Hypoxic preconditioning of myoblasts implanted in a tissue engineering chamber significantly increases local angiogenesis via regulation of angiogenic growth factors and miRNA

C.J. Taylor,[1,2,3,4] J.E. Church,[4] M.D. Williams,[1,2] Y-W. Gerrand,[1,3] E. Keramidaris,[1] J.A. Palmer,[1,3] L.A. Galea,[1] A.J. Pennington,[5] W.A. Morrison[1,2,3] and G.M. Mitchell,[1,2,3] [1]O'Brien Institute Department, St Vincent's Institute, Melbourne, VIC 3085, Australia, [2]University of Melbourne, Department of Surgery at St Vincent's Hospital, Melbourne, VIC 3085, Australia, [3]Faculty of Health Sciences, Australian Catholic University, Melbourne, VIC 3085, Australia, [4]Department of Physiology, Anatomy & Microbiology, School of Life Sciences, La Trobe University, Bundoora, VIC 3083, Australia and [5]Pediatric Plastic and Maxillofacial Surgery, Royal Children's Hospital, 50 Flemington Road, Parkville, VIC 3052, Australia.

A major impediment to progress in tissue engineering (TE) techniques using *in vivo* chambers is the formation of timely vascularization to the implanted TE constructs. While hypoxic pre-conditioning before cell implantation can enhance angiogenesis in constructs formed from stem cells (Lokmic *et al.*, 2007), the effectiveness of hypoxic pre-conditioning has not been studied in tissue constructs from other cell types. This study investigated the effect of hypoxic preconditioning of primary rat myoblasts *in vitro* and its influence on local angiogenesis post-myoblast implantation in a mouse tissue engineering chamber. Animal experiments were approved by the Animal Ethics Committee of St. Vincent's Hospital and conducted in accordance with the codes of practice stipulated by the National Health and Medical Research Council (Australia). Primary rat myoblasts were isolated from neonatal (3-week-old) inbred Sprague-Dawley rats. Animals were anaesthetized (75mg.kg^{-1} ketamine and 10mg.kg^{-1} xylazine, *i.p.*) and the skeletal muscle from the pectoral, abdomen and hind limbs was dissected under aseptic conditions. Tissue was cleared of connective tissue and associated fascia, and myoblast cultures generated using an isolation and enrichment technique described previously (Tilkorn et al 2010). Cells were subjected to either a 90 minute hypoxic preconditioning insult (experimental group) or 90 minutes normoxia (control group) period, followed by a 24 hour rest phase in normoxic culture conditions, prior to implantation. For implantation, nine adult male severe combined immunodeficient (SCID) mice were anaesthetized (4 mg/g chloral hydrate; *i.p.*) and bilateral chambers were created around each of the epigastric vascular pedicles in the groin as previously described (Cronin *et al.*, 2004). Each chamber was filled with 500,000 cells suspended in 45μL of full Matrigel and the open chamber end was closed with bone wax. The skin wounds were closed with metal clips and the mice were allowed to recover. After 14 days animals were anaesthetized again (4 mg/g chloral hydrate; *i.p.*) and euthanized with Lethabarb® before chambers were harvested and processed for immunohistological analysis. For *in vitro* signalling studies, total RNA (including miRNA) was isolated using TriReagent either immediately following the 90 min of hypoxia or normoxia, or following the subsequent 24-hour rest phase. RNA was transcribed to cDNA using a Quantitect Reverse Trancriptase Kit and expression was quantified using a QuantiFast SYBR Green PCR kit, while miRNA was transcribed using a miScript® II RT kit and expression was quantified using a miScript SYBR® Green PCR Kit with mScript Primer Assays (All PCR reagents were obtained from Qiagen). We found that the initial hypoxic insult resulted in significant signalling changes including downregulation of both miR-206 and miR-1, along with upregulation of VEGF-A and downregulation of Angiopoietin-1, and that both MiR-1 and Ang 1 remained significantly downregulated after the 24 hour rest phase. Analysis of tissue chambers 14 days after cell implantation demonstrated that preconditioning did not increase myoblast survival, however there was a significant increase in the percent volume of new blood vessels in chamber tissue indicating that more angiogenesis had occurred in the pre-conditioned constructs. We conclude that preconditioned myoblasts promote vascularization of tissue engineering constructs *via* changes in angiogenic signalling pathways, and that hypoxic pre-conditioning of implanted cells has broad, future applications in tissue engineering and regenerative medicine as a technique to enhance local angiogenesis.

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