samples were digested overnight at 60°C in buffer containing papain (0.2M NaH_2PO_4, 0.01M EDTA2H_2O, 0.01M cysteine HCl, 250 µg/mL papain (Sigma; Zwijndrecht, Netherlands), pH 6) and stored at -20°C until processed. Samples for gene expression analysis were harvested after 2, 5, 7, 10, and 14 days of chondrogenic differentiation. Samples were homogenised in 350 µL trizol (Gibco; Bleiswijk, Netherlands) and stored at -80°C until processed.

Fibrin encapsulation

Pellets and µpellets were collected 24h post pelleting and suspended in 100 µL human fibrinogen (40 mg/mL dissolved in 0.9% NaCl; Sigma; Zwijndrecht, Netherlands) at a density of 60 µpellets or 3 standard pellets per 100 µL fibrinogen. Human thrombin (Sigma; Zwijndrecht, Netherlands) was added at a ratio of 0.5 IU to 1 mg fibrinogen, crosslinking took between 15-20min. Samples for implantation and *in vitro* analysis were maintained in complete chondrogenic medium supplemented with 0.0875IU/mL bovine aprotinin (Sigma; Zwijndrecht, Netherlands) for 7 or 28 days to slow the degradation of the fibrin material by cell constructs during *in vitro* culture. All medium was refreshed twice a week and maintained in 5% CO_2 at 37°C.

Subcutaneous implantation

All experiments were approved by the Animal Experiments Committee at the Erasmus Medical Centre, Rotterdam (DEC protocol 116-12-08). 3 loose pellets, 60 loose µpellets, 3 fibrin encapsulated pellets, and 60 fibrin encapsulated µpellets (600,000 cells total per pocket) were implanted subcutaneously in 10 (7 day *in vitro* primed samples) or 13 week-old (28 day *in vitro* primed samples) male athymic nude mice (Balb/c nudes, Charles River; Sulzfeld, Germany), which were housed in groups of three under standard light dark cycles with access to ad libitum food and water (18 mice in total). Pre-operatively animals were given pain medication (buprenorphine 0.05mg/kg bodyweight) under general anaesthesia (1-3.5% isoflurane). Two incisions were created dorsally (one between the shoulder blades and the other between the hip bones) per animal, with two subcutaneous pockets created per incision. One of the following conditions: 3 standard pellets, 3 standard pellets encapsulated in fibrin, 60 loose µpellets, or 60 µpellets encapsulated in fibrin, were implanted per pocket (implantation randomised between pockets and animals). 3 replicates per condition were implanted per MSC donor (3 MSC donors total). Eight weeks post implantation, animals were euthanised by CO_2 asphyxiation and constructs were retrieved.

Micro CT imaging

µCT scans were performed and reconstructed at the Applied Molecular Imaging Erasmus MC facility using the Quantum FX (Perkin-Elmer; Groningen, Netherlands). Ex vivo scans were acquired using a field of view of 10mm (90kV/160mA, 3min). Scans were quantified using Analyze 11.0 software (AnalyzeDirect; Nieuwe Niedorp, Netherlands). Calcified tissue was quantified by converting original linear attenuation coefficient measurements, by linear transformation, to Hounsfield units (HU). Global thresholding was applied to all scans and determined by visual inspection. Values over 400HU, corresponding to 0.133g/cm^3 (as determined by phantom scans), were segmented out and independently quantified as calcified tissue.

Histology

Samples were fixed in 4% (w/v) formaldehyde in PBS for 2h (*in vitro*) or 24 h (*in vivo*) and *in vivo* samples were decalcified in 10% w/v EDTA in PBS for 7 to 10 days refreshing every other day then paraffin embedded. 6 µm-thick sections were cut, deparaffinised and stained with haematoxylin-eosin (H&E), safranin O, or tartrate-resistant acid phosphatase (TRAP). 4-5 sections were used per sample. H&E staining was performed by incubating deparaffinised samples in 100% Gil's haematoxylin (Sigma; Zwijndrecht, Netherlands) for 5min followed by 7min incubation in non-distilled water. After washing with distilled water samples were incubated in 2% Eosin (Merck; Amsterdam, Netherlands; in 50% ethanol, 0.5% acetic acid) for 45sec, 70% ethanol for 10sec, then

rehydrated (100% ethanol for 1min, xylene for 1min) and mounted. H&E stained sections were used to quantify the percent of bone, calcified cartilage, and bone marrow using morphologically characteristics to determine boundaries. Aforementioned areas were segmented and then quantified using Image J (National Institute of Health; Maryland, USA). Safranin O staining was performed by incubating deparaffinised samples in 0.05% light green solution (in distilled water; Sigma ; Zwijndrecht, Netherlands) for 8min followed by a rinse in 1% acetic acid (in distilled water; Fluka; Zwijndrecht, Netherlands) and a 12min incubation in 0.1% Safranin O solution (in distilled water; Fluka; Zwijndrecht, Netherlands). Samples were then rinsed with 96% ethanol for 30sec, rehydrated (as previously mentioned) and mounted. TRAP staining was performed by incubating deparaffinised samples in 0.2M acetate buffer (0.2M sodium acetate (Sigma; Zwijndrecht, Netherlands), 100 nM L (+) tartaric acid (Acros; Antwerp, Belgium); pH 5) for 20min. Following incubation 0.5 mg/ml naphtol AS-BI phosphate (Sigma; Zwijndrecht, Netherlands) and 1.1 mg/ml fast red TR salt (Sigma; Zwijndrecht, Netherlands) was added to the acetate buffer and incubated for 1hr at 37^{o}C. Collagen type II samples were rinsed in distilled water and counterstained with haematoxylin as previously described.

Immunohistochemistry

Samples were prepared as previously described (26). Briefly, Collagen type II stained samples were incubated in 1mg/ml pronase (Sigma; Zwijndrecht, Netherlands) and 10mg/ml hyaluronidase (Sigma; Zwijndrecht, Netherlands) for 30min at 37^{o}C. 10% normal goat serum (Southern Biotech; Uithoorn, Netherlands) was used to block non-specific antibody binding. Sections were incubated with 0.4mg/ml collagen type II antibody (II-II/II6B3; Developmental Studies Hybridoma Bank, University of Iowa; Iowa City, USA). Collagen type X staining was performed by incubating samples with 0.1% pepsin (Sigma; Zwijndrecht, Netherlands) in 0.5 M acetic acid (pH 2.0) followed by 10mg/ml hyaluronidase treatment. Rat knees which were decalcified in 10% EDTA (w/v in distilled water; Sigma; Zwijndrecht, Netherlands) for 2 weeks. Sections were incubated with 1: 10 or 1:100 diluted collagen type X antibody (in PBS/1%BSA; X53;Quartett; Berlin, Germany) for 16hr. All stainings were incubated with 2.2 mg/ml biotin-SP F (ab) 2 goat-α-mouse (diluted in PBS/1%BSA; Jackson; Huissen, Netherlands) for 30min and washed in PBS. Samples were then incubated with streptavidin-AP for 30min and washed in PBS (diluted 1:50 in PBS/1%BSA; Biogenex; Uithoorn; Netherlands) All slides were incubated 0.2 M Tris-HCL substrate (pH 8.5; containing 1g/25ml Neu Fuchsin (in 2M HCl ;1B467, Fisher Scientific; Landsmeer; Netherlands;), $NaNO_2$ (4% in distilled water; Sigma; Zwijndrecht, Netherlands), 0.3mg/ml Naphtol AS-MX phosphate (Sigma; Zwijndrecht, Netherlands), 33 µl/ml Dimethylformamide (Sigma; Zwijndrecht, Netherlands), 0.25 mg/ml Levamisole (Sigma; Zwijndrecht, Netherlands) for 30min.

Collagen type II samples were rinsed in PBS followed by haematoxylin counterstaining. Matching mouse IgG1 (X0931; Dako; Amstelveen, Netherlands) isotype controls were performed for each staining.

Biochemical assays

Samples were digested overnight at 60°C in buffer containing papain (as described above). Glycosaminoglycan (GAG) content was quantified using a dimethylmethylene blue (DMB, pH 3; Sigma; Zwijndrecht, Netherlands) assay. Chondroitin sulphate C (Sigma; Zwijndrecht, Netherlands) was used to create a standard for this experiment and A530:A590 ratio was used to determine GAG content. DNA was quantified from papain digested samples using ethidium bromide with calf thymus DNA (Sigma; Zwijndrecht, Netherlands) as a standard.

Gene expression

Samples were homogenised in 350 µ trizol (Gibco; Bleiswijk, Netherlands; Bleiswijk, Netherlands). 70 µl 100% chloroform was added and samples were agitated, incubated for 10min at room temperature, and the aqueous phase was transferred to Rneasy® kit columns. RNA was purified using RNeasy® microkit (Qiagen; Venlo, Netherlands) and cDNA was reverse transcribed using a First Strand cDNA Synthesis Kit (RevertAid; Bleiswijk, Netherlands; MBI Fermentas; St. Leon-Rot, Germany) as per manufactures instructions. Real-time PCR was performed using 10ng of cDNA. Samples were amplified using either SYBR Green I dye (Eurogentec; Seraing, Belgium) or TAQman 2xReagent (Applied Biosystems; Nieuwerkerk a/d Ijssel, Netherlands) in 10 µL PCR mix reactions containing 10 µM forward and reverse primers for GAPDH, ColII, ColX, COLI, VEGF, MMP13 or BMP2 (Table 1) for a maximum of 40 cycles. For gene expression analysis 3 different MSC donors were analysed with three pellets per donor. Genes are expressed as delta CT.

Primer Name	Forward sequence (5'- 3')	Reverse sequence (5'- 3')
GAPDH	ATGGGGAAGGTGAAGGTCG	TAAAAGCAGCCCTGGTGACC
VEGFa	CTTGCCTTGCTGCTCTACC	CACACAGGATGGCTTGAAG
COLI	CAGCCGCTTCACCTACAGC	TTTTGTATTCAATCACTGTCTTGCC
COLII	CCCCATCTGCCCAACTGA	CTCCTTTCTGTCCCTTTGGT
COLX	ACTTCTCTTACCACATACACG	CCAGGTAGCCCTTGATGATGTACT
BMP2	AACACTGTGCGCAGCTTCC	CTCCGGGTTGTTTTCCCAC
MMP13	AAGGAGCATGGCGACTTCT	TGGCCCAGGAGGAAAAGC

Abbreviations: *GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; VEGFa, Vascular endothelial growth factor a; COLI, Collagen type I; COLII, Collagen type II; COLX, Collagen type X; BMP2, Bone morphogenetic protein 2; MMP13, Matrix metallopeptidase 13*

RESULTS

MSCs display chondrogenic characteristics during the first week of *in vitro* priming

The effects of *in vitro* chondrogenic priming time were investigated in order to determine the earliest stable chondrogenic induction time which could be utilised for *in vivo* bone formation studies. Time course experiments, in which MSC pellets were differentiated for 2, 5, 7, 10, 14, 21 or 28 days showed GAGs were present early during priming and increased greatly by 7 days of *in vitro* culture compared to earlier time points (figure 1a, b). Increased expression of chondrogenic genes including ColII and ColX was observed by 7 days and continued to rise thereafter compared to time point 0 (figure 1c). As chondrogenic characteristics were observed to be stably and reproducibly increased compared to earlier time points after 7 days of *in vitro* priming we chose to continue with this time point during *in vivo* studies.

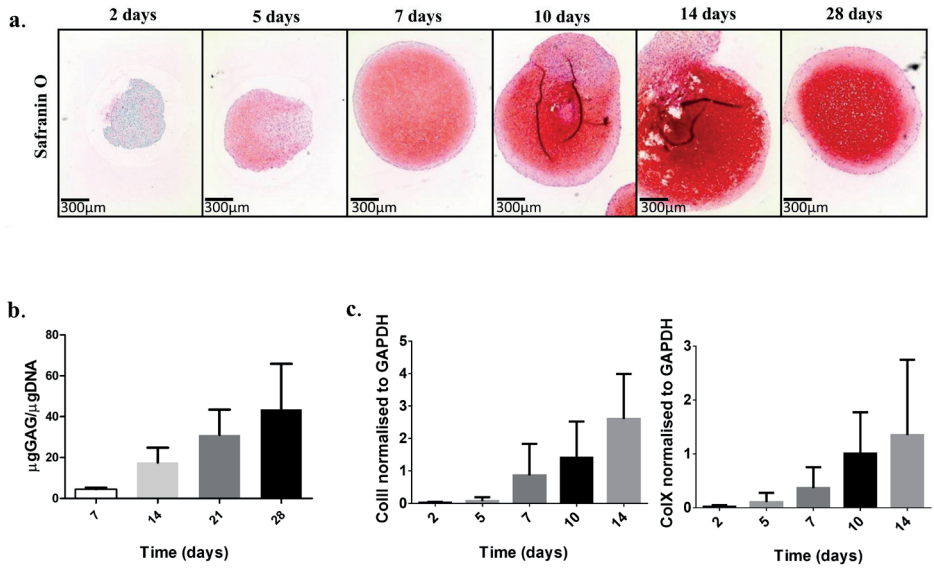

Figure 1: Chondrogenic markers are upregulated after 7 days of in vitro priming. ***(a)*** *Safranin O staining (donor 1) and* ***(b)*** *GAG (corrected for DNA) results show increase in matrix GAG deposition after 7 days of culture (all 3 MSC donors, 3 pellets per donor).* ***(c)*** *Expression of ColII and ColX increased after 7 days of in vitro culture and continued to increase over time (all 3 MSC donors, 3 pellets per donor; graphs display mean and standard deviation).*

µpellet-fibrin constructs maintain chondrogenic potential compared to standard pellets

µpellets were constructed using roughly 10,000 cells per µpellet, 20 of these µpellets were equivalent to the cell density of our standard pellet which is typically utilised in tissue engineered EO studies. µpellets were encapsulated in a 40 mg/ml fibrin gel. To ensure fibrin was not inhibiting chondrogenesis pellets were also encapsulated for comparison. Pellets, pellet-fibrin, µpellets and µpellet-fibrin were cultured for either 7 or 28 days after which time they were harvested for *in vitro* analysis or subcutaneously implanted in nude mice. *In vitro*, µpellets on their own failed to chondrogenically differentiate after 7 or 28 days, fibrin encapsulation was necessary in order to achieve differentiation (figure 2). In donor 3, µpellets aggregated following prolonged culture (28 days) forming one pellet with a decreased chondrogenic potential to standard pellets cultured from the same donor (figure 2c). Safranin O staining suggest a similarity in proteoglycan content between pellet, pellet-fibrin and µpellet-fibrin constructs after either 7 or 28 days of priming as staining intensity is comparable between conditions, although in earlier primed samples it may appear the µpellet-fibrin condition is less intense, the pellets are spread over a larger area distributing the staining over the section. The cumulative staining is similar to that observed in the standard pellet culture. Between donors there is variation in staining intensity highlighting a degree of donor variability during chondrogenesis (figure 2).

Cell morphology for each donor is similar between conditions and time points (figure 3a) but after 7 days of culture GAG/DNA data for donor 2 shows that µpellet-fibrin constructs contained more GAG than pellet counterparts (3b; one donor 6 replicates). This difference is not observed after extended culture (figure 3c; one donor 6 replicates).

Expression of COLII, COLX and COLI was observed in all donors after either 7 or 28 days of *in vitro* culture however gene expression levels varied significantly between donors (figure 4). Samples collected from donor 1 after both 7 and 28 days of culture showed greater expression and staining of COLII (figure 4a; 5). COLX expression was higher in µpellet-fibrin compared to pellets alone at both 7 and 28 days (figure 4b). Fibrin encapsulation appeared to lead to a more intense COLII staining (figure 5) throughout the matrix after 7 or 28 days of culture as shown in immunohistochemistry. There was no clear difference in COLX protein expression between conditions (figure 6). In IgG control stainings, COLX appeared to slightly stain the fibrin material (figure 6 inserts in a). This background staining was far less intense than that observed within our constructs. Staining specificity shown in rat knee tissue, however the optimal staining concentration for rat tissue was found to be lower than that of human (figure 6d).

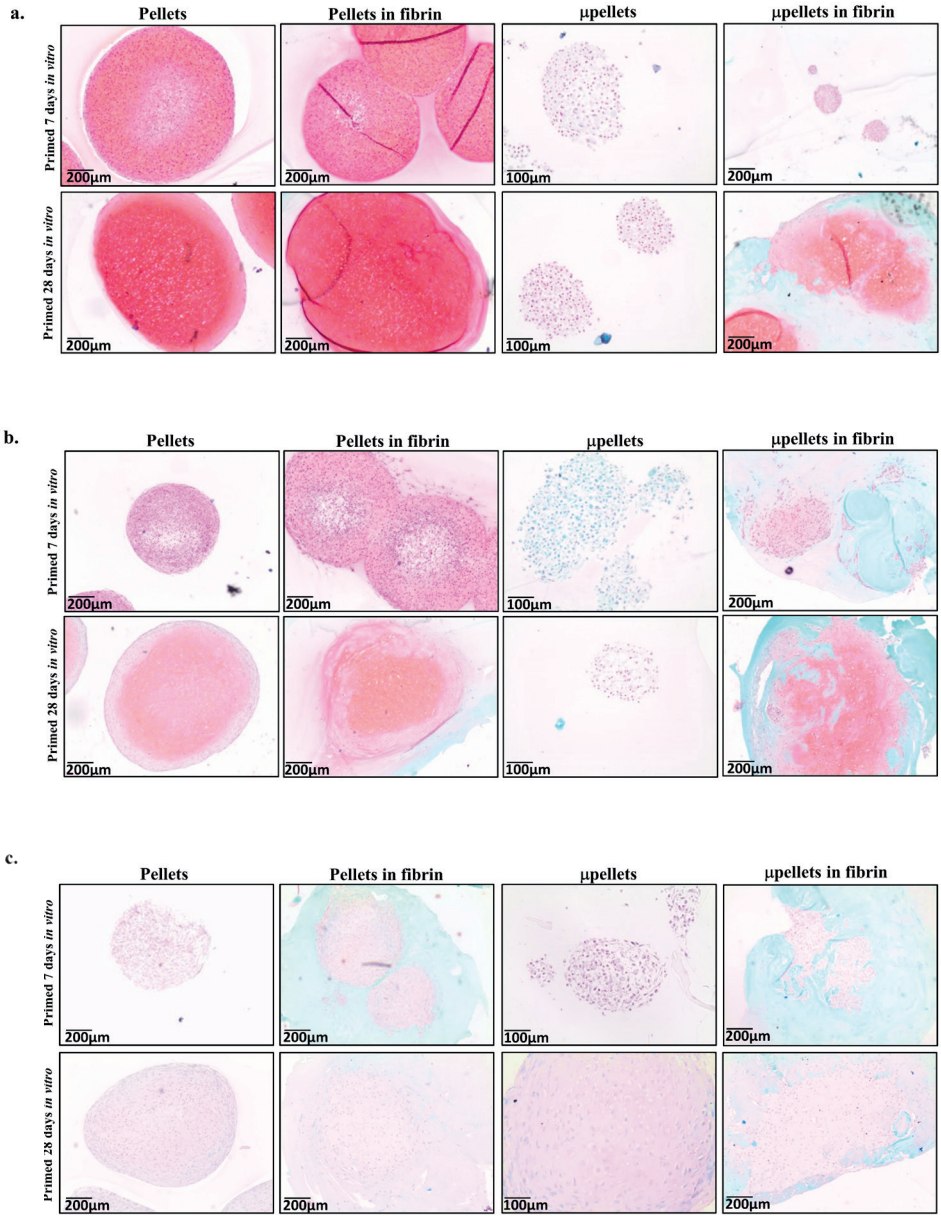

*Figure 2: **Non-encapsulated µpellets failed to chondrogenically differentiate.** Safranin O results show non-encapsulated µpellets exhibit no matrix GAG deposition after 7 or 28 days of culture (donors 1 **(a)** and 2 **(b)**) whereas other constructs show positive staining. Donor 3 **(c)** µpellets aggregated forming one pellet after 28 days in culture.*

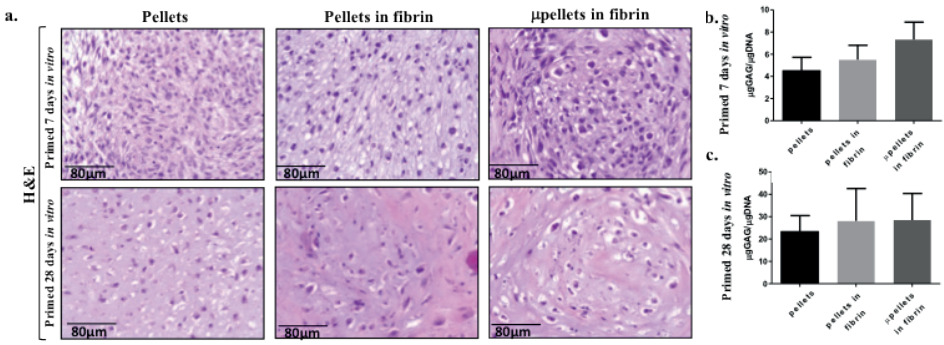

Figure 3: µpellet-fibrin constructs maintain cell morphology and exhibit chondrogenic potential. H&E staining showing morphology of chondrogenic pellet, pellet-fibrin and µpellet-fibrin (donor 2) is comparable between conditioned after 7 and 28 days of differentiated.

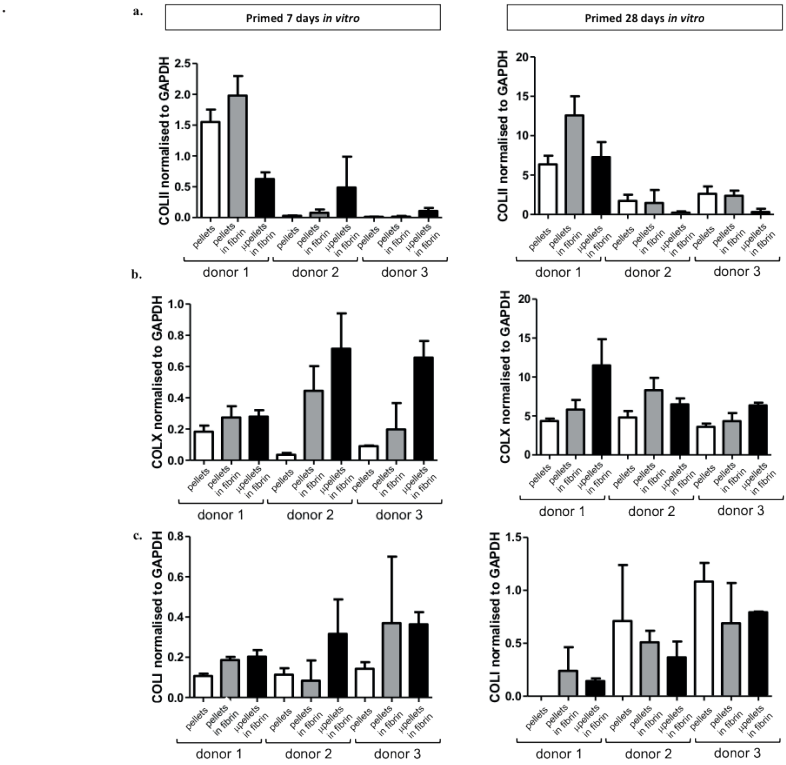

*Figure 4: Matrix components differentially affected between donors, however, hypertrophic marker COLX is consistently upregulated in µpellet-fibrin constructs cultured for 28 days. Expression of **(a)** ColII, **(b)** ColX and **(c)** ColI in pellet, pellet-fibrin and µpellet-fibrin constructs after 7 or 28 days of in vitro chondrogenic culture. Hypertrophic associated gene COLX was upregulated between pellet and µpellet-fibrin conditions cultured for 28 days.*

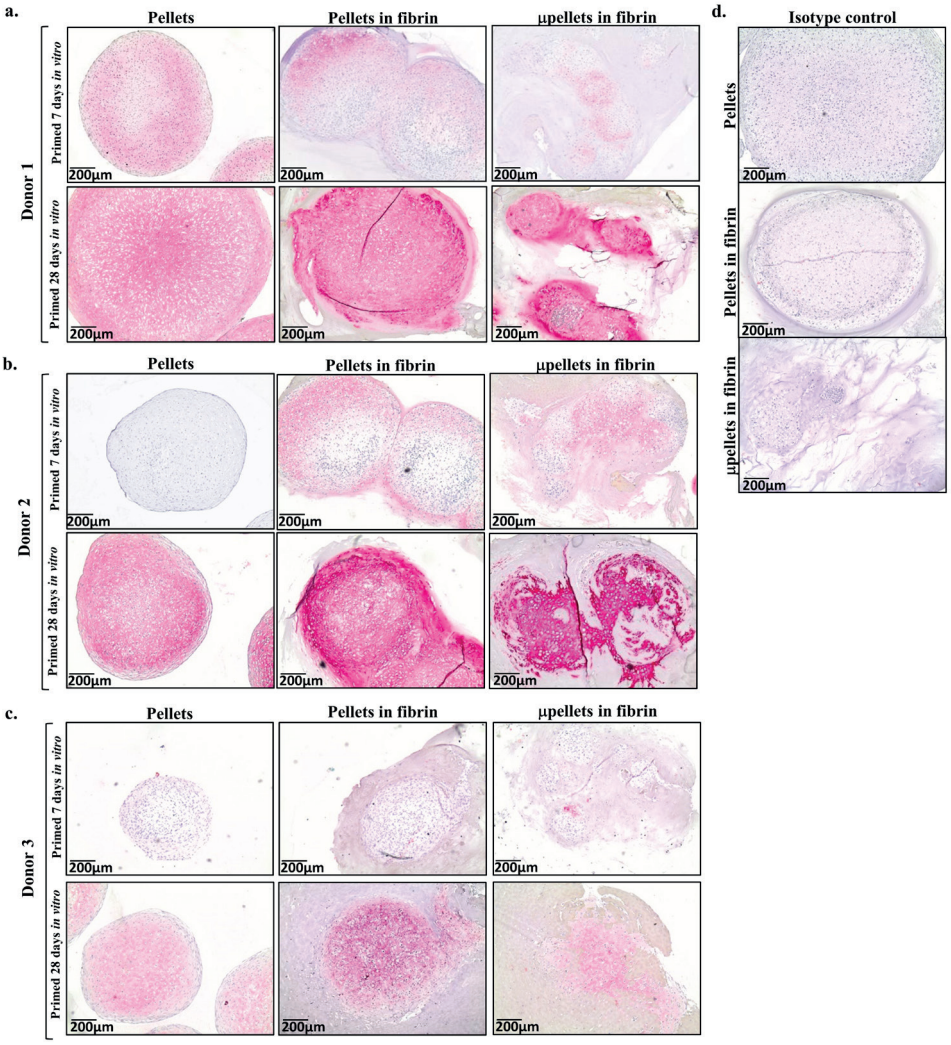

Figure 5: µpellet-fibrin constructs exhibit more homogenous collagen type II distribution after longer priming. COLII immunohistochemistry on chondrogenically differentiated pellet, pellet-fibrin and µpellet-fibrin constructs after 7 and 28 days of in vitro culture from MSC ***(a)*** *donor 1,* ***(b)*** *donor 2 and* ***(c)*** *donor 3* ***(d)*** *IgG controls performon donor 1 day 28 samples.*

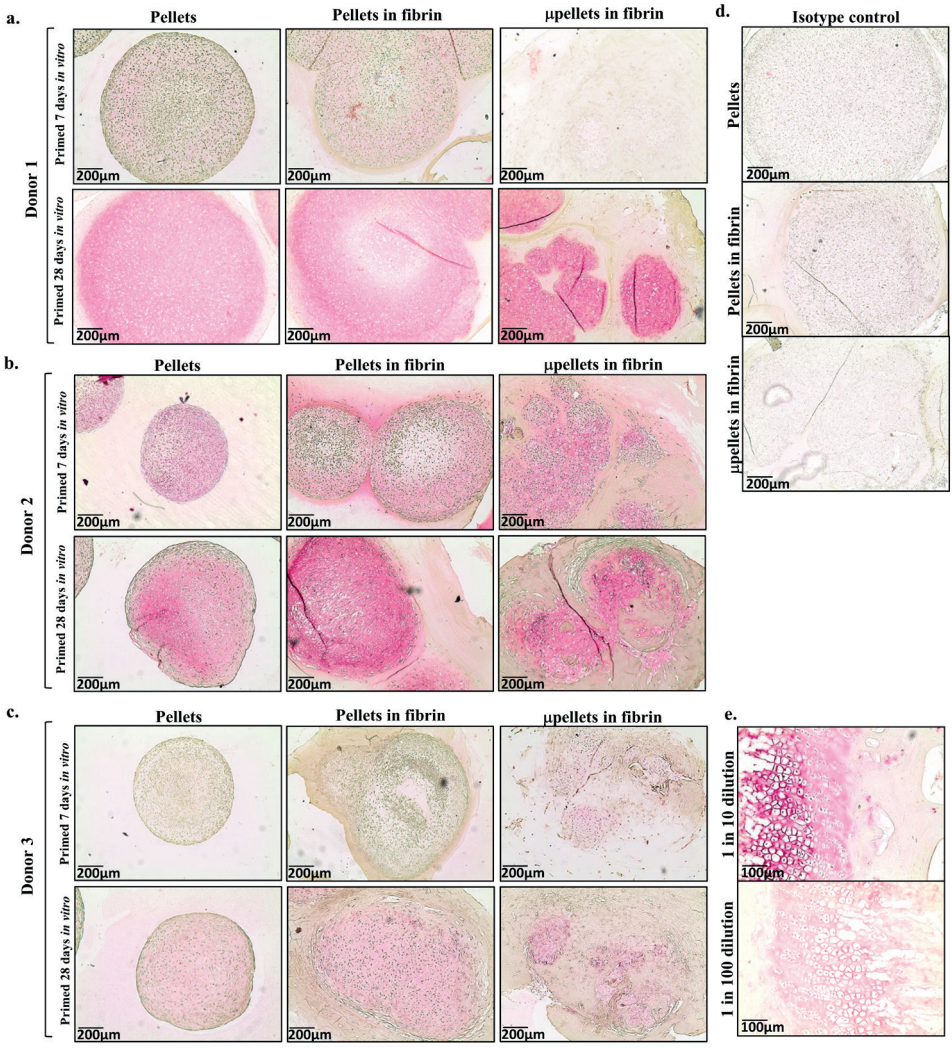

Figure 6: µpellet-fibrin constructs exhibit more homogenous collagen type X distribution after longer priming. *COLX immunohistochemistry on chondrogenically differentiated pellet, pellet-fibrin and µpellet-fibrin constructs after 7 and 28 days of in vitro culture from* **(a)** *donor 1,* **(b)** *donor 2 and* **(c)** *donor 3.* **(d)** *IgG controls are of day 28 samples from donor 1.* **(e)** *Rat knee positive control at 1:10 and1:100.*

µpellet-fibrin conditions generally had lower gene expression of VEGF compared to pellet controls after 7 days of *in vitro* culture, an effect that was not observed after 28 days of culturing (figure 7). In donor 1, VEGF expression was slightlydownregulated when compared to donors 2 and 3 after 28 days in culture (figure 7a). Other secreted factors including BMP2 and MMP13 were differentially affected between conditions and donors, but were present in all samples. Downscaling the standard pellet to µpellet-fibrin format did not appear to hinder the expression of these markers (figure 7).

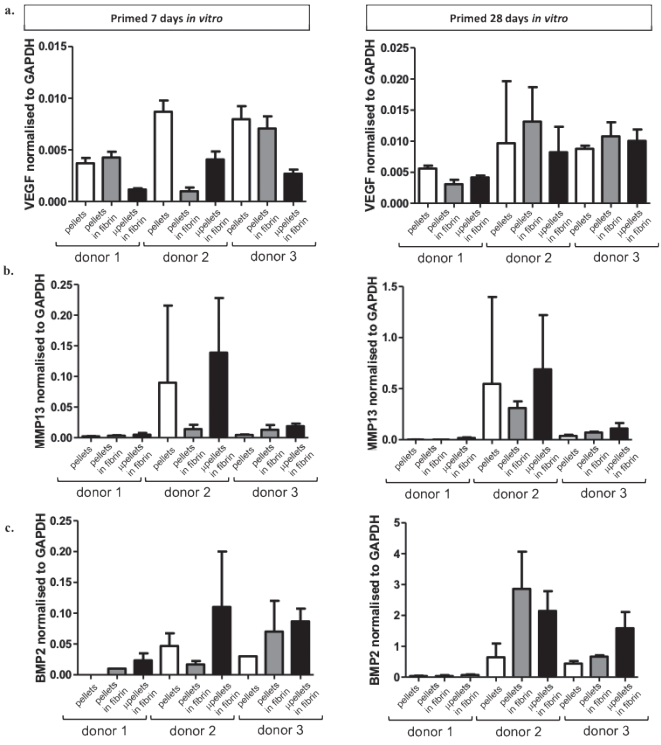

Figure 7: Gene expression of secreted factors is differentially affected across donors and is maintained in µpellet-fibrin constructs. *Expression of* ***(a)*** *VEGF,* ***(b)*** *MMP13 and* ***(c)*** *BMP-2 in pellet, pellet-fibrin and µpellet-fibrin constructs after 7 or 28 days of in vitro chondrogenic culture. Hypertrophic associated gene BMP2 was upregulated between pellet and µpellet-fibrin conditions in some donors where as it was relatively unaffected in others. Other markers were differentially affected depending on the donor.*

7 days of *in vitro* chondrogenic priming results in tissue calcification *in vivo*

In addition to *in vitro* characterisation pellets, pellet-fibrin, µpellets and µpellet-fibrin constructs were subcutaneously implanted in nude mice for 8 weeks and harvested for volumetric analysis and histology (figure 8a). Non-encapsulated µpellets could not be located following implantation and thus could not be analysed. Between the two *in vitro* priming times, 7 or 28 days, there was significantly more calcified tissue present in conditions primed for 28 days *in vitro* as shown in the µCT (figure 8 b, c). Fibrin encapsulation of pellets lead to greater calcification *in vivo* than standard pellets for all donors cultured for 7 days (figure 8c) however fibrin encapsulation does not appear to affect calcification when constructs are cultured for 28 days. Pellets that were not encapsulated in fibrin were only retrievable from 1 donor after implantation following 7 days of *in vitro* culture, and for this donor only 1 pellet was often retrieved of the 3 implanted. Regardless of the donor 7 day primed pellet-fibrin and µpellet-fibrin constructs were always retrieved and always showed calcification on µCT after *in vivo* implantation (figure 8b, c). 28 primed pellets, pellet-fibrin and µpellet-fibrin constructs were consistently retrieved from donors 1, 2 and 3 and always showed comparable calcification between donors (figure 8b, c). In some donors as shown in the µCT image (figure 8) pellets or µpellets had merged forming one larger construct. This was observed within some fibrin encapsulated samples only and was not observed consistently with a specific donor, priming time or condition.

Bone formation *in vivo* is dependent on extracellular matrix quality pre-implantation

Bone and calcified cartilage were observed in all harvested samples. Calcified tissue was quantified by µCT (figure 8), however non-encapsulated pellets primed *in vitro* for 7 days could only be retrieved from donor 1 and the quantity of bone and calcified cartilage observed at the time of harvest in H&E sections varied between donors. Donor 1 had a denser GAG rich matrix pre-implantation and higher COLII and COLX expression/production (figure 2, 4, 5, 6) which resulted in an almost complete absence of bone marrow and only a small recruitment of matrix remodelling osteoclasts after 8 weeks *in vivo* (figure 9-11) compared to donor 2 and 3 that had a less GAG rich matrix pre-implantation and lower COLII and COLX expression/production (figure 2, 4, 5, 6). This resulted in a greater percentage of bone marrow elements and greater osteoclast recruitment after *in vivo* implantation for the same time (figure 9, 10, 12, 13). These differences are observed most clearly in the histological sections as it was not possible to segment out calcified cartilage from bone in the µCT.

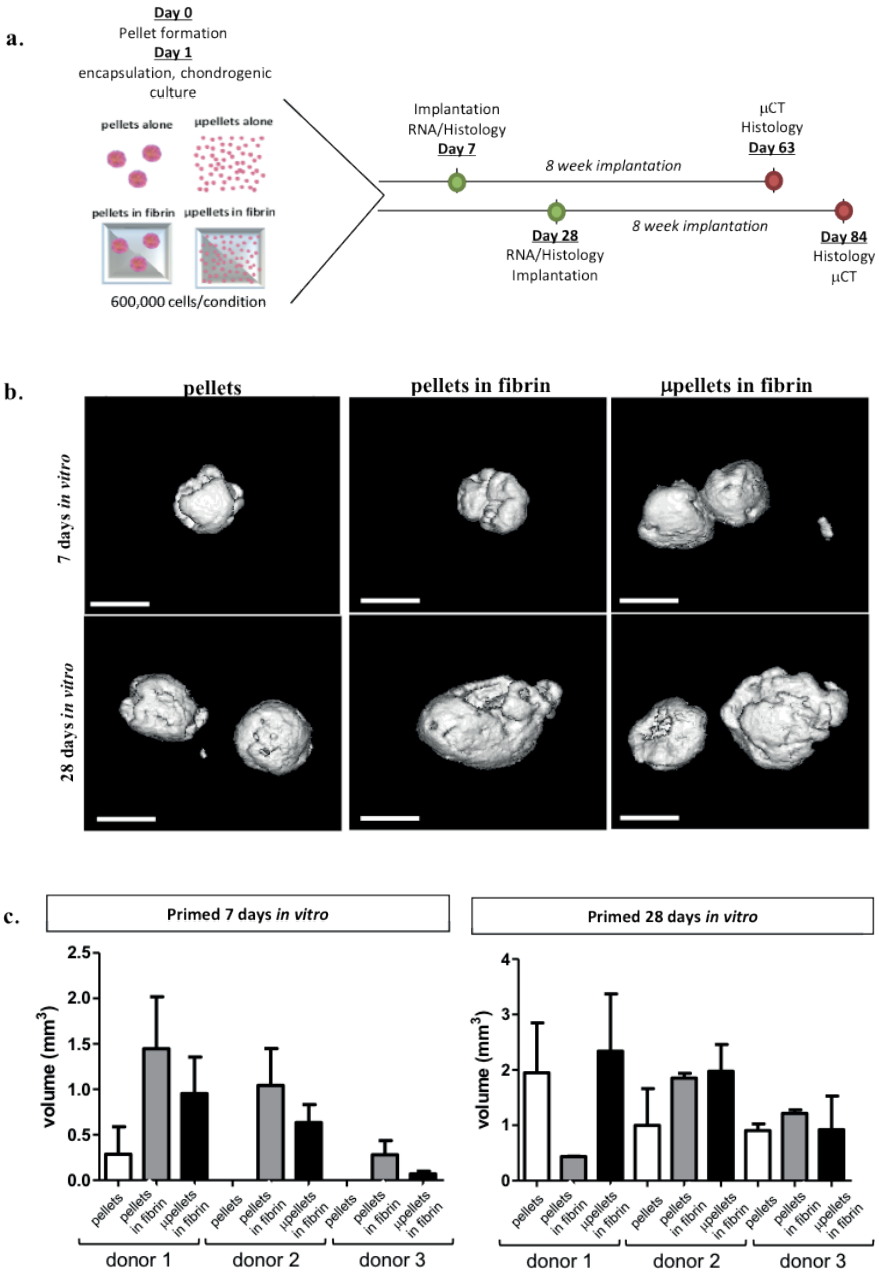

Figure 8: 7 day primed samples and µpellet constructs form mineralised tissue after in vivo implantation (a) In vivo study outline **(b)** *µCT scans of constructs primed in vitro for 7 or 28 days after 8 weeks in vivo implantation. (Donor 1; white bar indicates same length in all µCT images for scale).* **(c)** *Volume of mineralised tissue obtained from ex vivo µCT scans shows donor related differences in mineralisation.*

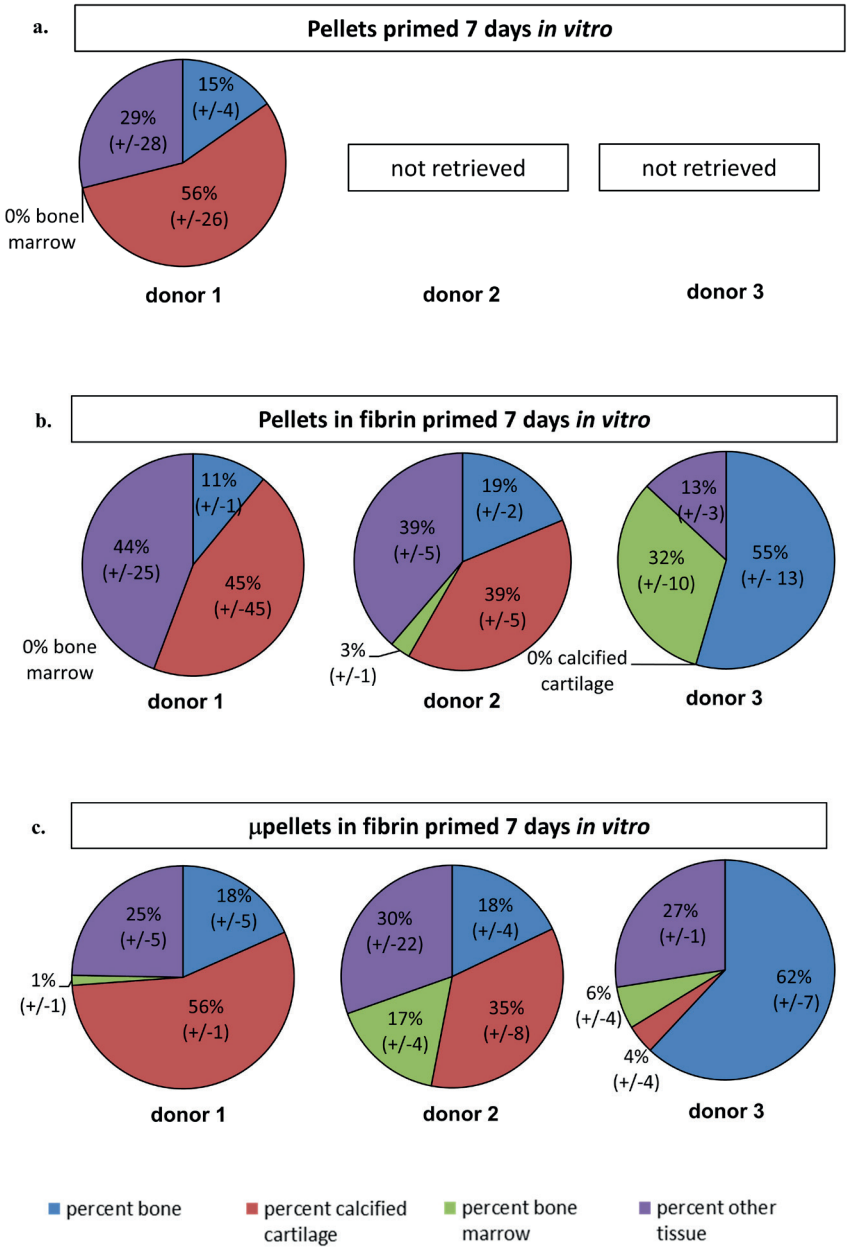

Figure 9: Quantity of bone and bone marrow following 7 days of in vitro priming is donor dependent. Percentage of bone, bone marrow, calcified cartilage and other tissue (not bone, bone marrow, or calcified cartilage) found in samples primed in vitro for 7 days following 8 weeks in vivo. Quantification for ***(a)*** *pellets,* ***(b)*** *pellet-fibrin and* ***(c)*** *µpellet-fibrin (3 constructs per condition per donor; 3 MSC donors used) was completed using high magnification images of H&E staining and segmenting areas based on morphology in ImageJ.*

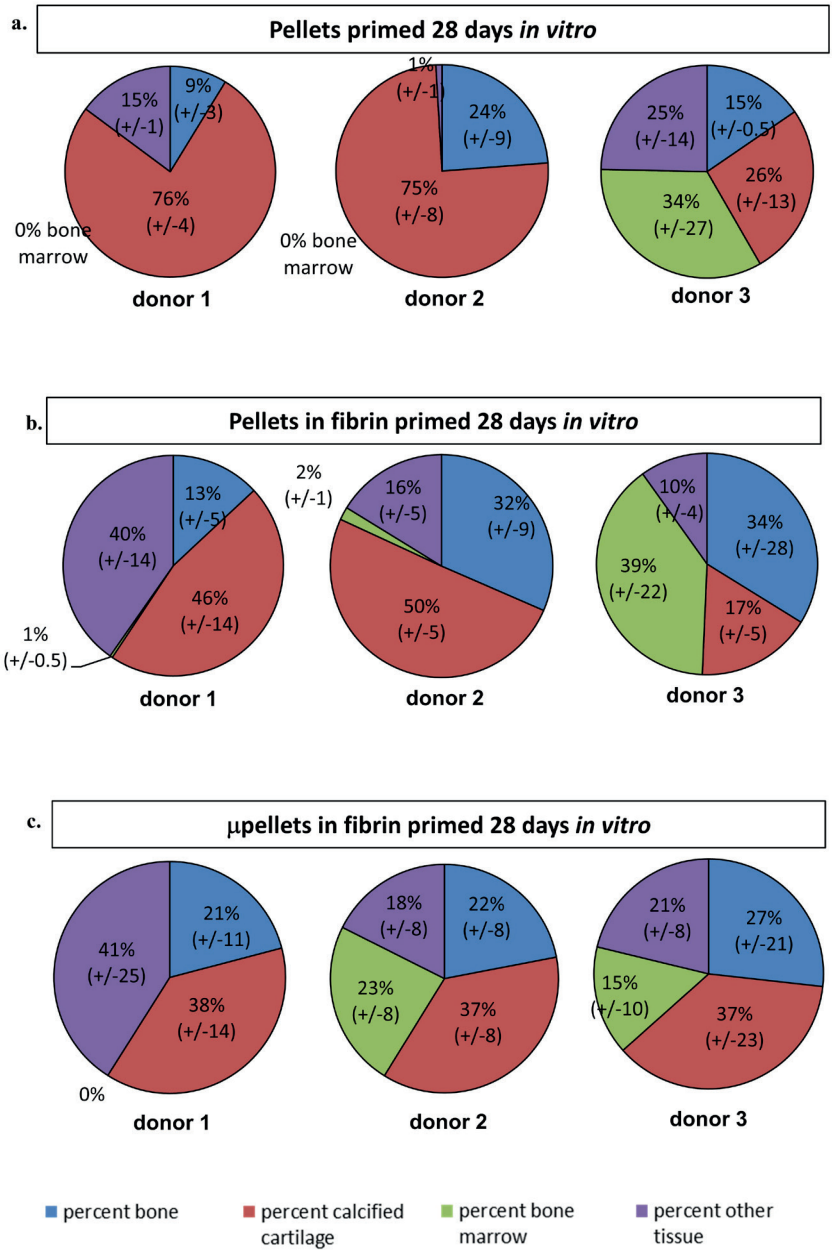

Figure 10: Quantity of bone and bone marrow following 7 days of in vitro priming is donor dependent. Percentage of bone, bone marrow, calcified cartilage and other tissue (not bone, bone marrow, or calcified cartilage) found in samples primed in vitro for 28 days following 8 weeks in vivo. Quantification for (a) pellets, (b) pellet-fibrin and (c) µpellet-fibrin (3 constructs per condition per donor; 3 MSC donors used) was completed using high magnification images of H&E staining and segmenting areas based on morphology in ImageJ.

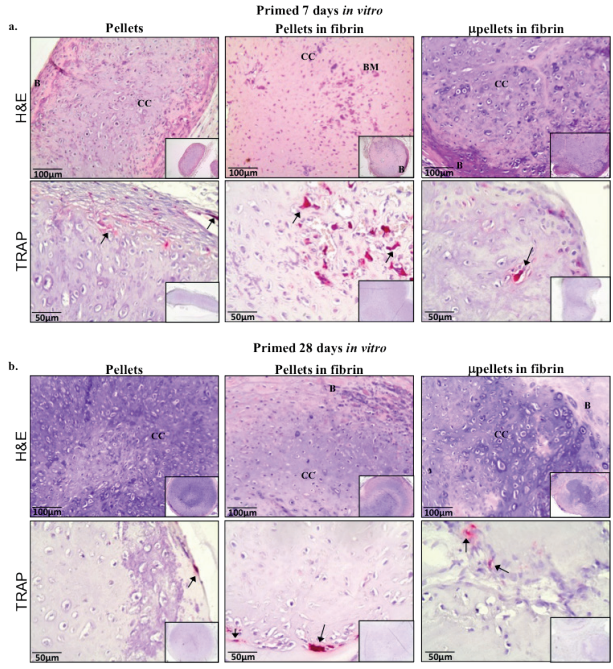

Figure 11: High GAG density in vitro results in slower remodeling and bone formation in vivo. (a) Day 7 and (b) 28 in vivo H&E and TRAP staining for donor 1. The intensity of the safranin O staining (figure 2) showed the quantity of GAGs is high pre-implantation. Denser GAG-rich matrices appear to be harder for cells to penetrate as evident by a decrease in marrow formation and osteoclast infiltration. (In H&E stains CC = calcified cartilage, BM = bone marrow, B = bone marrow; in TRAP staining arrows indicate areas of TRAP positivity).

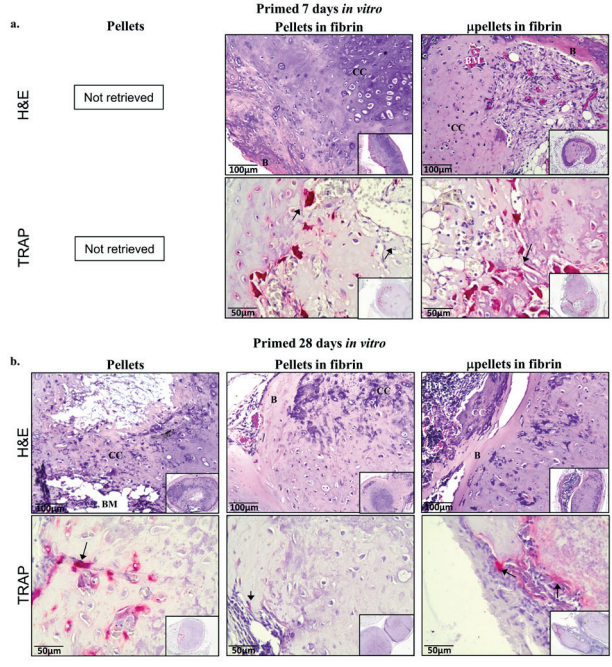

Figure 12: GAG density in vitro affects the rate of remodeling and bone formation in vivo. (a) Day 7 and (b) 28 in vivo H&E and TRAP staining for donor 2. The intensity of the safranin O staining (figure 2) showed a moderate quantity of GAGs pre-implantation. In vivo there was a varying degree of calcification, marrow space development and osteoclast infiltration which appeared correlated to in vitro GAG deposition pre-implantation. (In H&E stains CC = calcified cartilage, BM = bone marrow, B = bone marrow; in TRAP staining arrows indicate areas of TRAP positivity).

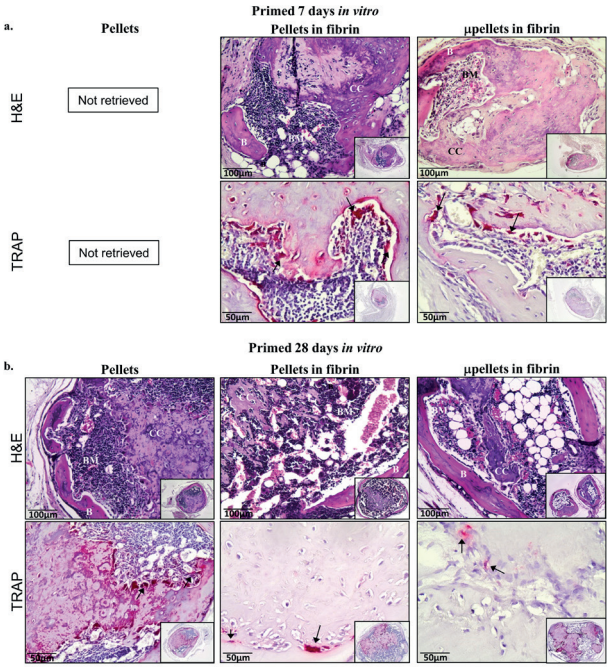

Figure 13: Lower GAG density in vitro results accelerated remodeling and bone formation in vivo. (a) Day 7 and (b) 28 in vivo H&E and TRAP staining for donor 3. The intensity of the safranin O staining (figure 2) showed the quantity of GAGs is low pre-implantation. Less dense GAG-rich matrices appear to be easier for cells to penetrate as evident by a increased in marrow formation and osteoclast infiltration. (In H&E stains CC =calcified cartilage, BM = bone marrow, B = bone marrow; in TRAP staining arrows indicate areas of TRAP positivity).

DISCUSSION

Priming time and reproducible bone formation represent two of the major hurdles for translation of tissue engineering based approaches to the clinic. In this manuscript we demonstrate that 7 day chondrogenic priming coupled with encapsulation in a fibrin gel is sufficient to allow bone formation to take place *in vivo*. Following 7 days of *in vitro* priming pellets reproducibly produced a more chondrogenic GAG rich matrix from the earlier priming times. At this time point hypertrophic markers were also consistently upregulated from the original starting time point. However, without encapsulation only one donor was observed to calcify *in vivo* after being primed for 7 days *in vitro*. This donor produced no bone marrow elements. Given the lack of bone formation the chondrogenic markers we chose are most likely insufficient predictors of *in vivo* bone formation.

Novel µpellet-fibrin constructs were created and characterised to ensure downscaling the standard pellet culture to a µpellet format would not inhibit chondrogenesis and subsequent bone formation. We observed that µpellet-fibrin constructs showed equivalent matrix production and bone formation compared to standard pellet culture counterparts. In a recent predictive model it was shown that matrix deposition and collagen distribution tends to accumulate around the outer periphery of pellets where nutrient supply is

abundant (27). They predict that uniform distribution of proteoglycans and collagens could be maintained be decreasing pellet size which would make nutrients and factors more available to individual differentiating cells. This could indeed explain why we saw an increase in some matrix related genes in µpellet constructs. Decreased VEGF gene expression after 7 days of culture in µpellet-fibrin constructs compared to standard pellets was also observed, but this decrease was not observed after 28 days of *in vitro* priming and did not appear to hinder *in vivo* bone forming capacity in µpellet-fibrin constructs.

In vivo results confirmed bone formation can be achieved by chondrogenically priming constructs for only 7 days *in vitro* using either standard or µpellet-fibrin constructs. Non-encapsulated µpellet constructs failed to differentiate in all donors and time points except for non-encapsulated µpellets cultured for 28 days from donor 3. These non-encapsulated µpellets aggregated forming one pellet after 28 days of culture. This aggregated pellet and all other non-encapsulated µpellets that were implanted did not result in bone formation indicating that µpellets alone and aggregated µpellets are not capable of bone formation. It appears that µpellets require encapsulation during *in vitro* differentiation or during implantation in order for bone formation to be achieved. We observed that fibrin encapsulation increased bone formation when priming time was reduced to 7 days in subcutaneously implanted pellet constructs. 7 day non-fibrin encapsulated pellets were rarely retrieved (one in three donors) and all retrieved pellets lacked bone marrow elements. Given the lack of bone formation it is probable that other chondrogenic markers may more accurately predict *in vivo* bone formation than the ones we had selected. It is possible that pellets that did not calcify or form bone were simply absorbed by the host or perhaps the addition of fibrin prevented this absorption long enough for remodelling and bone formation to take place.

Often fibrin encapsulated samples exhibited fusion *in vivo*, resulting in a fused mass of pellets/µpellets versus individual pellets on the µCT. This fusion was not consistently observed between conditions or donors but was only observed in fibrin encapsulated conditions. It could be the fibrin contracted bringing the pellets in closer proximity allowing for this fusion to take place. Fibrin has been shown to reduce oxygen diffusion and tension, leading to a hypoxic environment (28). This hypoxic environment is favourable for bone formation and has been shown to increase osteoclast and osteoblast activity.This may explain how fibrin was able to enhance bone formation *in vivo* (29-31). However in a prior pilot study, pellets primed *in vitro* for 7 days in the absence of fibrin did form bone containing mature marrow cavities (data not shown). This highlights a degree of variability in graft performance based on inherent donor differences which have been previously reported (1, 21). Indeed in this study we observed major differences between donors influenced the degree of bone formation observed following implantation.

In our study we observed a difference in chondrogenic differentiation and subsequent bone formation between all three donors. Samples retrieved from donor 1 often lacked

bone marrow elements following implantation. *In vitro* analysis from donor 1 showed a decrease in both VEGF, and COLX gene expression compared to the other two donors. VEGF is known to be involved in vascularisation which means this decreased expression of VEGF could have attributed to the lack of bone marrow elements we observed following *in vivo* implantation (32). Additionally, donor 1 showed greater COLII expression and GAG accumulation as well as decreased COLX gene expression compared to donors 2 and 3. Histologically we observed that GAG and other matrix collagens production pre-implantation appeared to be related to bone formation *in vivo*. A denser matrix containing more collagen elements and GAGs resulted in less mature bone formation, more incomplete remodelled calcified cartilage and a decrease in the presence of matrix remodelling osteoclasts. These differences are best observed in the H&E stained sections, as the µCT quantifies the entire area of calcified tissues and would not be able to show quantities of bone versus calcified cartilage. We hypothesise that denser matrices takes longer to be remodelled *in vivo*, as vessel and cell invasion is more difficult in a dense environment which results in delayed bone formation, whereas a less dense matrix can be invaded more easily, leading to faster vascularisation and remodelling, thus resulting in quicker bone formation. It has previously been shown that GAG depletion of articular cartilage leads to an increase in blood vessel invasion which contributes to osteoarthritis and bone development in diseased cartilage, which supports our hypothesis (33, 34). However, we also hypothesise that a minimum level matrix production is necessary for bone formation to occur and to prevent pellets from being resorbed or lost *in vivo* as evidenced by the fact that 7 day primed pellets that had extremely low levels of GAG production pre-implantation were unable to be retrieved (donor 2 & 3). It is difficult to say that variation in matrix elements alone attributed to the difference we observed in bone formation. In order to properly investigate this a larger number of donors needs to be investigated which we did not have available to us, however, it does present an interesting area of research for future studies.

It is also possible the model used here could have hindered or slowed matrix remodelling of more dense matrices as T-lymphocytes are known to degrade GAGs *in vivo* and were absent from our model (35). Had these T-lymphocytes been present faster bone formation from samples which had a more GAG rich matrix may have been observed, however this would require an immunocompetent model in order to verify. We have used immunocompromised mice, lacking functioning T and B cells, a model which is often employed in ectopic bone formation studies (36, 37). Our group has previously shown that it is possible to take chondrogenically primed MSCs from rats and implant them into immunocompetent animals to achieve bone formation (20). It has not been studied yet whether human MSCs would make bone in an immunocompetent animal. As we implant human MSC pellets in a mouse environment we utilised an immunodeficient mouse model to avoid xenographic rejection. This study was a proof of principle study in order

to characterise and determine the de novo bone forming potential of the µpellet-fibrin constructs we implanted our constructs in a subcutaneous environment which we felt was the best environment to determine this. We chose to do this instead of a large bone defect as we were able to better adhere to the 3R principle (38); safely implanting more conditions per animal allowing for fewer animals with minimal discomfort to be used to complete our study. Subcutaneous implantation of our relatively small constructs did not hinder their movement or range of motion. Constructs were not in an environment where bone forms naturally and this may have limited the bone formation and remodelling we observed within our constructs. Future studies which investigate these constructs in a defect environment will be useful.

µpellet-fibrin constructs were found to have equivalent calcification volumes (as assessed by µCT) after *in vivo* implantation when compared to pellets cultured for the same time. These volumes even after 28 days of culture pre-implanation are still far from sufficient to properly heal a large bone defect. As we have not studied how these constructs perform in a large defect we cannot say how they would calcify and integrate with the host bone tissue. Furthermore the inherent donor differences we observed meant that although samples were primed for the same time, the ultimate bone formation varied after implantation. This donor variation is an area of active research and by furthering our understanding of what is crucial for endochondral bone formation to take place *in vivo* in response to certain *in vitro* produced ques is essential for this research to move forward. In addition there is often a lack of bridging between standard pellets which needs to be addressed for proper integration of such constructs with host tissue. Scale-up approaches are still an area of active research in this field and in the future we hope to investigate these µpellets using bioactive injectable matrices in large bone defects to increase bone formation. Additionally, understanding the relationship between matrix composition pre-implantation and the subsequent effect on bone formation *in vivo* is important and merits further investigation, as determining an optimal set of criteria for chondrogenesis pre-implantation to achieve bone formation *in vivo* will improve future research. This is also important because identifying such criteria would result in more reproducible outcomes which is crucial for the translation of such constructs to the clinic. Still we find the bone forming potential of these novel µpellet constructs to be promising. We believe with further optimisation of *in vitro* culture we could optimise these constructs as an injectable bone construct which would be advantageous to treat bone defects.

CONCLUSIONS

This study has shown it is possible to greatly decrease *in vitro* culturing for pellet and µpellet constructs and still retrieve bone after implantation. With further optimisation

of culture conditions and material parameters µpellets could be offer a promising alternative to current clinical treatment options for bone defects. We also believe µpellets, due to their small size, could be easily optimised as an injectable therapy using either a thermoreversable gel or injectable fibrin-based material allowing for the creation of a customisable void filling bone substitute. Given the shorter priming time required coupled with injectability, this approach could offer promise for a minimally invasive therapy to replace some autologous bone transplantation procedures.

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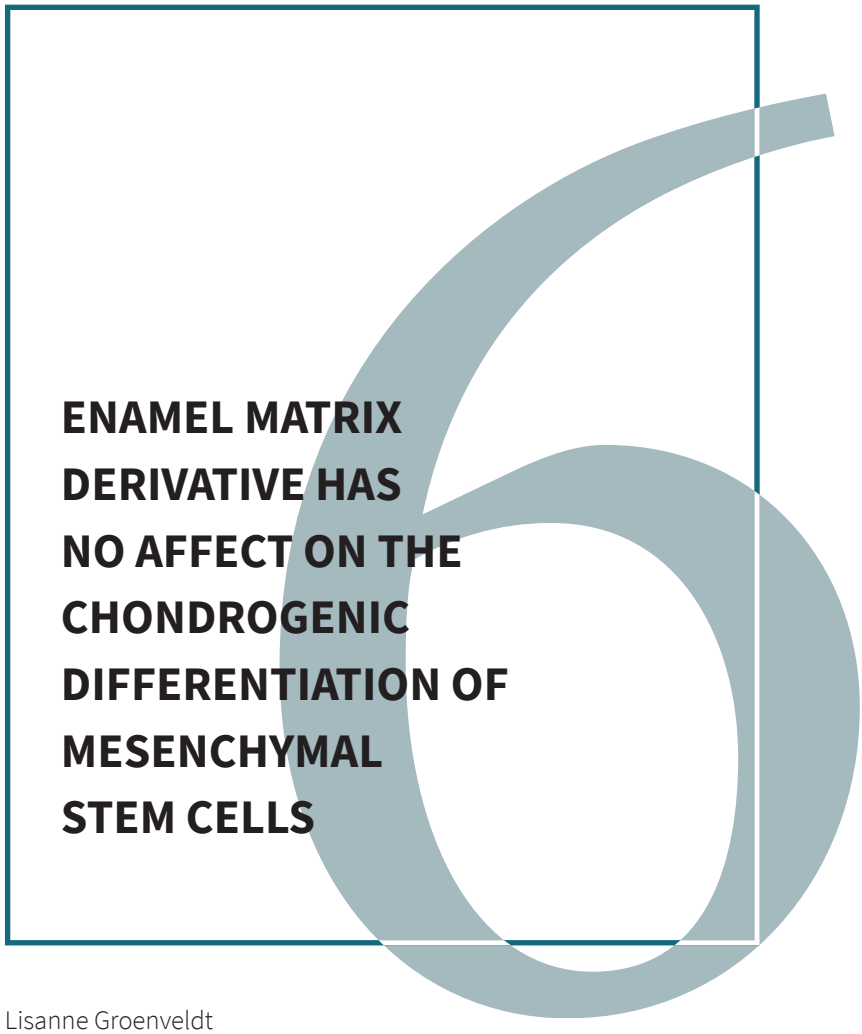
References

1. Amini, A.R., C.T. Laurencin, and S.P. Nukavarapu, Bone tissue engineering: recent advances and challenges. Crit Rev Biomed Eng, 2012. 40(5): p. 363-408.
2. Damien, C.J. and J.R. Parsons, Bone graft and bone graft substitutes: a review of current technology and applications. J Appl Biomater, 1991. 2(3): p. 187-208.
3. Gawlitta, D., et al., Modulating endochondral ossification of multipotent stromal cells for bone regeneration. Tissue Eng Part B Rev, 2010. 16(4): p. 385-95.
4. Dimitriou, R., E. Tsiridis, and P.V. Giannoudis, Current concepts of molecular aspects of bone healing. Injury, 2005. 36(12): p. 1392-1404.
5. Badylak, S.F., Regenerative medicine and developmental biology: The role of the extracellular matrix. The Anatomical Record Part B: The New Anatomist, 2005. 287B(1): p. 36-41.
6. Tortelli, F., et al., The development of tissue-engineered bone of different origin through endochondral and intramembranous ossification following the implantation of mesenchymal stem cells and osteoblasts in a murine model. Biomaterials, 2010. 31(2): p. 242-9.
7. Olsen, B.R., A.M. Reginato, and W. Wang, BONE DEVELOPMENT. Annual Review of Cell and Developmental Biology, 2000. 16(1): p. 191-220.
8. Huang, J.I., et al., Lunate arthroplasty with autologous mesenchymal stem cells in a rabbit model. J Bone Joint Surg Am, 2006. 88(4): p. 744-52.
9. Bahney, C.S., et al., Stem cell–derived endochondral cartilage stimulates bone healing by tissue transformation. Journal of Bone and Mineral Research, 2014. 29(5): p. 1269-1282.
10. Sheehy, E.J., et al., Engineering osteochondral constructs through spatial regulation of endochondral ossification. Acta Biomaterialia, 2013. 9(3): p. 5484-5492.
11. Bianco, P., et al., Bone formation via cartilage models: The “borderline” chondrocyte. Matrix Biology, 1998. 17(3): p. 185-192.
12. Maes, C., et al., Osteoblast precursors, but not mature osteoblasts, move into developing and fractured bones along with invading blood vessels. Dev Cell, 2010. 19(2): p. 329-44.
13. Kronenberg, H.M., Developmental regulation of the growth plate. 2003. 423(6937): p. 332-336.
14. Ortega, N., D.J. Behonick, and Z. Werb, Matrix remodeling during endochondral ossification. Trends in cell biology, 2004. 14(2): p. 86-93.
15. Street, J., et al., Vascular endothelial growth factor stimulates bone repair by promoting angiogenesis and bone turnover. Proceedings of the National Academy of Sciences, 2002. 99(15): p. 9656-9661.
16. Nagai, H. and M. Aoki, Inhibition of growth plate angiogenesis and endochondral ossification with diminished expression of MMP-13 in hypertrophic chondrocytes in FGF-2-treated rats. Journal of Bone and Mineral Metabolism, 2002. 20(3): p. 142-147.
17. Boyan, B.D., Z. Schwartz, and L.D. Swain, In Vitro Studies on the Regulation of Endochondral Ossification by Vitamin D. Critical Reviews in Oral Biology & Medicine, 1992. 3(1): p. 15-30.

18. Chen, G., C. Deng, and Y.-P. Li, TGF-β and BMP Signaling in Osteoblast Differentiation and Bone Formation. International Journal of Biological Sciences, 2012. 8(2): p. 272-288.
19. Gerber HP, V.T., Ryan AM, Kowalski J, Werb Z, Ferrara N., VEGF couples hypertrophic cartilage remodeling, ossification and angiogenesis during endochondral bone formation. 1999.
20. Farrell, E., et al., In-vivo generation of bone via endochondral ossification by in-vitro chondrogenic priming of adult human and rat mesenchymal stem cells. BMC Musculoskeletal Disorders, 2011. 12: p. 31-31.
21. van der Stok, J., et al. Chondrogenically differentiated mesenchymal stromal cell pellets stimulate endochondral bone regeneration in critical-sized bone defects. European cells & materials, 2014. 27, 137-48; discussion 148.
22. Yang, W., et al., Effects of in vitro chondrogenic priming time of bone-marrow-derived mesenchymal stromal cells on in vivo endochondral bone formation. Acta Biomater, 2014.
23. Yang, W., et al., Effects of in vitro chondrogenic priming time of bone-marrow-derived mesenchymal stromal cells on in vivo endochondral bone formation. Acta Biomaterialia, 2015. 13: p. 254-265.
24. Scotti, C., et al., Recapitulation of endochondral bone formation using human adult mesenchymal stem cells as a paradigm for developmental engineering. Proceedings of the National Academy of Sciences, 2010. 107(16): p. 7251-7256.
25. Osinga, R., et al., Generation of a Bone Organ by Human Adipose-Derived Stromal Cells Through Endochondral Ossification. STEM CELLS Translational Medicine, 2016. 5(8): p. 1090-1097.
26. Hellingman, C.A., et al., Fibroblast growth factor receptors in in vitro and in vivo chondrogenesis: relating tissue engineering using adult mesenchymal stem cells to embryonic development. Tissue Eng Part A, 2010. 16(2): p. 545-56.
27. Lewis, M.C., et al., Extracellular Matrix Deposition in Engineered Micromass Cartilage Pellet Cultures: Measurements and Modelling. PLoS ONE, 2016. 11(2): p. e0147302.
28. Demol, J., et al., Towards a quantitative understanding of oxygen tension and cell density evolution in fibrin hydrogels. Biomaterials, 2011. 32(1): p. 107-118.
29. Arnett, T.R., et al., Hypoxia is a major stimulator of osteoclast formation and bone resorption. Journal of cellular physiology, 2003. 196(1): p. 2-8.
30. Hung, S.-P., et al., Hypoxia promotes proliferation and osteogenic differentiation potentials of human mesenchymal stem cells. Journal of Orthopaedic Research, 2012. 30(2): p. 260-266.
31. Steinbrech, D.S., et al., Hypoxia Regulates VEGF Expression and Cellular Proliferation by Osteoblasts in Vitro. Plastic and Reconstructive Surgery, 1999. 104(3).
32. Yang, Y.-Q., et al., The role of vascular endothelial growth factor in ossification. International Journal of Oral Science, 2012. 4(2): p. 64-68.
33. Akkiraju, H. and A. Nohe, Role of Chondrocytes in Cartilage Formation, Progression of Osteoarthritis and Cartilage Regeneration. Journal of developmental biology, 2015. 3(4): p. 177-192.

34. Connelly, J.T., C.G. Wilson, and M.E. Levenston, Characterization of Proteoglycan Production and Processing by Chondrocytes and BMSCs in Tissue Engineered Constructs. Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society, 2008. 16(9): p. 1092-1100.
35. Savion, N., I. Vlodavsky, and Z. Fuks, Interaction of T lymphocytes and macrophages with cultured vascular endothelial cells: Attachment, invasion, and subsequent degradation of the subendothelial extracellular matrix. Journal of Cellular Physiology, 1984. 118(2): p. 169-178.
36. Scotti, C., et al., Engineering of a functional bone organ through endochondral ossification. Proceedings of the National Academy of Sciences, 2013. 110(10): p. 3997-4002.
37. Jukes, J.M., et al., Endochondral bone tissue engineering using embryonic stem cells. Proceedings of the National Academy of Sciences of the United States of America, 2008. 105(19): p. 6840-6845.
38. Kilkenny, C., et al., Animal research: reporting in vivo experiments—the ARRIVE guidelines. 2011, SAGE Publications Sage UK: London, England.

Frontiers in bioengineering and biotechnology. Sep. 2014. 2:29



6

ENAMEL MATRIX DERIVATIVE HAS NO AFFECT ON THE CHONDROGENIC DIFFERENTIATION OF MESENCHYMAL STEM CELLS

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Eric Farrell

Abstract

Treatment of large bone defects due to trauma, tumor resection, or congenital abnormalities is challenging. Bone tissue engineering using mesenchymal stem cells (MSCs) represents a promising treatment option. However, the quantity and quality of engineered bone tissue are not sufficient to fill large bone defects. The aim of this study was to determine if the addition of enamel matrix derivative (EMD) improves *in vitro* chondrogenic priming of MSCs to ultimately improve *in vivo* MSC mediated endochondral bone formation. MSCs were chondrogenically differentiated in medium supplemented with TGFβ3 in the absence or presence of 1, 10, or 100 µg/mL EMD. Samples were analyzed for gene expression, glycoaminoglycan (GAG) production, and histologically. Osteogenic and adipogenic differentiation capacity were also assessed. The addition of EMD did not negatively effect chondrogenic differentiation of adult human MSCs. EMD did not appear to alter GAG production or expression of chondrogenic genes. Osteogenic and adipogenic differentiation were likewise unaffected though a trend toward decreased adipogenic gene expression was observed. EMD does not effect chondrogenic differentiation of adult human MSCs. As such the use of EMD in combination with chondrogenically primed MSCs for periodontal bone tissue repair is unlikely to have negative effects on MSC differentiation.

INTRODUCTION

Trauma, tumor resection or congenital abnormalities can result in large bone defects. Treatment options for such defects include the use of autologous bone grafts, allogenic bone or other substitute material (1, 2). Autologous bone is preferred clinically; however, harvesting of material can result in secondary site morbidity and an increased risk of infection (1). In addition, tissue availability is limited, increasing the demand for an alternative graft substitute (3).

Tissue engineering represents a promising alternative treatment option for bone defects. Mesenchymal stem cells (MSCs), available from sources including bone marrow and adipose tissue, are multipotent cells that can be differentiated towards the osteogenic and chondrogenic lineages (10) making them an attractive cell source for bone tissue engineered constructs.

Multiple approaches have been taken to improve osteogenic differentiation of MSCs, mimicking the process of intramembranous ossification, including manipulating growth factors, scaffolds, and environmental parameters (e.g., oxygen and pressure) (5). Unfortunately, due to insufficient vascularisation of the MSC based implant necrosis, improper nutrient delivery, and inadequate waste removal occur, ultimately resulting in graft failure.

Bone tissue constructs modeled after the process of endochondral ossification (EO) may result in more promising outcomes as the initial tissue (cartilage) would be better suited to survive the initial avascular implantation site. During EO, cartilage is formed by chondrogenic differentiation of MSCs *in vitro*. Since chondrocytes reside in an avascular environment, they can survive the initial hypoxic insult following implantation (6). As the chondrocytes mature, become hypertrophic and apoptose, blood vessels invade and the cartilage rich matrix is mineralised ultimately serves as a template for future bone development. Several groups have produced promising results using MSCs guided bone formation along the process of EO *in vivo* (7-13). Van der stok et al. demonstrated the ability of such constructs to repair a long bone defect in recent proof of principle studies (13). Despite the promise of this approach, the resulting bone is often not sufficient to fill large clinically relevant defects, indicating a need to improve current techniques to optimize bone formation. Many researchers have investigated combining MSC with clinically relevant compounds to improve *in vivo* bone formation.

Enamel matrix derivative (EMD) is an extracellular matrix derivative obtained from porcine tooth buds. It contains amelogenin and proteins that belong to the amelogenin

family (>90%) (14). Sold commercially as Emdogain, EMD in a single dose syringe dissolved in propylene glycol alginate. Emdogain is used clinically to stimulate the regeneration of periodontal tissues. Combining EMD with surgical periodontal therapy (surgical therapy of the tissue surrounding or encasing teeth) of deep intrabony defects lead to improved clinical parameters compared to surgical therapy alone (2). Studies have shown that EMD stimulates the proliferation and osteogenic differentiation of MSCs (14-17). However, many groups used only specific proteins that are included in EMD or based their results on cells obtained from animals or cell-lines. Narukawa et al. found a stimulatory effect of Emdogain on the expression of chondrogenesis related transcription factors in chondrogenically primed MSCs. Utilizing a chondrogenic cell line, the group observed an increase in glycosaminoglycans (GAGs) quantity in the extracellular matrix (15, 18). EMD was also shown to increase the proliferation of early chondrocytes derived from rats and inhibited maturation. In mature chondrocytes, EMD enhanced proliferation and did not inhibited differentiation (19). Due to its clinical relevance and previous evidence suggesting an effect of EMD on chondrogenesis EMD was hypothesized to improve *in vitro* chondrogenic priming of human MSCs. These chondrogenically primed human MSCs could be utilised as a tissue engineered MSC based endochondral bone graft. The aim of this research was to determine if EMD enhanced chondrogenesis in human MSCs and to determine if EMD improves the quantity and quality of the chondrogenic matrix production. In order to compare with previous research, we also assessed the osteogenic capacity of MSCs in the presence of varying doses of MSCs as well as their adipogenic differentiation capability.

MATERIALS AND METHODS

EMD Enamel matrix derivative was supplied as a freeze dried preparation by Straumann Company. It was reconstituted in 50 mM acetic acid to 10 mg/mL and further diluted to the working concentrations below in the appropriate culture medium.

Mesenchymal stem cell isolation

MSCs were isolated from three human bone marrow samples aspirated from the greater trochanter major from patients undergoing total hip arthroplasty, after informed consent (METC 2004-142) from two females (aged 20 and 60) and one male (aged 54). MSCs showed similar growth and differentiation characteristics. MSCs were maintained in expansion medium (α-mem (Gibco) containing 10% FCS (Lonza), supplemented with 1 ng/mL FGF2, 25 µg/mL ascorbic acid) at 37°C and 5% CO_2 as described previously (20).

Adipogenic differentiation

MSCs were cultured in 12-well plates at a density of $2.1x10^4$ cells/cm^2. Cells were cultured for 14 days at 37°C and 5% CO_2 in adipogenic induction medium (DMEM containing 10% FCS, supplemented with 1 µM dexamethasone, 0.2 mM indo-methacin, 0.01 mg/mL insulin, 0.5 mM 3-isobutyl-l-methyl-xanthine (Sigma)). EMD treated samples were cultured in 1, 10, or 100 µg/mL EMD or vehicle alone (0.5 mM acetic acid). Medium was replaced twice a week.

Osteogenic differentiation

MSCs were cultured in 12-well plates at a density of $3x10^3$ cells/cm^2. Cells were cultured for 15–19 days at 37°C and 5% CO_2 in osteogenic induction medium (high-glucose DMEM (Invitrogen) with addition of 10% FCS, 50 µg/mL gentamycin (Invitrogen), 1.5 µg/mL fungizone (Invitrogen), 10 mM glycerol 2-phosphate (Sigma), 0.1 µM dexamethasone (Sigma), 0.1 mM ascorbic acid (Sigma)). EMD treated samples were cultured in 1, 10, or 100 µg/mL EMD or vehicle. Medium was replaced twice a week. Samples were harvested at the latest point, prior to detachment of the cells from the surface of the tissue culture plastic, as occurs during osteogenic differentiation in monolayer. This varied from 15–19 days between donors.

Chondrogenic differentiation

MSCs were cultured for 21 or 35 days in pellets of 2.0×10^5 cells in 500 µl chondrogenic medium (high-glucose DMEM supplemented with 50 µg/mL gentamycin (Invitrogen), 1.5 µg/mL fungizone (Invitrogen), 1 mM sodium pyruvate (Invitrogen), 40 µg/mL proline (Sigma), 1:100v/v insulin-transferrinselenium (ITS; BD Biosciences), 10 ng/mL transforming growth factor β1 (R&D Systems), 25 µg/mL ascorbic acid (Sigma), 100 nM dexamethasone (Sigma)). EMD treated samples were cultured in 1, 10, or 100 µg/mL EMD or vehicle alone. Medium was replaced twice a week.

Oil red O staining

Lipid droplets were stained by Oil Red O. Cells in monolayer were washed in 0.9% NaCl and fixed for 1 h in 4% paraformaldehyde. Cells were stained with Oil Red O (0.3% w/v in distilled water; Sigma) for 10–15 min and washed with distilled water. Cells were mounted with Vectamount.

von Kossa staining

Monolayers were washed in 0.9% NaCl, fixed with 4% formaldehyde for 1 h and stained with von Kossa staining. Cells were incubated in 5% silver nitrate and placed on a light box for 15 min. Excess silver nitrate was washed using distilled water and cells were placed on a light box for another 10 min. Cells were washed in distilled water and counterstained with thionine for 5 min. Cells were dehydrated in 70% (10 s), 96% (30 s), and 100% ethanol (2 min) and mounted with Vectamount.

Scaffold seeding

Collagen-GAG scaffolds were cut in 8 mm squares, placed in 6-well plates coated with 2% agarose (LE- analytical grade, Promega). Scaffolds were seeded with 5×10^5 cells in 150 µL culture medium on one side, incubated for 30 min then overturned and seeded again with the same cell number and volume. After another 30 min, the well was filled with 3 mL of culture medium. Constructs were cultured in chondrogenic medium with the addition or absence of 10 ng/mL transforming growth factor-β 1 (TGF-β1) and/or 100 µg/mL EMD. Samples were cultured at 37°C and 5% CO_2.

Gene expression analysis

RNA was isolated from chondrogenic pellets by homogenising samples with a Eppendorf-potter in 350 µL RNAbee (Freund Can Company). Adipogenic and osteogenic primed MSCs cultured in monolayers were harvested as follows: 2-wells were combined in 300 µL RNAbee, samples and stored at -80°C. RNA isolation, cDNA synthesis, and measurement of gene expression levels on 8–15 ng cDNA were performed as described before (200, 201). Primers and probes used for alkaline phosphatase (ALPL), Gamma-carboxyglutamic acid-containing protein (BGLAP), Integrin-binding sialoprotein (IBSP), Collagen type I (COLI), Peroxisome proliferator-activated receptor γ (PPARγ), Fatty acid-binding protein 4 (FABP4), Runt-related transcription factor 2 (RUNX2), Collagen type II (COL II), Collagen type X (COL X), Sex determining region Y-box 9 (SOX 9), and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Biochemical assay

Pellets and scaffolds were digested in 150 µL papaine digestion solution in combination with 150 µL sodium citrate buffer. GAGs were measured and adjusted to the amount of DNA present in each pellet or scaffold as described before using heparin (Leo Pharmaceutical Products BV), RNAse (Ribonuclease type III-A; Sigma), and ethidium bromide (GibcoBR1) (22).

Histology sample preparation

Pellets and scaffolds were fixed in 4% paraformaldehyde for 1 h, embedded in liquid paraffin wax, and cut into 5 µm sections using a microtome (Leica RM2135). Sections were placed onto SuperStar® microscope slides and de-waxed by soaking sequentially in xylene and 100, 96, and 70% ethanol (5 min each).

Histology

GAG formation was determined by 0.1% safranin O staining and cell morphology was determined utilizing H&E staining. Stainings were performed as described previously (23, 24).

Immunohistology

Antigen retrieval was performed using 0.1% pronase and 1% hyaluronidase. Sections were incubated with 1:100 mouse monoclonal antibody against collagen type II and stained by an ALPL substrate as described before (23).

Statistics

Data are presented as mean values±SD. Statistical analysis was carried out using repeated measures ANOVA test followed by Tukey post hoc correction using a statistical software package (Prism 5.00, Graphpad Software). Results were considered statistically significant at $p < 0.05$.

RESULTS

EMD does not effect the osteogenic differentiation capacity of human MSCs

Osteogenic genes ALPL, BGLAP, IBSP, and COL I were analyzed after 15–19 days by real-time PCR. No differences were observed between osteogenic control, vehicle, and the different doses of EMD (p-values respectively 0.1600, 0.2578, 0.6016, and 0.5673; figure 1).

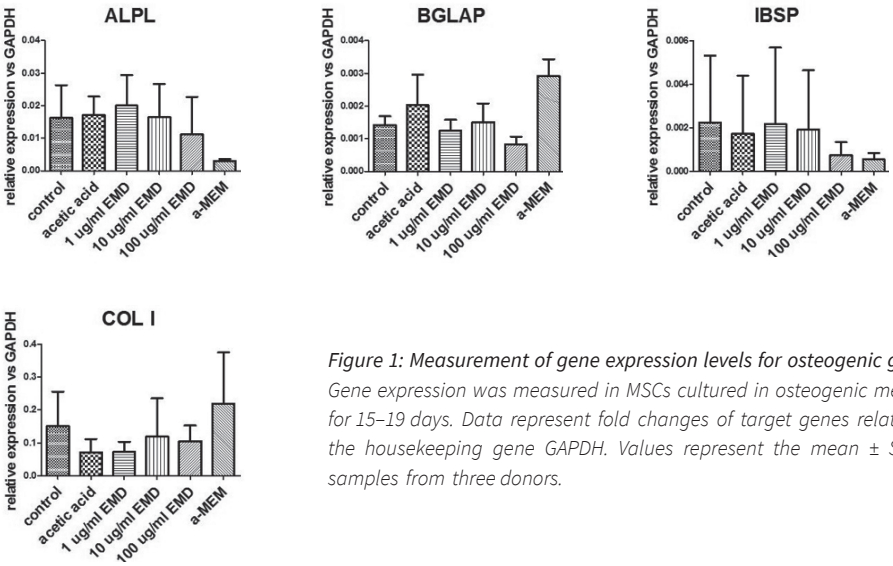

Figure 1: Measurement of gene expression levels for osteogenic genes. Gene expression was measured in MSCs cultured in osteogenic medium for 15–19 days. Data represent fold changes of target genes relative to the housekeeping gene GAPDH. Values represent the mean ± SD for samples from three donors.

Despite inter-donor variability, no differences were observed in the amount of calcium phosphate-nodules formed at the macroscopic level (figure 2). This suggests that EMD had no effect on the osteogenic differentiation of MSCs.

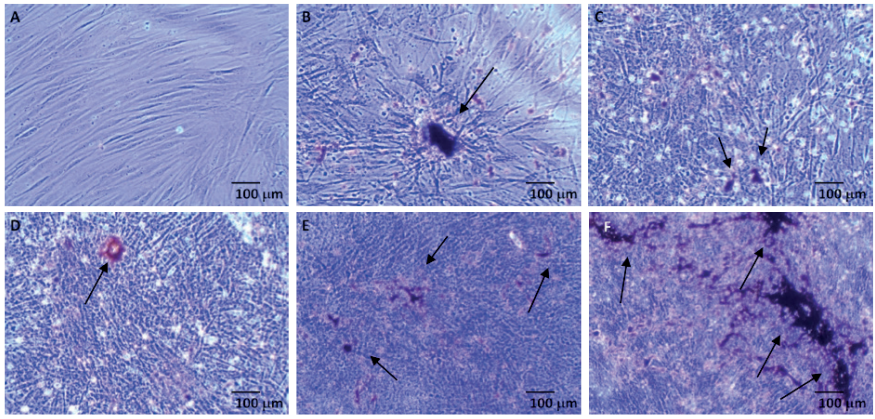

*Figure 2: Evidence of osteogenic differentiation of MSCs exposed to osteogenic factors for 19 days. Images represent von Kossa staining for MSCs of one donor cultured in the non-differentiation medium α-MEM as a negative control **(A)** MSCs cultured in osteogenic differentiation only **(B)**, MSCs cultured in osteogenic differentiation medium in presence of the vehicle **(C)**, and MSCs cultured in osteogenic differentiation medium in presence of 1, 10, or 100 µg/mL EMD **(D–F)**Arrows indicate calcium phosphate-containing nodules.*

EMD has no effect on the adipogenic differentiation of human MSCs

Adipogenic genes, FABP4 and PPARγ, were investigated for three donors by real-time PCR after 14 days to determine the role of EMD on adipogenesis. Cells cultured in the high dose EMD (100 µg/mL) showed a trend toward inhibition of gene expression compared to vehicle and adipogenic control. However, given the large inter-donor variability, this difference was not statistically significant for FABP4 ($p = 0.4835$) or PPARγ ($p = 0.1063$; figure 3).

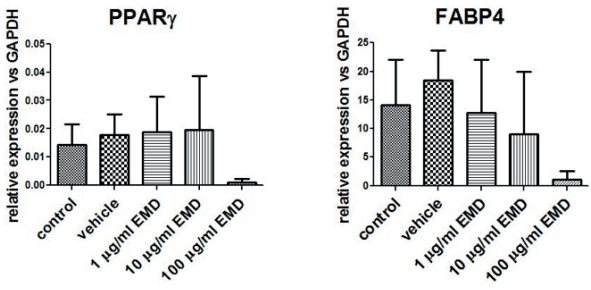

Figure 3: Measurement of gene expression levels for PPARγ and FABP4. Gene expression was measured in MSCs cultured in adipogenic differentiation medium for 14 days with addition of vehicle or different doses of EMD (1, 10, or 100 µg/mL). Data represent fold changes of target genes relative to the housekeeping gene GAPDH. Values represent the mean ± SD for samples from three donors.

The effects of vehicle and EMD on adipogenic differentiation were also assessed by Oil Red O staining of fat-containing droplets. No cells cultured in the expansion medium (used as a negative control) showed evidence of fat-containing droplets (figure 4A). When MSCs were cultured in all other treatment conditions, cells positively stained in all conditions (Figures 4B–F). Staining was slightly reduced in the high dose EMD (100 µg/mL) compared to adipogenic control or vehicle across all donors and wells. This suggests, together with the results for FABP4 and PPARγ, a potentially inhibitory effect of EMD on adipogenic differentiation of MSCs at the highest dose. However, this effect was minimal as determined by staining.

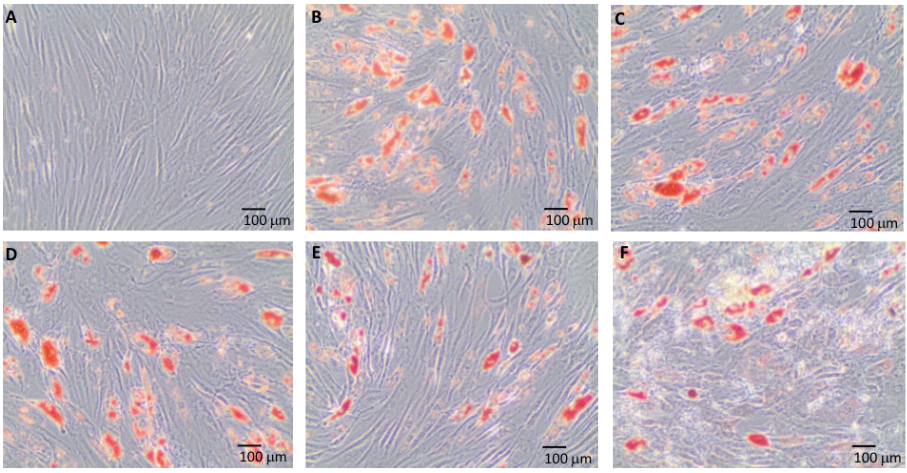

*Figure 4: Oil Red O staining illustrates adipogenic differentiation of MSCs exposed to adipogenic factors for 14 days. Images represent MSCs cultured in the non-differentiation medium α-MEM **(A)**, MSCs cultures in adipogenic differentiation medium only **(B)**, MSCs cultured in adipogenic differentiation medium in addition of vehicle only (0.5 mM acetic acid) **(C)**, and MSCs cultured in adipogenic differentiation medium in addition of 1, 10, or 100 µg/mL EMD **(D–F)**.*

EMD does not effect the chondrogenic differentiation capacity of human MSCs

Chondrogenically primed cell pellets were analyzed by real-time PCR after 21 days for three donors. Four different chondrogenic genes were analyzed; COL II, COL X, SOX 9, and RUNX2 (figure 5). After treatment with vehicle only or EMD, no statistical significant differences in COL II ($p = 0.0538$), COL X ($p = 0.2457$), SOX 9 ($p = 0.7458$), or RUNX2 ($p = 0.5863$) mRNA levels were observed between groups. GAG-production measured in control was approximately 40 µg GAG per microgram DNA. There was no effect of EMD at any concentration on the quantity of GAG production ($p = 0.8989$; figure 5B).

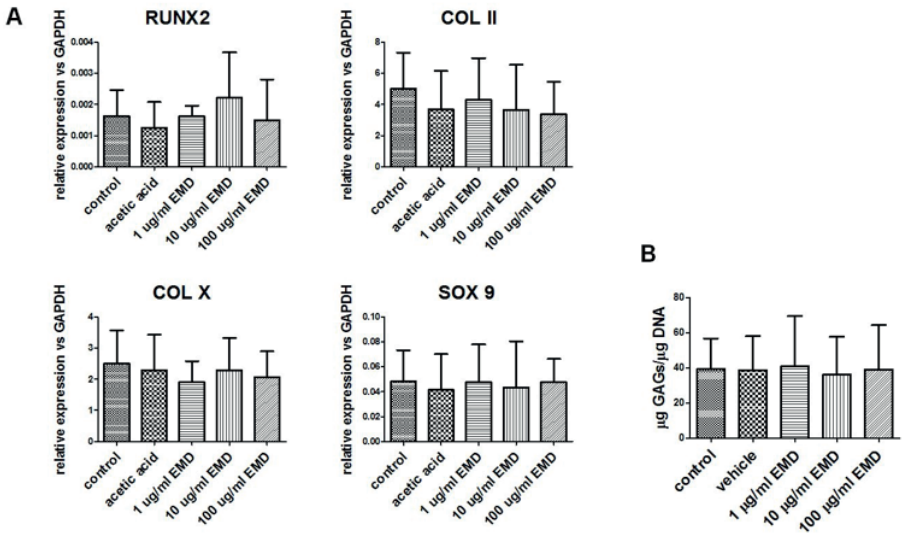

Figure 5: Gene expression levels of chondrogenic genes following differentiation. (A) Gene expression was measured in MSCs cultured in chondrogenic differentiation medium treated with vehicle only or different doses of EMD (1, 10, or 100 µg/mL) for 35 days. Data represent fold changes of target genes relative to the housekeeping gene GAPDH. Values represent the mean ± SD for samples from three donors. (B) Quantification of GAGs in MSCs cultured with chondrogenic factors. Data represent amount of GAGs normalized to DNA content in each pellet

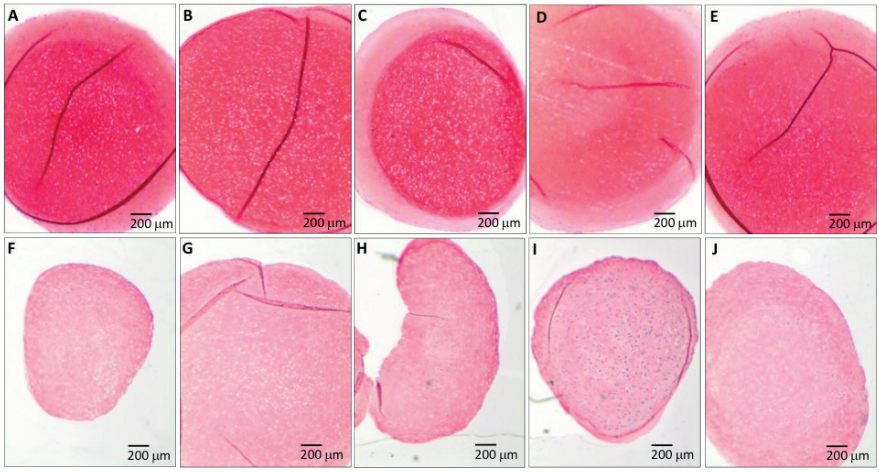

Figure 6: Histological analysis of chondrogenic differentiation of MSCs exposed to chondrogenic factors for 35 days. Images represent MSCs cultured in chondrogenic differentiation medium only (A, F), MSCs cultured in chondrogenic differentiation medium in addition of vehicle only (B, G), and MSCs cultured in chondrogenic differentiation medium in addition of 1 µg/mL (C, H), 10 µg/mL (D, I), or 100 µg/mL EMD (E, J). GAGs were stained by Safranin O (A–E), COL II immunohistochemistry was performed for images (F–J).

Following 35 days of culture in chondrogenic medium, or in the presence of vehicle, or EMD, chondrogenic pellets were stained with safranin O (Figures 6A–E). Immunohistochemical staining for COL II was also performed on these pellets (Figures 6F–J). All pellets demonstrated high quantities of GAGs stained by safranin O and collagen type II. However, no differences in staining were observed between pellets in the chondrogenic control conditions or in the presence of different doses of EMD (1, 10, or 100 µg/mL).

MSCs in 3D culture

In order to assess the effects of EMD on the cell distribution and chondrogenic differentiation in a 3D environment, two collagen-GAG scaffolds were seeded with human MSCs and cultured in the presence or absence of TGFβ1 (10 ng/mL) and/or EMD (100 µg/mL). Hematoxylin and eosin staining demonstrated similar cellular distribution in both conditions (Figures 7A–D). Thionine staining illustrated the presence of GAGs in both conditions (Figures 7E–H).

Upon quantification of the amount of GAG production in two scaffolds per condition, less GAG/DNA was produced in the TGFβ1 + EMD condition (figure 8). As this was only performed with cells from one donor, it was not possible to statistically analyze these results.

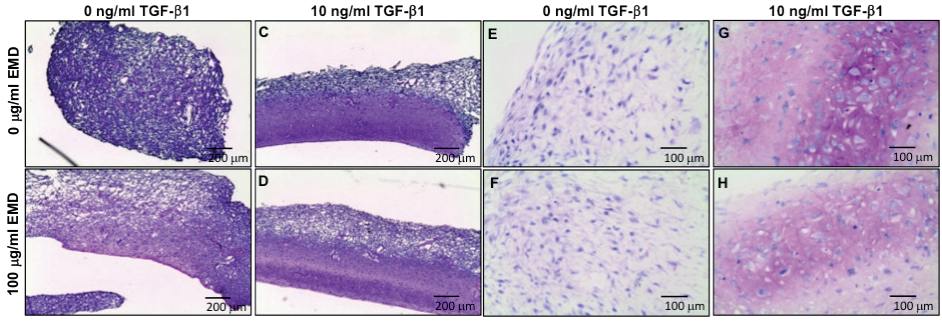

*Figure 7: **(A–D)** Distribution of MSCs through the collagen-GAG scaffolds cultured under the four different conditions (H&E staining). **(E–H)** Staining for GAGs produced by MSCs seeded on collagen-GAG scaffolds cultured whether or not in presence of TGF-β and/or EMD (thionine staining).*

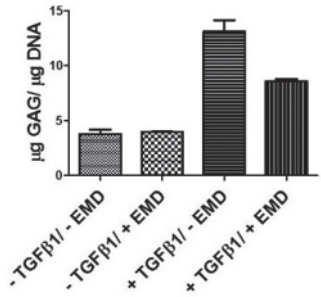

Figure 8: Quantification of GAGs in MSCs seeded on scaffolds cultured with chondrogenic factors for 21 days (two samples from one donor for each condition).

DISCUSSION

Current treatment options for the treatment of large bone defects, such as autologous bone or bone substitutes, are often accompanied by limitations and serious complications, highlighting the necessity for an alternative treatment option (1, 3, 25, 26). Reports of EMD on MSC osteogenesis are mixed (10, 14, 16, 18, 27) while little is known about the effects of EMD on chondrogenesis of human MSCs. Tissue engineered bone formation modeled after EO, in which MSCs are chondrogenically differentiated *in vitro* and then implanted, represents a promising avenue for bone tissue engineering (10). We hypothesised that, given the reported abilities of EMD to improve cell proliferation, migration, and differentiation (particularly during osteogeneic differentiation) (15-17, 27), EMD might also improve the chondrogenic priming of human MSCs. In this study, we focused on chondrogenic differentiation of adult human MSCs as a first step to tissue engineering bone via the process of EO. In order to put the work in the context of prior research, we also assessed osteogenic and adipogenic differentiation of these cells in the presence of EMD.

No differences were observed in GAG production nor in COL II expression in any of the conditions. While the group of Narukawa found an upregulation of COL II, COL X, and SOX 9, as well as increased GAG production following chondrogenic treatment of the ATDC5 hypertrophic cell line in the presence of Emdogain (15), we observed no effects on chrondrogenic differentiation in primary human MSCs. Given the natural tendency of ATDC5 cells to progress along the chondrogenic lineage toward hypertrophy, it is hard to directly compare the two cell types. The effect of EMD on cell migration and chondrogenesis in a 3D environment, a collagen-GAG scaffold, was also analysed in this study. This was performed using cells from a single donor on two scaffolds per condition. On histology, no differences were observed between chondrogenically treated groups. However, while chondrogenisis did occur, there was a trend toward decreased GAG production in the EMD treated samples. This experiment would require repetition with MSCs from several donors to confirm if this is the case.

We observed no effect of EMD on extracellular matrix production or on gene level when stimulated osteogenically. This is in agreement with the work of some other groups (28, 29). However, other groups also stated EMD, or components of it, stimulated the differentiation of MSCs toward osteocytes (15, 30-32). These utilised both cell lines and rat derived MSCs, as well as only selective proteins found in EMD, which may explain the differences observed. Considering published work and our results, we have no evidence to support the idea that EMD would negatively influence osteogenic differentiation of human MSCs. The *in vivo* effects of EMD on ossification remain unclear. Some groups reported enhanced bone induction *in vivo* in animal models and humans (33-35) while others showed no effect of EMD on the formation of mineralized bone (36). Yagi et al. showed that EMD inhibits RANKL expression, resulting in inhibited osteoclast formation, the cells that are responsible for bone resorption (37). The variability in these results could be caused by numerous factors including variable biological characteristics due to of the defect and patient variability (38). However, these results are based on bone formation by surrounding cells instead of implanted chondrogenically primed cells. It is difficult to extrapolate the results observed in this study to an *in vivo*/clinical situation. We observed a mild trend toward inhibition of adipogenic differentiation at the highest dose of EMD on human MSCs. No tests have been performed to determine the effects of EMD on adipogenic differentiation of MSCs previously. The decreasing trend toward adipogenic differentiation of MSCs, in this proposed application, could be considered a positive outcome suggesting undesirable fat tissue formation is unlikely, or could be that in adipogeneic differentiation EMD at high doses is mildly toxic.

Enamel matrix derivative does not appear to effect the multilineage differentiation of human MSCs. There may be a slight inhibitory effect of EMD, at the highest dose, on adipogenesis. However, this was not proven to be statistically significant. While this work suggests that EMD would not increase the chondrogenic potential of MSCs, which could be utilised in a bone tissue construct via EO for the treatment of large bone defects, there is also no evidence that tissue formation/bone formation would be inhibited if EMD was used in combination with MSCs for the repair of minor bone defects or periodontal tissue repair.

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Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

References

1. Arrington ED, Smith WJ, Chambers HG, Bucknell AL, Davino NA. Complications of iliac crest bone graft harvesting. Clinical Orthopaedics and Related Research®. 1996;329:300-9.
2. Froum SJ, Weinberg MA, Rosenberg E, Tarnow D. A comparative study utilizing open flap debridement with and without enamel matrix derivative in the treatment of periodontal intrabony defects: a 12-month re-entry study. Journal of Periodontology. 2001;72(1):25-34.
3. Meijer GJ, de Bruijn JD, Koole R, van Blitterswijk CA. Cell based bone tissue engineering in jaw defects. Biomaterials. 2008;29(21):3053-61.
4. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, et al. Multilineage potential of adult human mesenchymal stem cells. science. 1999;284(5411):143-7.
5. Salgado AJ, Coutinho OP, Reis RL. Bone tissue engineering: state of the art and future trends. Macromolecular bioscience. 2004;4(8):743-65.
6. Coyle CH, Izzo NJ, Chu CR. Sustained hypoxia enhances chondrocyte matrix synthesis. Journal of Orthopaedic Research. 2009;27(6):793-9.
7. Huang JI, Durbhakula MM, Angele P, Johnstone B, Yoo JU. Lunate arthroplasty with autologous mesenchymal stem cells in a rabbit model. JBJS. 2006;88(4):744-52.
8. Farrell E, van der Jagt OP, Koevoet W, Kops N, Van Manen CJ, Hellingman CA, et al. Chondrogenic priming of human bone marrow stromal cells: a better route to bone repair? Tissue Engineering Part C: Methods. 2008;15(2):285-95.
9. Farrell E, Both SK, Odörfer KI, Koevoet W, Kops N, O'Brien FJ, et al. In-vivo generation of bone via endochondral ossification by in-vitro chondrogenic priming of adult human and rat mesenchymal stem cells. BMC musculoskeletal disorders. 2011;12(1):31.
10. Gawlitta D, Farrell E, Malda J, Creemers LB, Alblas J, Dhert WJ. Modulating endochondral ossification of multipotent stromal cells for bone regeneration. Tissue Engineering Part B: Reviews. 2010;16(4):385-95.
11. Janicki P, Kasten P, Kleinschmidt K, Luginbuehl R, Richter W. Chondrogenic pre-induction of human mesenchymal stem cells on β-TCP: enhanced bone quality by endochondral heterotopic bone formation. Acta biomaterialia. 2010;6(8):3292-301.
12. Thompson EM, Matsiko A, Farrell E, Kelly DJ, O'Brien FJ. Recapitulating endochondral ossification: a promising route to in vivo bone regeneration. Journal of tissue engineering and regenerative medicine. 2015;9(8):889-902.
13. van der Stok J, Koolen M, Jahr H, Kops N, Waarsing J, Weinans H, et al. Chondrogenically differentiated mesenchymal stromal cell pellets stimulate endochondral bone regeneration in critical-sized bone defects. Eur Cell Mater. 2014;27(137):e48.
14. Grandin HM, Gemperli AC, Dard M. Enamel matrix derivative: a review of cellular effects in vitro and a model of molecular arrangement and functioning. Tissue Engineering Part B: Reviews. 2011;18(3):181-202.

15. Narukawa M, Suzuki N, Takayama T, Shoji T, Otsuka K, Ito K. Enamel matrix derivative stimulates chondrogenic differentiation of ATDC5 cells. Journal of periodontal research. 2007;42(2):131-7.
16. Jue SS, Lee WY, Kwon YD, Kim YR, Pae A, Lee B. The effects of enamel matrix derivative on the proliferation and differentiation of human mesenchymal stem cells. Clinical oral implants research. 2010;21(7):741-6.
17. Song Z, Shu R, Zhang X. Cellular responses and expression profiling of human bone marrow stromal cells stimulated with enamel matrix proteins in vitro. Cell proliferation. 2010;43(1):84-94.
18. Narukawa M, Suzuki N, Takayama T, Yamashita Y, Otsuka K, Ito K. Enamel matrix derivative stimulates osteogenesis- and chondrogenesis-related transcription factors in C3H10T1/2 cells. Acta biochimica et biophysica Sinica. 2007;39(1):1-7.
19. Dean D, Lohmann C, Sylvia V, Cochran D, Liu Y, Boyan B, et al. Effect of porcine fetal enamel matrix derivative on chondrocyte proliferation, differentiation, and local factor production is dependent on cell maturation state. Cells Tissues Organs. 2002;171(2-3):117-27.
20. Leijs MJ, Van Buul GM, Lubberts E, Bos PK, Verhaar JA, Hoogduijn MJ, et al. Effect of arthritic synovial fluids on the expression of immunomodulatory factors by mesenchymal stem cells: an explorative in vitro study. Frontiers in immunology. 2012;3:231.
21. Verseijden F, Jahr H, Posthumus-van Sluijs SJ, Hagen TLT, Hovius SE, Seynhaeve AL, et al. Angiogenic capacity of human adipose-derived stromal cells during adipogenic differentiation: an in vitro study. Tissue Engineering Part A. 2008;15(2):445-52.
22. Clockaerts S, Bastiaansen-Jenniskens Y, Feijt C, Verhaar J, Somville J, De Clerck L, et al. Peroxisome proliferator activated receptor alpha activation decreases inflammatory and destructive responses in osteoarthritic cartilage. Osteoarthritis and cartilage. 2011;19(7):895-902.
23. Narcisi R, Signorile L, Verhaar J, Giannoni P, Van Osch G. TGFβ inhibition during expansion phase increases the chondrogenic re-differentiation capacity of human articular chondrocytes. Osteoarthritis and cartilage. 2012;20(10):1152-60.
24. de Vries–van Melle ML, Narcisi R, Kops N, Koevoet WJ, Bos PK, Murphy JM, et al. Chondrogenesis of mesenchymal stem cells in an osteochondral environment is mediated by the subchondral bone. Tissue Engineering Part A. 2013;20(1-2):23-33.
25. Vahabi S, Amirizadeh N, Shokrgozar M, Mofeed R, Mashhadi A, Aghaloo M, et al. A comparison between the efficacy of Bio-Oss, hydroxyapatite tricalcium phosphate and combination of mesenchymal stem cells in inducing bone regeneration. Chang Gung medical journal. 2012;35(1):28-37.
26. Wiggins A, Austerberry R, Morrison D, Ho KM, Honeybul S. Cranioplasty with custom-made titanium plates—14 years experience. Neurosurgery. 2012;72(2):248-56.

27. Qu Z, Laky M, Ulm C, Matejka M, Dard M, Andrukhov O, et al. Effect of Emdogain on proliferation and migration of different periodontal tissue–associated cells. Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontology. 2010;109(6):924-31.
28. Mrozik KM, Gronthos S, Menicanin D, Marino V, Bartold PM. Effect of coating Straumann® Bone Ceramic with Emdogain on mesenchymal stromal cell hard tissue formation. Clinical oral investigations. 2012;16(3):867-78.
29. Van den Dolder J, Vloon A, Jansen J. The effect of Emdogain® on the growth and differentiation of rat bone marrow cells. Journal of periodontal research. 2006;41(5):471-6.
30. Keila S, Nemcovsky C, Moses O, Artzi Z, Weinreb M. In vitro effects of enamel matrix proteins on rat bone marrow cells and gingival fibroblasts. Journal of dental research. 2004;83(2):134-8.
31. Itoh N, Kasai H, Ariyoshi W, Harada E, Yokota M, Nishihara T. Mechanisms involved in the enhancement of osteoclast formation by enamel matrix derivative. Journal of periodontal research. 2006;41(4):273-9.
32. Amin HD, Olsen I, Knowles JC, Donos N. Differential effect of amelogenin peptides on osteogenic differentiation in vitro: identification of possible new drugs for bone repair and regeneration. Tissue Engineering Part A. 2012;18(11-12):1193-202.
33. Hammarstrom L, Heijl L, Gestrelius S. Periodontal regeneration in a buccal dehiscence model in monkeys after application of enamel matrix proteins. Journal of clinical periodontology. 1997;24(9 Pt 2):669-77.
34. Boyan BD, Weesner TC, Lohmann CH, Andreacchio D, Carnes DL, Dean DD, et al. Porcine fetal enamel matrix derivative enhances bone formation induced by demineralized freeze dried bone allograft in vivo. J Periodontol. 2000;71(8):1278-86.
35. Jaiswal R, Deo V. Evaluation of the effectiveness of enamel matrix derivative, bone grafts, and membrane in the treatment of mandibular Class II furcation defects. The International journal of periodontics & restorative dentistry. 2013;33(2):e58-64.
36. Schneider D, Weber FE, Hammerle CH, Feloutzis A, Jung RE. Bone regeneration using a synthetic matrix containing enamel matrix derivate. Clin Oral Implants Res. 2011;22(2):214-22.
37. Yagi Y, Suda N, Yamakoshi Y, Baba O, Moriyama K. In vivo application of amelogenin suppresses root resorption. J Dent Res. 2009;88(2):176-81.
38. Venezia E, Goldstein M, Boyan BD, Schwartz Z. The use of enamel matrix derivative in the treatment of periodontal defects: a literature review and meta-analysis. Critical reviews in oral biology and medicine : an official publication of the American Association of Oral Biologists. 2004;15(6):382-402.

7

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

OVERVIEW

Large bone defects are challenging to treat clinically, often relying on the use of autologous bone grafts (ABGs), which are in limited supply (1-3). As early as the 18th century surgeons had already begun investigating different alternative bone substitutes (3); some of the methodologies tested then, such as the use of decalcified bone (4), still persist today. However, understanding of defect repair and regeneration has since improved, resulting in better approaches to tissue engineered (TE) bone formation.

Cell based approaches to bone repair are often utilised in tissue engineering, usually modelled after one of the developmental pathways of bone formation, with mesenchymal stem cells (MSC) typically the preferred cell source due to their chondrogenic and osteogenic differentiation potential (5, 6). As mentioned in this thesis, MSC constructs modeled after intramembranous ossification are often unfavorable due to poor survival and integration rates (7) and as such the research in this thesis exploited the endochondral ossification (EO) pathway. The cartilage intermediate often employed in TE EO is thought to be well suited to survive in the initial avascular, hypoxic environment (8), and through the secretion of bioactive molecules and a biologically relevant extracellular matrix (ECM) these constructs can recruit cells from the host important for vascularization (9, 10), matrix remodeling (11-14) and ultimately bone formation (12, 15).

The aim of this thesis was to improve TE EO constructs and our understanding of how they work as well as to investigate how current constructs could be improved to meet clinical needs. Specifically, by identifying a superior cell source compared to the gold standard adult MSCs (A-MSCs) we present a more reliable and culture sustainable cell source, ideal for tissue engineering (**chapter 3**). We then investigated the role of collagen type X (COLX) produced by MSCs which appeared essential to the process but had never previously been investigated in a TE setting. In this chapter we prove the importance of this collagen not only during *in vitro* cartilage formation but also subsequent bone formation (**chapter 4**).

After identifying a more reproducible cell source and discovering the importance of COLX in TE EO, we started to investigate ways we could improve chondrogenic MSC constructs to meet clinical needs and expectations. First we created a clinically relevant micropellet system for use, ultimately, as an injectable bone substitute to allow for a minimally invasive, easily applicable TE bone treatment option for clinicians (**chapter 5**) (16). However, as is often the case, bone defects occur concurrently with soft tissue damage which also requires surgical intervention to heal properly. By investigating the combination of MSCs with a commercially available product, enamel matrix derivative

(EMD), used in the regeneration of soft tissue surrounding the bone defect site, (**chapter 6**) we showed with further development there may be potential to combine the injectable micropellet constructs (**chapter 5**) with EMD to allow for the treatment of both bone and soft tissue damage concurrently. Together, these studies improve the construction and understanding of TE EO, and also highlight new clinical application possibilities for the future. Below, we discuss the new findings presented in this thesis as well as focus on future applications and perspectives for EO grafts.

IMPROVED CELL SOURCES FOR TISSUE ENGINEERED ENDOCHONDRAL OSSIFICATION

MSCs are an attractive cell source for TE and regenerative medicine, however their unpredictable behaviour and tissue culture induced changes limit their applications (17, 18). Adult bone marrow derived MSCs (A-MSCs) have been the golden standard cell source for many years despite their unpredictable nature. Paediatric MSCs (P-MSCs), however, can be easily isolated from surplus iliac crest bone chips by an easily reproduced protocol. As opposed to A-MSCs these P-MSCs showed better expansive properties, yielding more MSCs with less senescence per passage than adult counterparts. The compromised regenerative capacity of aged cells may be due to the accumulation of senescent cells (18, 19); so, from a tissue engineering perspective, it may be advantageous to have a lower percentage of senescent cells in the overall population. Lehmann et al. have shown senescent MSCs within the starting population can induce senescence in the non-senescent cells present in culture as expansion continues (in preparation; abstract presented during International Cartilage Regeneration and Joint Prevention Society in 2018). This will lead to poor cell expansion rates as the cells continue to be passaged. For experiments requiring large cell numbers, or those which require extensive passaging of MSCs as is the case with lentivirus work (**chapter 4**) our P-MSCs are an attractive cell source.

Here we show even after more extensive passaging (passage 5 in **chapter 3**; passage 7 in **chapter 4**) P-MSCs exhibit/maintain their chondrogenic differentiation capacity, and we show they are capable of more consistent trilineage differentiation (**chapter 3**) which outperformed the capacity of A-MSCs at earlier passages. As pointed out by Arnold Caplan, the developmental demands on the body of a newborn versus that of a 70 year old are different, and these differences more than likely directly contribute to the age related changes we see in MSCs (20). As our P-MSCs are obtained from younger patients, who are still thought to be actively developing endochondral bone into their late 20s to early 30s (21)(22), it may reason that they would be better at regenerating these tissues than cells from patients well beyond this developmental stage. Additionally, as many adult MSCs are affected by age-related cell dysregulations (20), our MSCs from a paediatric source

are likely not to be affected by these problems, which could have contributed to their enhanced culture characteristics. Although their superior proliferation and differentiation capacity is clear, it must be pointed out that P-MSCs are still susceptible to a certain degree of donor-variation (**chapter 3**, figure 6), which will result in varying *in vivo* results (**chapter 4** & **5**). Identifying which subset of MSCs is responsible for chondrogenic differentiation may reduce this variability, and remains the focus of many research groups (23). Moreover, isolating such subsets from even younger cell sources, such as foetal or embryonic, could provide significant improvements compared to P-MSCs, although this can be difficult as these materials are in limited supply. As P-MSCs can be easily isolated from a source that would otherwise be deemed clinical waste we have found a way to not only reduce waste but utilise it in a meaningful way. By identifying this cell source we have provided those in the regenerative medicine community with a more reproducible and reliable cell source compared to the current gold standard.

INVESTIGATING MSC MEDIATED ENDOCHONDRAL OSSIFICATION

Identifying an ideal MSC cell source or chondrogenic cell subset is just the beginning. Our understanding of *in vitro* chondrogenic differentiation for reproducible *in vivo* bone formation must be improved; discovering what is essential for successful cartilaginous differentiation and subsequent *in vivo* bone formation will significantly improve what can be achieved in the field. Collagen type X (COLX) is an EO-associated collagen present both during *in vitro* chondrogenic induction (24) and *in vivo* bone formation (25) whose role in developmental EO is often debated (26, 27) but has never been studied in context of TE EO. COLX has been hypothesised to be vital for proper construction of the pericellular network (28, 29), adding structural support which is crucial during matrix remodeling (28), and is thought to play a role in initiating biomineralisation during bone formation by allowing selective binding of matrix vesicles (30-32). We show that COLX is expressed early and consistently during *in vitro* chondrogenic differentiation of MSCs (**chapter 5**) (24), suggesting it is important for successful chondrogenic differentiation.

Knocking-down COLX (**chapter 4**) altered chondrogenic induction of MSCs resulting in reduced levels of key ECM components and secreted factors. Interestingly in addition to the altered matrix formation, several secreted factors were found to be downregulated when COLX expression was decreased. FGF and PDGF, factors usually present during fracture repair, were among these factors. PDGF is important for EO as it plays a role in osteoblastic differentiation (33) and vessel stabilisation (34). FGF likewise plays an important role in EO, as it is essential for chondrocyte proliferation (16, 35). TNFα was also downregulated following COLX inhibition. This is of particular interest as TNFα is also found to play in osteoclastic recruitment and bone turnover (36, 37). Without COLX, not

only was the matrix greatly affected but the secreted profile, which also contributes to important cell recruitment and behaviour *in vivo*, are greatly impaired. MSCs with decreased COLX expression have decreased *in vivo* bone forming potential, further supporting the importance of COLX in MSC mediated EO. However, we also see in this experiment that there appears to be a certain threshold of COLX expression which is sufficient for chondrogenic differentiation and bone formation to occur. Determining this threshold is important for future studies as it can be used as a way to screen implants pre-implantation to determine the likelihood of bone formation *in vivo*. Additionally determining if greater COLX expression over this threshold has a positive effect on bone formation would likewise be interesting, perhaps an overexpression of COLX would improve bone formation.

The use of other knock-down methods, such as the CRISPR/cas system which is capable of targeted gene editing, would provide further support to our finding. This system, however, would require clonal expansion, a technique quite challenging to achieve with primary MSCs. Instead, an immortalised MSC cell line, such as TERT-MSCs (38), could be used with this technique. The use of a cell line would also help overcome the donor variability observed when using primary MSCs.

It would be interesting to see if the overexpression of COLX would improve *in vivo* bone formation as the knock-down has such negative effects. The exact timing of the over expression would need to be investigated however as inducing an over expression from the start may not be favourable as COLX is a hypertrophic associated collagen and early expression may hinder proper chondrogenic differentiation thus negatively affecting how the MSCs differentiate or produce the ECM. Regardless it is clear COLX plays an important role not only in chondrogenesis but also during bone formation by chondrogenic MSCs.

MODIFYING THE CURRENT MSC PELLET CULTURE SYSTEM FOR INJECTABILITY

Cell based TE EO is often achieved via *in vitro* chondrogenic differentiated MSCs in the form of pellets (**chapter 1**, figure 3). The resulting chondrogenic pellets (generally 200,000 cells/pellet) have been shown to form bone *in vivo* after 21-28 days of chondrogenic differentiation (39-41). However, when an irregular defect exists the current pellet system may not allow for sufficient treatment of the defect as they cannot sufficiently fill the defect site. This issue was addressed by creating a novel micropellet culture system (10,000 cells/pellet) which was shown to be capable of bone formation following only a week of chondrogenic differentiation. These micropellet constructs, which are capable of recruiting cells important for matrix remodelling, vessel invasion and bone formation (**chapter 5**), would allow for tailored void filling of irregular defects once optimised as an injectable bone substitute (24).

Before investigating the bone forming potential of micropellet constructs we needed to prove that the scaled down pellet constructs would maintain the chondrogenic potential observed in standard pellets. Chondrogenic priming time could be achieved in just one week, indicating the time required for *in vitro* culture before implantation could be substantially shortened, making future constructs available faster than previously thought. However bone formation was only reproducible when constructs were encapsulated in fibrin. Fibrin can create a hypoxic environment (42) which is favourable for both osteoclastic (43) and osteoblastic (44) activity, which may explain why when encapsulated in fibrin bone formation was possible. In our micropellet constructs fibrin allowed for a more three dimensional distribution of pellets within the fibrin. This could mean micropellets must be in closer proximity to one another to allow for better cross-talk between them. It could be thought the fibrin itself is the essential element, however, we also found when encapsulated in alginate chondrogenesis was also achievable which may indicate that oxygen tension is a more critical factor (unpublished). Further investigation into improving culture methodology, perhaps via a bioreactor, to ensure in the absence of fibrin chondrogenic differentiation can be achieved would be advantageous. Another avenue could be to use a thermo-reversible biomaterial which would allow the micropellets to stay in close proximity and still allow for injectability following differentiation. From a clinical standpoint however fibrin is advantageous as it is already a FDA approved material (45).

Optimising micropellets as an injectable substitute would be beneficial as it would be easily applied and offer a minimally invasive treatment option for clinicians. In the future, using micropellets in combination with functionalised injectable fibrin gels with proteins which are known to aid in the recruitment of cells required during EO or those known to improve bone formation such as BMP2 (discussed further in **chapter 2**) could result in greater bone formation which would allow for improved treatment. Additionally these micropellets could be used in combination with compounds such as Enamel matrix derivative (EMD) (**chapter 6**) to create an injectable treatment for both bone and soft tissue defects.

SOFT TISSUE REGENERATIVE COMPOUNDS ON MSC BEHAVIOUR, TOWARDS COMBINATIONAL THERAPEUTIC APPROACHES

Often when bone injuries occur, the surrounding soft tissue will be affected. Our constructs as presented here only form bone meaning other factor(s)/compound(s) would be required to regenerate the damaged soft tissues. EMD is a commercially available product for ligament repair that has previously been shown to improve stem cell proliferation and osteogenic differentiation (46-48). However if we were to use EMD in combination with

chondrogenic pellets, perhaps suspended around our pellet/micropellet constructs, we needed to determine if the addition of EMD to our system would have deleterious effects which would prevent its use in combinational therapy approaches.

We demonstrated that the trilineage differentiation potential of MSCs was not hindered by EMD (**chapter 6**). This is interesting as the addition of EMD to our system may not affect the trilineage differentiation potential of endogenous stem cells which are crucial for bone formation in our system. This suggests EMD would be a promising candidate for combinatorial therapy approaches using chondrogenically differentiated MSCs. However, further studies into the *in vivo* performance of the two together are necessary to ensure EMD does not hinder the bone forming potential of the chondrogenically differentiated MSCs and ensure there are truly no negative influences the endogeneous MSCs *in vivo*. This combinational therapy could be tested in an osteochondral defect model (49) in order to gauge the regenerative capacity of the construct in an area where both tissue types could be regenerated. As EMD is suspended in propylene glycol alginate (PGA) supplied as an injectable compound, it would be ideal to use in combination with an injectable chondrogenic construct, like the one developed in the **chapter 6**. Although just the beginning, the fact that EMD does not hinder the multilineage differentiation capacity of cells is promising and it will be interesting to see if/how EMD influences bone formation *in vivo* when added to our chondrogenic pellet constructs.

CONCLUSIONS, TOWARD CLINICAL TRANSLATION

Throughout this thesis we sought to improve TE EO. By identifying a more reproducible cell source we provided a better starting material with which to construct these grafts and by understanding the importance of COLX during chondrogenic differentiation and bone formation we improved our understanding of how these cells achieve EO. From a clinical perspective we develop a promising micropellet construct which could one day be optimised as an injectable bone substitute. By combining these constructs with a compound such as EMD it could be possible to create a construct which could improve both bone and soft tissue regeneration, making clinical treatment swifter and easier for clinicians. Although just the beginning of a long line of research, the results presented here are promising and with further development will highlight new ways in which researchers can improve bone formation output;. As these procedures become more standardised and routine this will drive down the cost, it requires to create TE EO constructs. As these avenues all merge and the field becomes more defined and understood a biologically relevant construct will emerge that everyone who has contributed to its advancement can be proud of.

References

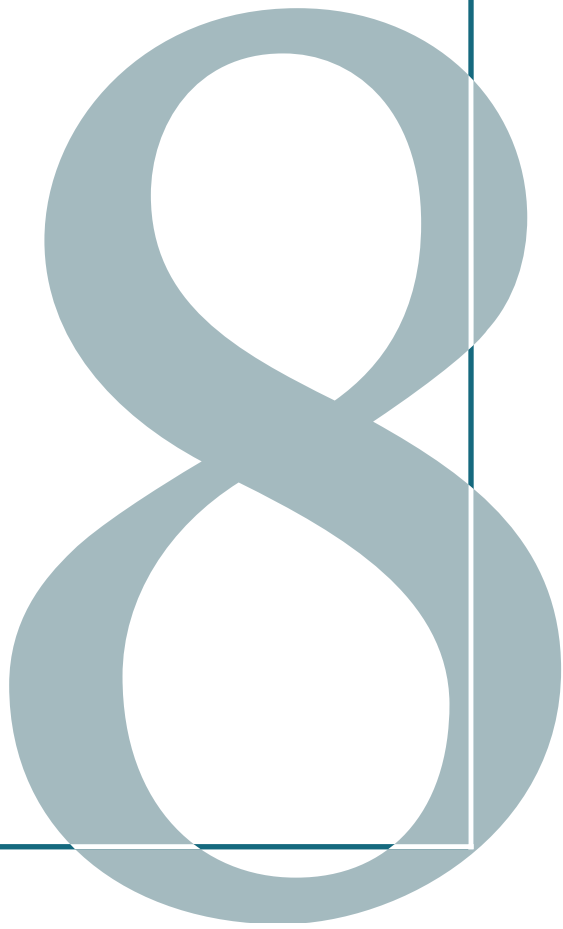
1. Lieberman JR, Friedlaender GE. Bone regeneration and repair: biology and clinical applications: Springer; 2005.
2. Frohlich M, Grayson WL, Wan LQ, Marolt D, Drobnic M, Vunjak-Novakovic G. Tissue engineered bone grafts: biological requirements, tissue culture and clinical relevance. Current stem cell research & therapy. 2008;3(4):254-64.
3. Lazzeri D, Gatti GL, Romeo G, Balmelli B, Massei A. Bone regeneration and periosteoplasty: a 250-year-long history. The Cleft Palate-Craniofacial Journal. 2009;46(6):621-8.
4. Senn N. On the healing of aseptic bone cavities by implantation of antiseptic decalcified bone. The American Journal of the Medical Sciences (1827-1924). 1889;98(3):219.
5. Lennon DP, Haynesworth SE, Young RG, Dennis JE, Caplan AI. A chemically defined medium supports in vitro proliferation and maintains the osteochondral potential of rat marrow-derived mesenchymal stem cells. Experimental cell research. 1995;219(1):211-22.
6. Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy. 2006;8(4):315-7.
7. Chatterjea A, Meijer G, van Blitterswijk C, de Boer J. Clinical application of human mesenchymal stromal cells for bone tissue engineering. Stem cells international. 2010;2010.
8. Farrell E, van der Jagt OP, Koevoet W, Kops N, Van Manen CJ, Hellingman CA, et al. Chondrogenic priming of human bone marrow stromal cells: a better route to bone repair? Tissue Engineering Part C: Methods. 2008;15(2):285-95.
9. Yang Q, McHugh KP, Patntirapong S, Gu X, Wunderlich L, Hauschka PV. VEGF enhancement of osteoclast survival and bone resorption involves VEGF receptor-2 signaling and β 3-integrin. Matrix Biology. 2008;27(7):589-99.
10. Knowles HJ, Athanasou NA. Hypoxia-inducible factor is expressed in giant cell tumour of bone and mediates paracrine effects of hypoxia on monocyte–osteoclast differentiation via induction of VEGF. The Journal of pathology. 2008;215(1):56-66.
11. Colnot CI, Helms JA. A molecular analysis of matrix remodeling and angiogenesis during long bone development. Mechanisms of development. 2001;100(2):245-50.
12. Gerber H-P, Vu TH, Ryan AM, Kowalski J, Werb Z, Ferrara N. VEGF couples hypertrophic cartilage remodeling, ossification and angiogenesis during endochondral bone formation. Nature medicine. 1999;5(6).
13. Ortega N, Behonick DJ, Werb Z. Matrix remodeling during endochondral ossification. Trends in cell biology. 2004;14(2):86-93.
14. Schindeler A, McDonald MM, Bokko P, Little DG, editors. Bone remodeling during fracture repair: the cellular picture. Seminars in cell & developmental biology; 2008: Elsevier.
15. Hu K, Olsen BR. Osteoblast-derived VEGF regulates osteoblast differentiation and bone formation during bone repair. The Journal of clinical investigation. 2016;126(2):509.

16. Ornitz DM, Marie PJ. FGF signaling pathways in endochondral and intramembranous bone development and human genetic disease. Genes & development. 2002;16(12):1446-65.
17. van der Stok J, Koolen M, Jahr H, Kops N, Waarsing J, Weinans H, et al. Chondrogenically differentiated mesenchymal stromal cell pellets stimulate endochondral bone regeneration in critical-sized bone defects. Eur Cell Mater. 2014;27(137):e48.
18. Wagner W, Bork S, Horn P, Krunic D, Walenda T, Diehlmann A, et al. Aging and replicative senescence have related effects on human stem and progenitor cells. PloS one. 2009;4(6):e5846.
19. Collado M, Blasco MA, Serrano M. Cellular senescence in cancer and aging. Cell. 2007;130(2):223-33.
20. Caplan AI. Adult mesenchymal stem cells for tissue engineering versus regenerative medicine. Journal of cellular physiology. 2007;213(2):341-7.
21. Baxter-Jones AD, Faulkner RA, Forwood MR, Mirwald RL, Bailey DA. Bone mineral accrual from 8 to 30 years of age: an estimation of peak bone mass. Journal of Bone and Mineral Research. 2011;26(8):1729-39.
22. Recker RR, Davies KM, Hinders SM, Heaney RP, Stegman MR, Kimmel DB. Bone gain in young adult women. Jama. 1992;268(17):2403-8.
23. Augello A, Kurth TB, De Bari C. Mesenchymal stem cells: a perspective from in vitro cultures to in vivo migration and niches. Eur Cell Mater. 2010;20(121):e33.
24. Knuth C, Witte-Bouma J, Ridwan Y, Wolvius E, Farrell E. Mesenchymal stem cell-mediated endochondral ossification utilising micropellets and brief chondrogenic priming. European cells & materials. 2017;34:142-61.
25. Yang L, Tsang KY, Tang HC, Chan D, Cheah KS. Hypertrophic chondrocytes can become osteoblasts and osteocytes in endochondral bone formation. Proceedings of the National Academy of Sciences. 2014;111(33):12097-102.
26. Rosati R, Horan GS, Pinero GJ, Garofalo S, Keene DR, Horton WA, et al. Normal long bone growth and development in type X collagen-null mice. Nature genetics. 1994;8(2):129.
27. Sweeney E, Campbell M, Watkins K, Hunter C, Jacenko O. Altered endochondral ossification in collagen X mouse models leads to impaired immune responses. Developmental Dynamics. 2008; 237(10):2693-704.
28. Shen G. The role of type X collagen in facilitating and regulating endochondral ossification of articular cartilage. Orthodontics & Craniofacial Research. 2005;8(1):11-7.
29. Schmid TM, Linsenmayer TF. Immunohistochemical localization of short chain cartilage collagen (type X) in avian tissues. The Journal of cell biology. 1985;100(2):598-605.
30. Kirsch T, Pfäffle M. Selective binding of anchorin CII (annexin V) to type II and X collagen and to chondrocalcin (C-propeptide of type II collagen) Implications for anchoring function between matrix vesicles and matrix proteins. FEBS letters. 1992;310(2):143-7.
31. Wu L, Genge B, Lloyd G, Wuthier R. Collagen-binding proteins in collagenase-released matrix vesicles from cartilage. Interaction between matrix vesicle proteins and different types of collagen. Journal of Biological Chemistry. 1991;266(2):1195-203.

32. Anderson HC, Garimella R, Tague SE. The role of matrix vesicles in growth plate development and biomineralization. Front Biosci. 2005;10(1):822-37.
33. Li A, Xia X, Yeh J, Kua H, Liu H, Mishina Y, et al. PDGF-AA promotes osteogenic differentiation and migration of mesenchymal stem cell by down-regulating PDGFRα and derepressing BMP-Smad1/5/8 signaling. PLoS One. 2014;9(12):e113785.
34. Caplan AI, Correa D. PDGF in bone formation and regeneration: new insights into a novel mechanism involving MSCs. Journal of Orthopaedic Research. 2011;29(12):1795-803.
35. Kronenberg HM. Developmental regulation of the growth plate. Nature. 2003;423(6937):332.
36. Bertolini DR, Nedwin GE, Bringman TS, Smith DD, Mundy GR. Stimulation of bone resorption and inhibition of bone formation in vitro by human tumour necrosis factors. Nature. 1986;319(6053):516.
37. Kimble RB, Bain S, Pacifici R. The functional block of TNF but not of IL-6 prevents bone loss in ovariectomized mice. Journal of Bone and Mineral Research. 1997;12(6):935-41.
38. Skårn M, Noordhuis P, Wang M-Y, Veuger M, Kresse SH, Egeland EV, et al. Generation and characterization of an immortalized human mesenchymal stromal cell line. Stem cells and development. 2014;23(19):2377-89.
39. Farrell E, Both SK, Odorfer KI, Koevoet W, Kops N, O'Brien FJ, et al. In-vivo generation of bone via endochondral ossification by in-vitro chondrogenic priming of adult human and rat mesenchymal stem cells. BMC Musculoskelet Disord. 2011;12:31.
40. Knuth CA W-BJ, Ridwan Y, Wolvius EB, Farrell E. Mesenchymal stem cell-mediated endochondral ossification utilising micropellets and brief chondrogenic priming. Eur Cell Mater. 2017;34:142-61.
41. van der Stok J, Koolen MKE, Jahr H, Kops N, Waarsing JH, Weinans H, et al. Chondrogenically differentiated mesenchymal stromal cell pellets stimulate endochondral bone regeneration in critical-sized bone defects. Eur Cell Mater [Internet]. 2014 2014; 27:[137-48; discussion 48 pp.]. Available from: http://europepmc.org/abstract/MED/24554271.
42. Demol J, Lambrechts D, Geris L, Schrooten J, Van Oosterwyck H. Towards a quantitative understanding of oxygen tension and cell density evolution in fibrin hydrogels. Biomaterials. 2011;32(1):107-18.
43. Arnett TR, Gibbons DC, Utting JC, Orriss IR, Hoebertz A, Rosendaal M, et al. Hypoxia is a major stimulator of osteoclast formation and bone resorption. Journal of cellular physiology. 2003;196(1):2-8.
44. Hung SP, Ho JH, Shih YRV, Lo T, Lee OK. Hypoxia promotes proliferation and osteogenic differentiation potentials of human mesenchymal stem cells. Journal of Orthopaedic Research. 2012;30(2):260-6.
45. Spotnitz WD. Fibrin sealant: past, present, and future: a brief review. World journal of surgery. 2010;34(4):632-4.

46. Grandin HM, Gemperli AC, Dard M. Enamel matrix derivative: a review of cellular effects in vitro and a model of molecular arrangement and functioning. Tissue Engineering Part B: Reviews. 2011;18(3):181-202.
47. Narukawa M, Suzuki N, Takayama T, Yamashita Y, Otsuka K, Ito K. Enamel matrix derivative stimulates osteogenesis-and chondrogenesis-related transcription factors in C3H10T1/2 cells. Acta biochimica et biophysica Sinica. 2007;39(1):1-7.
48. Jue SS, Lee WY, Kwon YD, Kim YR, Pae A, Lee B. The effects of enamel matrix derivative on the proliferation and differentiation of human mesenchymal stem cells. Clinical oral implants research. 2010;21(7):741-6.
49. De Vries-van Melle ML, Mandl EW, Kops N, Koevoet WJ, Verhaar JA, van Osch GJ. An osteochondral culture model to study mechanisms involved in articular cartilage repair. Tissue Engineering Part C: Methods. 2011;18(1):45-53.

SUMMARY



SUMMARY

The aim of this thesis was to investigate methods to improve the generation of mesenchymal stem cell (MSCs) based bone grafts via the process of endochondral ossification (EO) and to try to further understand some of the important aspects of this process. As there is a clear and present need for alternative bone substitutes to be developed I focused on achieving bone formation via the endochondral ossification pathway as I believe, compared to the intramembranous pathway, it to be the more promising route for tissue engineered (TE) bone regeneration.

In **chapter 1** an overview of developmental endochondral bone formation is given, specifically focusing on the construction of the cartilage template prior to bone formation and the overall processes that occur. Following the establishment and hypertrophic differentiation of the cartilage template, the chondrogenic matrix is remodeled, invaded with blood vessels and mineralised. These processes must be initiated by TE grafts in order for bone formation to be successful. We and others have shown how this process can be recapitulated utilising chondrogenically differentiated MSCs, which are capable of inducing bone and bone marrow formation following *in vivo* implantation (summarised in chapter 1, figure 3). How TE MSC based endochondral bone constructs achieve bone formation is reviewed in chapter 2. **Chapter 2** focused on how the TE donor components interact with the host to achieve bone formation and what efforts are being undertaken to improve knowledge about and formation of MSC endochondral bone grafts.

We and many others have found MSC based grafts are difficult to create as the inherent donor variation that exists in MSCs makes reproducibility difficult. In **chapter 3** I compared the "gold standard MSC cell source, adult bone marrow derived MSCs to a new cell source with improved expansion and differentiation capabilities. An easily replicated protocol to isolate MSCs from surplus human paediatric iliac crest material is described for other researchers to utilise. These cells were shown to be capable of faster proliferation, producing cells which more predictably underwent chondrogenic differentiation. I believe them to be a superior cell source compared to adult counterparts which improves the process of TE endochondral ossification by offering a more reliable cell source.

After identifying a more suitable starting cell population I investigated the importance of an extracellular matrix component, collagen type X (COLX), a collagen produced specifically

by hypertrophic chondrocytes during developmental EO. The importance of COLX during chondrogenic differentiation of MSCs and subsequent MSC mediated EO, had yet to be determined. In **chapter 4** I knocked down COLX expression utilising lentiviral delivered shRNA and showed how a significant decrease of COLX in MSCs resulted in a decreased chondrogenic differentiation potential. Not only was matrix formation greatly hindered but the secreted profile was also altered. These affects were found to be so detrimental that following *in vivo* implantation bone formation could not occur. In this way it was shown that COLX is important in both proper chondrogenic differentiation of MSCs and subsequent bone formation.

Also in this thesis I aimed to alter the current chondrogenic MSC format, the chondrogenic pellet, to create a construct which could ideally be further optimised as an injectable bone substitute. In **chapter 5** the construction of the standard MSC pellets was altered, downsizing them to only 20% of the original size to create "micropellets". In this chapter a straightforward protocol was described to create these micropellets and their chondrogenic potential after 7 and 28 days of differentiation was characterised. These micropellets were found to maintain their chondrogenic potential and bone forming capacity in a similar manner to standard pellets. With further optimisation these constructs would be ideal as an injectable bone substitute which would be easily applied in a clinical setting and allow for tailored void filling at the defect site.

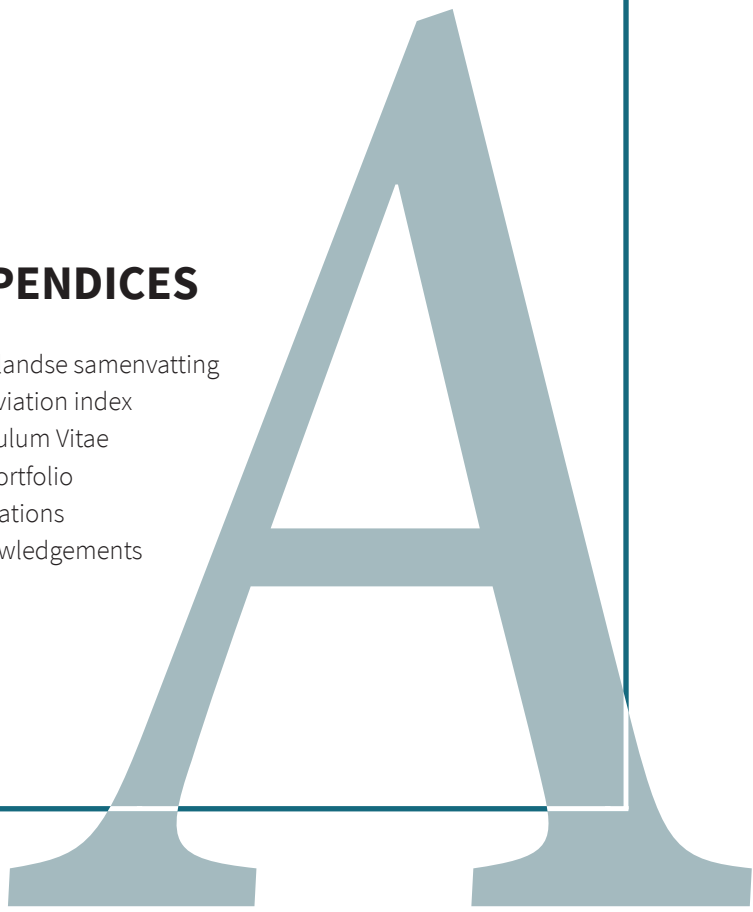
Although these MSC based constructs form bone, when a bone defect occurs it is often accompanied by soft tissue damage which also requires treatment. Enamel matrix derivative (EMD) can be utilised in ligament regeneration however it was unknown if this compound would have a negative effect on MSC behaviour, preventing EMD from being used in combination therapies to regenerate both tissue types simultaneously in the future. In **chapter 6** we showed EMD did not prevent the trilineage differentiation of MSCs. This not only means the compound should not hinder the differentiation of MSCs in our system, but also that it should not negatively affect the endogenous MSCs trilineage differentiation potential. This is important as host MSCs would contribute to bone formation in our system.

The findings of this thesis are more extensively discussed in **chapter 7** but briefly: I was able to identify a more reproducible cell source for use in TE endochondral bone formation, improving graft formation; by identifying the importance of COLX during both chondrogenic differentiation and bone formation I improved our understanding of how MSCs achieve EO; we showed the safety of EMD for use in combinational therapies with MSCs; and I created and characterised a novel micropellet construct which with further optimisation will offer an injectable cell based bone substitute for the treatment of bone

defects. Although these studies are in some cases just the beginning of a long line of research they all move these MSC based constructs forward, towards improved bone regeneration.

APPENDICES

Nederlandse samenvatting
Abbreviation index
Curriculum Vitae
PhD Portfolio
Publications
Acknowledgements



Nederlandse Samenvatting

Het doel van dit proefschrift is om inzicht te krijgen en het verbeteren van mesenchymale stam cel gebaseerde bot enten. Omdat er in de kliniek vraag is naar een alternatief voor vervangend bot heb ik mij gericht op de vorming van nieuw bot via de endochondrale ossificatie (EO) route, hierbij wordt kraakbeen omgevormd tot botweefsel. Ik meen dat EO van een kraakbeen-ent een veelbelovende optie is voor het verbeteren van de bot regeneratie (die na het ontstaan van een botdefect). Een ander alternatief is het aanmaken van bot via de intramembraneuze-route, waarbij bot wordt ontwikkeld vanuit bindweefselmembraan van mesenchymale stamcellen, die uiteindelijke gemineraliseerd worden, echter de vorming van nieuw botweefsel via de EO route is mijns inziens een veelbelovender optie .

In **hoofdstuk 1** wordt een overzicht geschetst van de ontwikkeling van botweefsel en de algehele processen die hierbij een rol spelen. Het proces waarbij kraakbeen wordt omgevormd tot botweefsel wordt ook wel endochondrale ossificatie genoemd. In hoofdstuk1 wordt de aanmaak van een sjabloon gemaakt van kraakbeen – dat uiteindelijk bot kan vormen – uitgelicht. Na de vorming en hypertrofe differentiatie van het kraakbeen sjabloon wordt de chondrogene matrix getransformeerd; er vindt minearalisatie van het kraakbeen plaats en er worden nieuwe bloedvaten gevormd. Dit proces wordt in werking gezet door gebruik te maken van de TE bot-enten. Wij en anderen hebben laten zien dat dit proces kan worden hervat door gebruik te maken van chondrogeen gedifferentieerde MSC's, die in staat zijn om - na in vivo implantatie - de vorming van bot en beenmerg te induceren (samengevat weergegeven in hoofdstuk 1, figuur 3). In **hoofdstuk 2** wordt een overzicht gegeven over de totstandkoming van het bot met behulp van TE-MSC gebaseerde endochondrale bot constructen. In hoofdstuk 2 wordt in meer detail beschreven hoe de TE donor componenten een interactie aangaan met de gastheer om bot te vormen, en de pogingen die zijn ondernomen om meer inzicht te krijgen in het construeren van MSC bot enten.

Wij, en vele anderen hebben ondervonden dat er veel haken en ogen zitten aan MSC gebaseerde enten, doordat donor variatie onafscheidelijk verbonden is aan de kwaliteit en reproduceerbaarheid van het kweken en differentiëren van MSCs. Hierdoor is het moeilijk om van iedere donor kwalitatief goed reproduceerbare constructen te kweken. In **hoofdstuk 3** vergelijk ik de "gouden standaard" MSC cel bron (MSC's verkregen uit volwassen beenmerg) met een nieuwe bron met verbeterde expansie en differentiatie mogelijkheden. Ik beschrijf in dit hoofdstuk een gemakkelijk reproduceerbaar protocol om MSC's te isoleren vanuit chirurgisch restmateriaal afkomstig van de bekkenkam van kinderen. Deze cellen zijn in staat om sneller te prolifereren, alsmede beter voorspelbaar

chondrogeen te differentiëren. Ik meen dat deze jonge stamcellen een betere bron zijn vergeleken met hun volwassen tegenhangers, waardoor het proces van TE endochondrale ossificatie verbeterd kan worden, immers deze stamcellen genereren een betrouwbaarder resultaat wat betreft de proliferatie en differentiatie capaciteiten.

Na het identificeren van een meer geschikt start materiaal heb ik onderzocht wat het belang is van een extracellulaire matrix component; collageen type X (COLX), een collageen die specifiek wordt aangemaakt door hypertrofe chondrocyten tijdens de ontwikkeling van EO. Het belang van COLX tijdens de chondrogene differentiatie van MSC's en de opvolgende MSC gemedieerde EO is nog onduidelijk. In **hoofdstuk 4**, heb ik de expressie van COLX door gebruik te maken van een lentiviraal shRNA construct – significant verlaagd in MSC's wat resulteert in een verminderde chondrogene differentiatie. De vorming van matrix wordt sterk verminderd, maar ook de door de cellen gemaakte factoren zijn veranderd. Deze effecten bleken zo sterk, dat na in vivo implantatie geen bot werd gevormd. Hieruit wordt geconcludeerd dat COLX een belangrijke rol speelt in zowel chondrogene differentiatie van MSC's alsmede in de opvolgende vorming van het bot.

Ook in dit proefschrift richt ik mij of de mogelijkheid om het huidige chondrogene MSC model, het chondrogene kraakbeen "bolletje," aan te passen tot een injecteerbaar bot substituut. Hiertoe wordt, in **hoofdstuk 5**, de samenstelling van een standaard MSC kraakbeen "bolletje", het zogenaamde MSC pellet, verkleind tot slechts 20% van de normale grootte; een "micropellet". Hierna werd onderzocht hoe de chondrogene differentiatie verliep na 7 en 28 dagen. Deze micropellets bleken op een vergelijkbare manier chondrogeen te worden, wanneer ze worden vergeleken met de standaard pellets. Na verdere optimalisatie zouden dit type constructen ideaal zijn als injecteerbaar plaatsvervangend "bot", een gemakkelijk toepasbare oplossing voor de patiënt, aangezien het de mogelijkheid bied van het opvullen van het botdefect met een passende, op de patiënt afgestelde oplossing.

Hoewel deze MSC gebaseerde constructen bot kunnen vormen, ontstaat een bot-defect meestal in combinatie met schade aan het omliggende zachte weefsel, dat ook behandeling nodig heeft. Enamel matrix derivaten (EMD) kunnen worden gebruikt bij het regenereren van de gewrichtsbanden. Echter omdat het onbekend is wat het effect van EMD is op het karakter van de MSC's, is het nog niet mogelijk is om EMD in combinatie therapieën te gebruiken waarbij zowel het bot, als de omliggende spierweefsels kunnen worden behandeld. In **hoofdstuk 6** laten we zien dat EMD geen negatief effect heeft op de drie verschillende differentiaties die MSC's kunnen ondergaan. Dit is belangrijk omdat MSC's van de gastheer een belangrijk aandeel hebben in de botvorming.

Concluderend, in dit proefschrift heb ik een beter reproduceerbare stamcel bron gevonden om beter reproduceerbare enten te verkrijgen, ten behoeve van TE-gemedieerde endochondrale botvorming. In **hoofdstuk 7** worden alle resultaten en conclusies uitgebreider toegelicht. Door het identificeren van de belangrijke rol van COLX tijdens zowel de chondrogene differentiatie alsmede de vorming van bot heb ik een beter inzicht verkregen in hoe MSC's EO tot stand brengen. Wij hebben de veiligheid van EMD voor het gebruik van combinatie therapieën met MSC's onderzocht. En tot slot heb ik een nieuw micropellet construct ontworpen en gekarakteriseerd. Na verdere optimalisatie bieden deze micropellets een injecteerbaar op stamcellen gebaseerd alternatief, dat na toediending kan worden gebruikt om plaatsvervangend bot aan te maken in een botdefect. Hoewel deze studies - in sommige gevallen - nog in de kinderschoenen staan en nog heel veel onderzoek nodig hebben, wijzen ze allemaal richting een verbeterde bot regeneratie met behulp van MSC's.

Abbreviation index

ABG	autologous bone grafts
ACAN	aggrecan
ALP(L)	alkaline phosphatase
A-MSCs	adult mesenchymal stem cells
ANG-1	angiopoietin 1
ATMPS	advanced therapeutic medical products
BGLAP	bone gamma carboxyglutamate protein
BM	bone marrow
BMP	bone morphogenetic protein
CCN2	connective tissue growth factor
COLI	collagen type I
COLII	collagen type II
COLX/COL10A1	collagen type X
CTACK	cutaneous T-cell attracting chemokine
DC	dendritic cells
DMB	dimethylmethylene blue
H&E	haemotoxylin and eosin
DNA	deoxyribonucleic acid
ECM	extracellular matrix
EMA	european medicines agency
EMD	enamel matrix derivative
EO	endochondral ossification
FABP4	fatty acid binding protein 4
FDA	federal drug administration
FGF	fibroblast growth factor
GAG	glycoaminoglycans
GDF5	growth differentiation factor 5
HC	hypertrophic chondrocytes
hPLAP	human placental alkaline phosphatase
HSPG	heparan sulfate proteoglycans
IBSP	integrin binding sialoprotein
Ihh	indian hedgehog
IL	interleukin
IMO	intramembranous ossification
KO	knock-out
MFI	mean fluorescent intensity
MHC	major histocompatibility complex

MMP	matrix metallopeptidase
MSC	mesenchymal stem cell/marrow stromal cell
NK	natural killer cells
PBMC	peripheral blood mononuclear cells
PCR	polymerase chain reaction
PDGRa	platelet-derived growth factor receptor A
PLGA	poly(D, L-lactic-co-glycolic acid
P-MSCs	paediatric mesenchymal stem cells
PPARg	peroxisome proliferator-activated receptor gamma
PRP	platelet-rich plasma
PTHrP	parathyroid hormone-related protein
RANKL	receptor activator of nuclear factor kappa-B ligand
RISC	RNA-induced silencing complex
RNA	ribonucleic acid
RUNX2	runt-related transcription factor 2
SMCD	schmid metaphyseal chondrodysplasia disorder
SOX9	sex- determining region Y box-9 protein
TE	tissue engineering
TERM	tissue engineering and regenerative medicine
TERT-MSC	telomerase reverse transcriptase mesenchymal stem cells
Tg	transgenic
TGF	transforming growth factor
TGFb	tumor growth factor beta
TIMP-1/2	tissue inhibitors of metalloproteinases ½
TNFa	tumor necrosis factor alpha
TRAP	tartrate-resistant acid phosphatase
VEGF	vascular endothelial growth factor
WNT	wingless-type MMTV integration site family
µCT	micro computed topography
µpellet	micropellet

Curriculum vitae (about the author)

Callie An Knuth was born the last of five children on August 27th 1988 alongside her twin brother Matthew in Pittsburgh, Pennsylvania (USA) to Judith and Andrew Knuth. Following her high school education at Bethel Park Senior High School (USA), where she was the 2005 PSFC girls' individual foil champion, Callie attended Penn State, the Behrend College in Erie, Pennsylvania (USA). From 2007 to 2011 she studied and received Bachelor's degrees in Cellular and Molecular Biology and Biochemistry. Alongside her studies Callie completed an independent research project under the instruction of Dr. Matthew Gruwell involving the identification of 3 new species and revision to the genus Mycetaspis, an invasive agricultural species. She presented this research during the Sigma Xi research conference and won an award for her oral presentation skills. During this time she was a teaching assistant for introduction to biology, summer school instructor in molecular biology techniques to visiting students from the University of Miami, summer school counselor at the Zion Lutheran Church and an avid hula hooper. Following her undergraduate degree Callie completed a Master's of research in Regenerative Medicine at the University of Newcastle upon Tyne in Newcastle (UK). During this study Callie completed an independent research project investigating the effects of low oxygen tension on novel haemarthrosis fluid derived MSCs under the supervision of Dr. Annette Meeson and Dr. Rachel Oldershaw. The findings from this research was presented at the North East Stem Cell Institute Conference where she was awarded the best poster presentation award. After the completion of her Master's degree Callie started her PhD (the findings of which make up this thesis) at Erasmus MC in Rotterdam (Netherlands) under the supervision of Dr. Eric Farrell, Professor Dr. Eppo Wolvius and Dr. Roberto Narcisi. During this time she presented findings throughout Europe, published several manuscripts, developed new technical skills and attended many wonderful festivals. Callie now lives in Den Haag with her boyfriend Stan and new Labrador puppy Cody who delayed the completion of this work by eating many drafts and important reference material but none the less is "nog steeds een brave hond".

Personal Details

Name	Callie An Knuth
Department	Dept. of Oral & Maxillofacial Surgery, Orthodontics & Special Dental Care
PhD Period	August 2013 – February 2018
Supervisor	Professor Eppo B. Wolvius, MD, PhD
Co-promotor(s)	Dr. Eric J. Farrell, PhD and Dr. Roberto Narcisi, PhD

Year	Courses	Workload (ECTS)
2013	Laboratory animal science	4
2014	Handling laboratory animals and introduction to IVC's (EDC)	0.3
2014	Research Integrity	0.3
2013	Photoshop & Illustrator CS6 workshop	0.3
2014	Basic introduction course to SPSS	1
2014	Research management for PhD/Post-Docs	1
2014	Translational imaging workshop	1.4
2014	Advanced immunology, short course	1
2015	Masterclass cell-based bone regeneration (Nijmegen)	1

Year	Conference presentations	Workload (ECTS)
2014	TERMIS (Genoa, Italy) Poster presentation	1
2014	NBTE (Lunteren, Netherlands) Oral presentation	1
2014	ECTS-IBMS (Rotterdam, Netherlands) Poster presentation	1
2015	Molecular medicine day (Rotterdam, Netherlands) Oral Presentation	1
2015	NBTE (Lunteren, Netherlands) Rapid fire oral & poster presentation	1

Year	Inter(national) symposiums	Workload (ECTS)
2014	Symposium NCMLS New Frontiers (Nijmegen)	1
2014	TERMIS (Genoa, Italy)	
2016	TERMIS (Uppsalla, Sweden)	

Year	Teaching and student supervision	Workload (ECTS)
2013	Supervising masters student, molecular medicine (part time)	3
2016	Supervising masters student, molecular medicine (full time)	5
2014	High school student presentation lecture	0.3
2017	SCORE meeting oral presentation	1

Year	Department meetings and presentations	Workload (ECTS)
2013-18	Journal club meetings (monthly)	1
2013-18	Lab meeting & presentations: department of orthopaedics (weekly)	2
2013-18	Research meeting & presentations: dept. of internal medicine (weekly)	2
2013-18	Research meeting & presentations: dept. of oral max surgery (monthly)	1

Year	Miscellaneous	Workload (ECTS)
2016	Organization lab day Orthopaedics/ENT	0.5
2013-2014	Dutch language classes, -A1 level (Rotterdam, Netherlands)	2
2017-2018	Dutch language classes, A1-A2, A2-B1 level (Den Haag, Netherlands)	4
	TOTAL ECTS	**38.1**

Publications

Knuth, C.A., Andreas Santre, E., Fahy, N.B., Witte-Bouma, J., Ridwan, Y., Strabbing, E., Koudstaal, M., van de Peppel, J., Wolvius, E.B., Narcisi, R., Farrell, E. “Collagen type X is essential for successful mesenchymal stem cell mediated cartilage formation and subsequent endochondral ossification.” Manuscript submitted.

Knuth, C.A., Kiernan, C., Wolvius, E.B., Narcisi, R., Farrell, E. “Unravelling tissue engineered endochondral ossification; towards improved bone regeneration.” Eur Cell Mater. 2019.

Kenswil, K., Ping, Z., Vanchin, B., **Knuth, C.A.**, Chen, S., van Dijk, C., Hoogenboezem, R., Jaramillo, A., Mylona, M., Adisty, M., Bindels, E., Bos, P., Cupedo, T., Farrell, E., Krenning, G., Raaijmakers, M. Identification of an endothelial cell capable of conversion to mesenchymal cell fates in the human bone marrow. Manuscript submitted.

Knuth, C.A., Kiernan, C., Palomares Cabeze, V., Lehmann, J., Witte-Bouma, J., ten Berg, D., Brama, P., Wolvius, E.B., Strabbing, E., Koudstaal, M., Narcisi, R., Farrell, E. "Isolating paediatric mesenchymal stem cells with enhanced expansion and differentiation capabilities." Tissue Engineering part C. 2018.

Kiernan, C, **Knuth, C.A.**, Farrell, E. "Chapter 6: Endochondral Ossification: Recapitulating Bone Development for Bone Defect Repair." Developmental Biology and Musculoskeletal Tissue Engineering. 2018.

Knuth, C.A., Witte-Bouma, J., Ridwan, Y., Wolvius, E.B., Farrell, E. "Mesenchymal stem cell-mediated endochondral ossification utilising micropellets and brief chondrogenic priming." Eur Cell Mater. 2017.

Rogers, C.M., Deehan, D.J., **Knuth, C.A.**, Rose, F.R., Shakesheff, K.M., Oldershaw, R.A. Biocompatibility and enhanced osteogenic differentiation of human mesenchymal stem cells in response to surface engineered poly (d, l-lactic-coglycolic acid).microparticles. Journal of Biomedical Materials Research Part A. 2014.

Groeneveldt, L. **Knuth, C.A.**, Witte-Bouma, J., O-Brian, F.J., Wolvius, E.B., Farrell, E. "Enamel matrix derivative has no effect on the chondrogenic differentiation of mesenchymal stem cells." Frontiers in bioengineering and biotechnology. 2014.

German, M.J., Osei-Bempong, C., **Knuth, C.A.**, Deehan, D.J., Oldershaw, R.A. Investigating the biological response of human mesenchymal stem cells to titanium surfaces. Journal of orthopaedic surgery and research. 2014.

Knuth, C.A., Clark, M.E., Meeson, A.P., Khan, S.K., Dowen, D.J., Deehan, D.J., Oldershaw, R.A. "Low oxygen tension is critical for the culture of human mesenchymal stem cells with strong osteogenic potential from haemarthrosis fluid." Stem Cell Reviews and Reports. 2013.

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