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PURPOSE: Vascularized composite allotransplantation (VCA) can include vascularized bone marrow (VBM) to potentially generate chimerism and subsequent tolerance in the recipient. While increasing ischemia time is known to be detrimental to free flaps and solid organ transplants, it is unknown whether increasing VCA ischemia time may diminish VBM’s capacity to achieve chimerism and tolerance. This study determines whether increasing cold ischemia time results in decreased peripheral chimerism levels, increasing loss of the donor bone marrow compartment, and increased skin paddle rejection in a murine VCA model.

METHODS: Twenty-three adult Lewis rats received osteomyocutaneous hind limb allografts from 3 groups of Brown-Norway rats. The groups underwent 0, 6, and 12 hours of cold ischemia at 4°C (n=8, 6, and 9 respectively). Immunosuppressive conditioning consisted of anti-lymphotoyte serum 1cc 3 days pre-operatively and on post-op day (POD) 1, tacrolimus 2mg/kg/day for PODs 1–7, and then concluded with rapamycin 3mg/kg/day for PODs 8–28. Peripheral chimerism levels were drawn after POD 30. Skin paddle was observed for 30 days after immunosuppression cessation for rejection or tolerance. Allograft was collected at POD 60 for flow cytometry and histologic analysis.

RESULTS: Peripheral chimerism levels in both myeloid and lymphoid lines were decreased in the 12 & 6 hours groups versus 0 hours (p<0.01). At POD 60, the amount of donor cells in the allograft bone marrow was significantly decreased at 12 & 6 hours versus the 0 hours group (p<0.05). At 12 hours, hematoxylin & eosin histology demonstrated significantly increased reactive lymphocytes, increased stroma, significantly decreased hematopoetic cells, and significant myelofibrosis. At 6 hours, moderate stromal increases, moderately decreased hematopoetic cells, and moderate myelofibrosis was observed. Trichrome staining revealed viable bone at all time points though with there were increasing amounts of collagen deposition with increasing ischemia and osteoslerosis was noted at 12 hours. Microvasculature was preserved at all time points. By POD 60, 0% of skin paddles survived in the 12 hours group, 17% at 6 hours, and 73% at 0 hours.

CONCLUSION: With increasing ischemia time prior to VCA re-vascularization, there is decreased peripheral chimerism levels, increased loss of donor hematopoetic cells in the donor bone marrow, and increased skin paddle rejection. These findings indicate that ischemia time may significantly limit the capacity for VBM to facilitate chimerism and subsequent VCA tolerance and thus strategies using VBM to induce tolerance may need to consider the additional obstacle of time. Prolonged ischemia time may be a contributing factor to the absence of stable chimerism and tolerance in human VBM-bearing VCA recipients.

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Human ARF Tumor Suppressor Suppresses Zebrafish Cardiac Regeneration

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PURPOSE: This study explores how the Alternative Reading Frame (ARF) tumor suppressor, while preventing oncogenesis, may simultaneously inhibit mammalian epimorphic regeneration. ARF is a tumor suppressor encoded by the Cdkn2a gene in mammals but not lower regenerative vertebrates, previously implicated as a context-sensitive suppressor of regeneration in murine skeletal muscle and humanized ARF-expressing zebrafish fins. We aim to characterize the impact and target of ARF during the more complex and clinically translatable processes of heart regeneration after massive myocardial infarction.

METHODS: Transgenic zebrafish lines expressing ARF under control of the heat shock promoter (hs:ARF) and natural human ARF promoter (ARF:ARF) were used. Heart cryoinjury with a liquid nitrogen probe was performed on anesthetized transgenic fish and wild type (WT) controls. Hearts were collected at various days post-injury (dpi) for analysis. Regenerative progress was analyzed using histology, immunofluorescence, and qPCR of tissue-specific regenerative markers.

RESULTS: ARF expression was upregulated during the cardiac regenerative process and slowed the rate of morphological recovery. In hs:ARF fish, AFOG and troponin staining revealed a 48.7% (p<0.01) reduction in myocardial recovery compared to WT fish. In ARF:ARF fish, myocardial recovery was reduced by 2.3% (p=0.96), 20.4% (p=0.47), 41.3% (p<0.01), 36.1% (p=0.05), and 24.3%
(p<0.01) at 1, 4, 7, 15, and 30 dpi respectively. A cardiomyocyte proliferation index generated by MEF2/PCNA staining confirmed cardiomyocyte-specific suppression in ARF:ARF heart regeneration by 46.6% (p=0.01) at 11 dpi. Tissue-specific regenerative gene expression was tracked by qPCR in ARF:ARF and WT fish. Fgf17b, vegfaa, and Twist1b were reduced by 42% (p<0.01), 43% (p<0.01), and 55% in ARF:ARF hearts at 11 dpi, reflective of decreases in myocardial regeneration, vascular regeneration, and epithelial-to-mesenchymal (EMT) transition respectively. There was no significant difference in fgfr2c expression (p=0.44), a marker of epicardial regeneration.

CONCLUSIONS: Understanding how ARF suppresses cardiac regeneration is important for promoting recovery after heart injury in humans. The timeline of recovery in ARF:ARF fish suggests that ARF does not affect the acute processes of scarring, but rather suppresses cardiomyocyte proliferation. ARF’s selective impact on myocardial regeneration, vascular regeneration, and EMT, while not affecting epicardial regeneration, elucidates that in the context of regeneration, ARF is not indiscriminately expressed in all proliferating cells, but is rather localized to cells undergoing dedifferentiation or transdifferentiation. Our findings show that ARF will require alteration in conjunction with other genes to permit regeneration.

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Extended Graft Survival After Cryopreservation and Storage Below -130°C in a Rat Orthotopic Hind Limb Model

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PURPOSE: Vascularized composite allotransplantation (VCA) is an increasingly used reconstructive option for devastating tissue defects of the face, hand, arm, and most recently the penis. Though highly successful cases have been reported, the growth of the field is restrained by the limited ischemia time that is tolerated by the graft. Long-term graft preservation would allow for transplantation across greater distances and thus improved organ sharing, better donor matching, and provide transplant teams with additional time to pre-condition recipients for novel immunomodulatory regimens. Here we present our first outcomes using cryopreservation in a rat hind limb transplant model.

METHODS: Lewis rats were used as both donors (7) and recipients (14) of orthotopic hind limb transplants. Limbs were flushed with Lactated Ringers containing Heparin, loaded with cryoprotectant formulation (10% DMSO in Culture Medium), cooled at 1°C/min to -90°C and then stored below -130°C for 1–2 weeks. Controls (N=2) were performed without cryopreservation. Intervention groups were A) controlled cooling rate frozen limbs with spontaneous nucleation (N=8) and B) similarly treated limbs with induced nucleation at -4°C (N=4). Transplants were thawed and tissue DMSO concentrations reduced by simultaneously soaking and perfusing the limbs with culture medium containing 0.5M mannitol. Preserved syngeneic hind limbs were then transplanted at the mid-thigh to the recipient animal using a non-suture cuff technique for vascular anastomosis. Recipients were monitored daily until the study endpoint of POD14. Biopsies were acquired at postoperative day (POD) 7 and endpoint. Samples were stained with hematoxylin and eosin for histopathology review. Control and treated limbs were also evaluated without implantation to determine tissue component viability using a metabolic resazurin assay.

RESULTS: Viability evaluation after rewarming demonstrated that the femoral arteries, skin and cartilage were >70% of fresh controls, while the muscle was 35–40% of controls (p<0.05). Blood flow was established in all transplanted limbs. Both control limb transplants were successful to POD14. Only one of eight limbs cryopreserved using spontaneous nucleation survived past POD7 and the recipient was euthanized on POD10. Histopathology revealed regeneration of skeletal myofibers and associated fibrosis. Two of four limbs cryopreserved using induced nucleation (50%) demonstrated gross signs of healing around the ankles and feet by POD7 and further skin and muscle regeneration between POD7-POD14.