Article type: Short Communication

An endochondral ossification approach to early-stage bone repair: Use of tissue-engineered hypertrophic cartilage constructs as primordial templates for weight-bearing bone repair

Authors:

Amos Matsiko[1, 2, 3, ‡], Emmet M. Thompson[1, 2, 3, ‡], Cai Lloyd-Griffith[1,2,3], Gráinne M. Cunniffe[2, 3, 4], Tatiana Vinardell[2,3,4], John P. Gleeson[1,2,3], Daniel J. Kelly[2, 3, 4], Fergal J. O'Brien[1, 2, 3]

[1] Tissue Engineering Research Group, Dept. of Anatomy, Royal College of Surgeons in Ireland (RCSI), 123 St. Stephen's Green, Dublin 2, Ireland.

[2] Trinity Centre for Bioengineering, Trinity Biomedical Sciences Institute, Trinity College Dublin (TCD), Dublin 2, Ireland.

[3] Advanced Materials and Bioengineering Research (AMBER) Centre, Dublin, Ireland

[4] Department of Mechanical and Manufacturing Engineering, School of Engineering, Trinity College Dublin (TCD), Dublin 2, Ireland.

‡ **These authors contributed equally to this work**

Corresponding author:

Prof. Fergal O'Brien,

Department of Anatomy,

Royal College of Surgeons in Ireland,

123 St. Stephen's Green,

Telephone Number: +353 (0)1402 2149

Email: fjobrien@rcsi.ie

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/term.2638

Abstract:

Mimicking endochondral ossification to engineer constructs offers a novel solution to overcoming the problems associated with poor vascularisation in bone repair. This can be achieved by harnessing the angiogenic potency of hypertrophic cartilage. In this study, we demonstrate that tissue-engineered hypertrophically-primed cartilage constructs can be developed from collagen-based scaffolds cultured with mesenchymal stem cells. These constructs were subsequently implanted into femoral defects in rats. It was evident that the constructs could support enhanced early-stage healing at 4 weeks of these weight-bearing femoral bone defects compared to untreated defects.

Main text:

Autologous grafts have been widely utilized in bone defect repair as a clinical gold standard due to their immuno-compatibility and osteo-inductivity. However, due to limitations of donor-site morbidity and limited supply, alternative strategies have been explored, including tissue-engineered bone grafts. The development of these tissue-engineered bone graft substitutes remains a major challenge since poor vascularisation following implantation can limit their success (Ko et al., 2007). Bone formation and fracture healing which occur by endochondral ossification (ECO) are characterised by the release of pro-angiogenic signals from a hypertrophic cartilage anlage which acts as a primordial template to facilitate invasion of blood vessels that ultimately regulates bone formation (Gerstenfeld et al., 2003; Marsell and Einhorn, 2011). In bone tissue engineering, researchers typically utilise an approach of stimulating in vitro mesenchymal stem cell osteogenesis within scaffolds prior to implantation, mimicking the embryological process of intramembranous ossification (IMO). However, such approaches often fail once the constructs are implanted within defects in vivo due to the problem of avascular necrosis and core degradation (Lyons et al., 2010). Inspired by the embryological process, recapitulating ECO has generated a growing interest due to the ability of hypertrophic cartilage in secreting angiogenic factors that enhances bone repair (Thompson et al., 2015).

The majority of studies that have adopted the ECO approach to develop tissue-engineered constructs have evaluated their potential using in vivo subcutaneous models (Pelttari et al., 2006). Whilst such an ectopic model is useful for the investigation of vascular invasion and mineralisation, it does not offer an ideal model for the assessment of efficacy of a construct for bone defect repair. Weight-bearing bone defect are more suitable models and have been utilised for the assessment of a range of constructs *in vivo* due to the physiological relevance in terms of mechanical loads experiences within the defect space compared to non-weight-bearing subcutaneous models (Kempen et al., 2009; Meinel et al., 2006) . A recent study (Harada et al., 2014) utilised a large 15 mm femoral defect model within immuno-competent rats treated with a PLGA scaffold cultured with MSC-derived chondrocytes which were capable of eliciting bone healing after 16 weeks thereby providing evidence of the potential of mimicking the ECO process for bone repair. Further studies have also utilised critically-sized weight-bearing bone defects in the assessment of chondrogenically-primed constructs in bone defect repair (van der Stok et al., 2014; Bernhard et al., 2017).

In our laboratory, we adopt a biomimetic approach to develop biomaterials for tissue regeneration by utilising components of the extracellular matrix. Recent research has led to the development of CHyA scaffolds which have shown the potential to support MSC chondrogenesis and cartilage-like tissue formation *in vitro* (Matsiko et al., 2015). In this study, we propose to use these scaffolds as templates for engineering hypertrophically-primed constructs with mesenchymal stem cells (MSCs) in order to repair large weight-bearing defects. We have recently demonstrated that using an ECO-based approach, CHyA scaffolds were capable of supporting *in vitro* MSC-mediated chondrogenesis with resulting production of a hypertrophic cartilaginous matrix, secretion of the pro-angiogenic factor vascular endothelial growth factor (VEGF) and subsequent mineral deposition. Moreover, in the same study, we also demonstrated the ability of these hypertrophic cartilaginous constructs to support *de novo* bone formation within critically-sized cranial defects *in vivo* which may be attributed to VEGF production (Thompson et al., 2016). In this context, this current study is focused on the investigation of the *in vivo* potential of ECO-based hypertrophic cartilage constructs developed from collagen-based scaffolds and MSCs, for the repair of weight-bearing femoral bone defects.

The overall aim of this study was thus to investigate the potential of tissue engineered ECO-based hypertrophic constructs to enhance early-stage bone healing in a rat critical-sized weight-bearing mid-diaphyseal bone defect. The specific focus was to determine whether MSC-seeded CHyA constructs cultured using an ECO-based approach were capable of enhancing bone healing *in vivo* within a 5 mm femoral defect. The ECO-based hypertrophic construct comprised of the CHyA scaffold cultured with rat MSCs in the presence of chondrogenic factors for 21 days followed by a switch to hypertrophic factors for 14 days (ECO construct). An empty defect group, which comprised of an untreated defect was utilised as a control. Following ethical approval from the Research Ethics Committee of RCSI, the study was carried out on skeletally mature Fischer rats and a 5 mm mid-diaphyseal defect was created on the right femur of the animals which was either left untreated or treated with tissue-engineered constructs. The defect was secured with a custom-designed polyether ether ketone (PEEK) internal fixator which was attached to the anterolateral femur with screws. This internal fixators were obtained under collaboration with the University of Michigan (Filion et al., 2011). We chose rats as an initial proof of concept and previous literature in the field has demonstrated this animal model to be sufficient to investigate bone formation in weight-bearing defects although we acknowledge the need for larger animal models in future studies. It was clear that all the animals tolerated the procedure and completed the study with no signs of significant distress. Good health was evidenced by progressive weight gain and appropriate behaviour over the experimental period. No wound infections or adverse local tissue response were observed at the implant site upon retrieval of the tissue after 4 weeks.

Qualitative micro-CT analysis as well as quantitative assessment of the ratio of bone volume-to-tissue volume was carried out to determine the level of healing within the defect (**Figure 1(i)**). There was limited new bone tissue within the defect space that was left untreated which served as a control group. The micro-CT qualitative assessment also revealed that the ECO constructs supported mineralised tissue and bone formation in the defects with evidence of early stages of defect bridging. Quantitative micro-CT was carried out to assess the volume of new bone within the volume of interest within the defect space (**Figure 1(ii)**). It was evident that the ECO constructs supported higher new bone formation compared to the control group as demonstrated by the higher bone volume per tissue volume ($p<0.01$). This was approximately 15-fold higher than the untreated empty defect.

Histological assessment was also carried out using Masson's trichrome and hematoxylin and eosin to determine the quality of tissue formed within the defects (**Figure**

2). It was evident that there was lower mineralised tissue in the empty defect group. The defect space was predominantly occupied by fibrous tissue indicating poor attempts at regeneration. The tissue-engineered construct supported greater dense mineralised tissue formation within the defect space compared to the empty defect control although at 4 weeks, it is clear that this is not fully mature bone tissue bridging within the defect space. There was limited residual scaffold within the ECO group suggesting that the ECO-based tissue-engineered constructs were capable of biodegradation as early as 4 weeks.

The *in vitro* culture regime used prior to implantation may have influenced stimulation of hypertrophy, angiogenesis and subsequent bone healing. We have adopted a method of stimulating MSC chondrogenesis on the scaffolds followed by culture with L-thyroxine and betaglycerolphosphate to support hypertrophy and mineral deposition (Thompson et al., 2016). This regime induces expression of collagen type X (COLX) and vascular endothelial growth factor (VEGF) gene expression and protein production and has also been successfully utilised in other studies (Sheehy et al., 2013). In the current study, it was clear that the ECO construct was capable of supporting greater bone formation than the control group. The CHyA scaffolds were previously shown to support chondrogenesis due to their composition, pore architecture and mechanical properties and are thus suitable for this application (Matsiko et al., 2012; Matsiko et al., 2015).

In conclusion, we have shown the ability of collagen-based scaffolds to act as suitable templates for the development of tissue-engineered constructs capable of supporting early-stage bone repair. It was evident that mimicking ECO using a collagen-hyaluronic acid scaffold optimised for cartilage repair, cultured with MSCs in a regime designed to induce hypertrophic chondrogenesis, led to bone formation as early as 4 weeks. This current study provides further evidence in support of previous literature suggesting the potential of endochondral ossification-based strategies in enhancing bone repair in weight-bearing defects (Harada et al., 2014; van der Stok et al., 2014; Bernhard et al., 2017). While only one time-point was assessed, this 4 week study was carried out as a proof of principle and we believe that this approach substantiates the concept of recapitulating endochondral ossification. Learning from developmental biology to advance the field of bone tissue engineering demonstrates a paradigm shift and may ultimately open new avenues in tissue repair, ultimately presenting more promising clinical prognosis.

Acknowledgements

We acknowledge the support of Prof. Steven A. Goldstein for his advice on the use of the femoral defect model. Funding was provided by the Health Research Board of Ireland (Grant No. HRA_POR/2011/27) and the European Research Council (ERC Grant Agreement No. 239685).

References

Bernhard, J., Ferguson, J., Rieder, B., Heimel, P., Nau, T., Tangl, S., Redl, H. and Vunjak-Novakovic, G., 2017. Tissue-engineered hypertrophic chondrocyte grafts enhanced long bone repair. *Biomaterials*.

Filion, T. M., Li, X., Mason-Savas, A., Kreider, J. M., Goldstein, S. A., Ayers, D. C. & Song, J. 2011. Elastomeric Osteoconductive Synthetic Scaffolds with Acquired Osteoinductivity Expedite the Repair of Critical Femoral Defects in Rats. *Tissue Engineering. Part A,* 17**,** 503-511.

Gerstenfeld, L. C., Cullinane, D. M., Barnes, G. L., Graves, D. T. & Einhorn, T. A. 2003. Fracture healing as a post-natal developmental process: molecular, spatial, and temporal aspects of its regulation. *Journal of Cellular Biochemistry,* 88**,** 873-84.

Harada, N., Watanabe, Y., Sato, K., Abe, S., Yamanaka, K., Sakai, Y., Kaneko, T. & Matsushita, T. 2014. Bone regeneration in a massive rat femur defect through endochondral ossification achieved with chondrogenically differentiated MSCs in a degradable scaffold. *Biomaterials,* 35**,** 7800-10.

Kempen, D. H. R., Lu, L., Heijink, A., Hefferan, T. E., Creemers, L. B., Maran, A., Yaszemski, M. J. & Dhert, W. J. A. 2009. Effect of local sequential VEGF and BMP-2 delivery on ectopic and orthotopic bone regeneration. *Biomaterials,* 30**,** 2816-2825.

Ko, H. C., Milthorpe, B. K. & McFarland, C. D. 2007. Engineering thick tissues--the vascularisation problem. *European Cells and Materials,* 14**,** 1-18; discussion 18-9.

Lyons, F. G., Al-Munajjed, A. A., Kieran, S. M., Toner, M. E., Murphy, C. M., Duffy, G. P. & O'Brien, F. J. 2010. The healing of bony defects by cell-free collagen-based scaffolds compared to stem cell-seeded tissue engineered constructs. *Biomaterials,* 31**,** 9232-9243.

Marsell, R. & Einhorn, T. A. 2011. The biology of fracture healing. *Bone Regeneration in the 21st Century,* 42**,** 551-555.

Matsiko, A., Gleeson, J. P. & O'Brien, F. J. 2015. Scaffold mean pore size influences mesenchymal stem cell chondrogenic differentiation and matrix deposition. *Tissue Engineering - Part A,* 21**,** 486-497.

Matsiko, A., Levingstone, T. J., O'Brien, F. J. & Gleeson, J. P. 2012. Addition of hyaluronic acid improves cellular infiltration and promotes early-stage chondrogenesis in a collagen-based scaffold for cartilage tissue engineering. *Journal of the Mechanical Behavior of Biomedical Materials,* 11**,** 12.

Meinel, L., Betz, O., Fajardo, R., Hofmann, S., Nazarian, A., Cory, E., Hilbe, M., McCool, J., Langer, R., Vunjak-Novakovic, G., Merkle, H. P., Rechenberg, B., Kaplan, D. L. & Kirker-Head, C. 2006. Silk based biomaterials to heal critical sized femur defects. *Bone,* 39**,** 922-931.

Pelttari, K., Winter, A., Steck, E., Goetzke, K., Hennig, T., Ochs, B. G., Aigner, T. & Richter, W. 2006. 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 and Rheumatism,* 54**,** 3254-66.

Sheehy, E. J., Vinardell, T., Buckley, C. T. & Kelly, D. J. 2013. Engineering osteochondral constructs through spatial regulation of endochondral ossification. *Acta Biomaterialia,* 9**,** 5484-92.

Thompson, E. M., Matsiko, A., Farrell, E., Kelly, D. J. & O'Brien, F. J. 2015. Recapitulating endochondral ossification: a promising route to in vivo bone regeneration. *Journal of Tissue Engineering and Regenerative Medicine,* 9**,** 889–902.

Thompson, E. M., Matsiko, A., Kelly, D. J., Gleeson, J. P. & O'Brien, F. J. 2016. 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,* 22**,** 556-567.

van der Stok, J., Koolen, M.K., Jahr, H., Kops, N., Waarsing, J.H., Weinans, H. and Van der Jagt, O.P., 2014. Chondrogenically differentiated mesenchymal stromal cell pellets stimulate endochondral bone regeneration in critical-sized bone defects. *Eur Cells Mater*, *27*, pp.137-48.

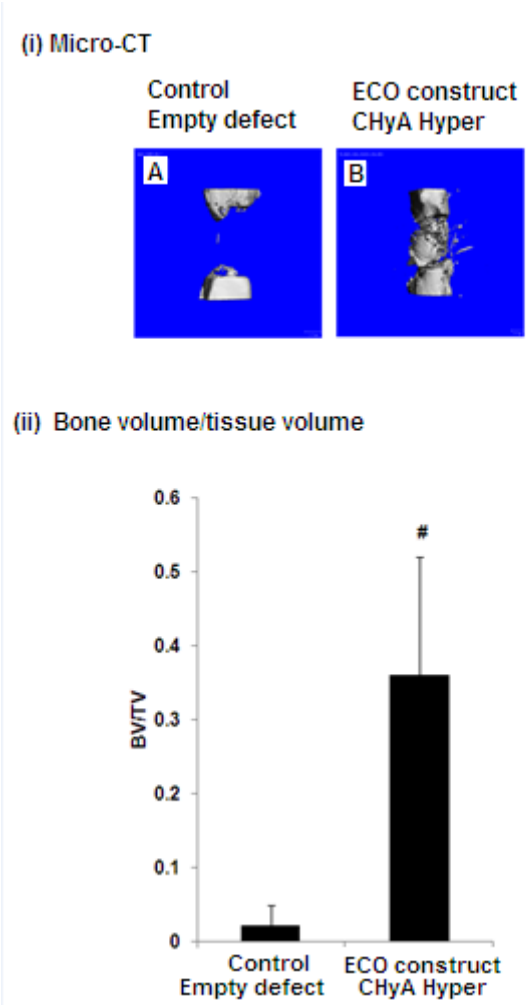

Figure 1: (i) Qualitative micro-CT assessment of early-stage bone formation within a femoral defect either left untreated – empty defect (A), or treated with the ECO construct (B) after 4 weeks in vivo. **(ii)** Quantitative assessment of the ratio of bone volume to tissue volume of the femoral defect either left untreated or treated with ECO construct. There was significantly higher bone volume/tissue volume in the ECO construct group compared to the control group. # denotes $p<0.01$ statistical significant difference.

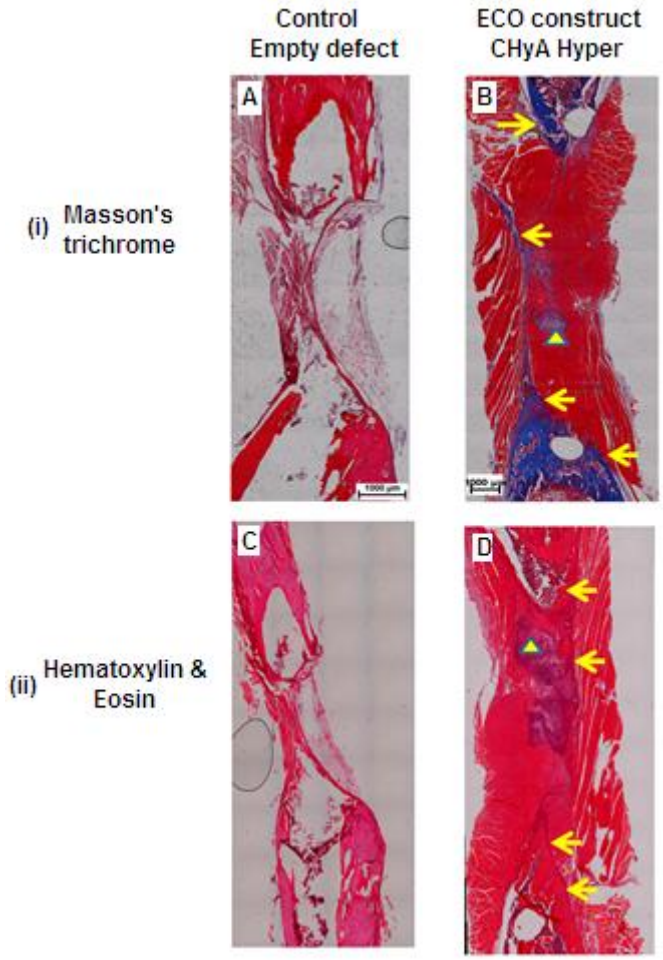

Figure 2: (i) Masson's trichrome staining and **(ii)** Hematoxylin and Eosin staining of femoral defects either left untreated (A, C) or treated with the ECO construct (B, D) after 4 weeks in vivo. The ECO group showed greater mineralised tissue within the defects compared to the other groups. Yellow triangles represent undegraded scaffold matrix within the defect. Yellow arrows represent newly formed mineralised tissue.