In vivo Recruitment of Two "Waves" of Host's Stem/Progenitor Cells by Exogenous Mesenchymal Stem Cells Seeded onto Porous Ceramic Scaffolds and Role of Endothelial Progenitors in the New Bone Formation.

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INTRODUCTION: A better understanding of cell interactions and events occurring within the bone stem cell niche is essential for the comprehension of the cellular and molecular mechanisms governing bone regeneration. It is broadly accepted that vascular development always precedes the osteogenesis process. Inadequate vascularization still remains one of the major limitations in bone development and repair repair and in particular in bone tissue engineering. Indeed, the ultimate efficiency of an artificial bone construct depends on timely delivery, exchange of oxygen and nutrients from surrounding blood vessels to the MSC and removal of waste products. Therefore, mastering the control of angiogenesis as a microvascular network with properly structured spatial organization will be crucial to any attempts to obtain bone regeneration/repair by a tissue engineering approach.

RESULTS: Standard X-ray micro-tomography (microCT) is a technique that allows a good visualization of the structure of mineralized tissues and biomaterials, but it fails to finely discern soft tissues. We used X-ray synchrotron radiation holotomography to visualize at three-dimensional (3D) level a microvascular networks for the first time with no need for contrast agents, and to extract quantitative structural data in a bone engineered construct implanted in a mouse and to investigate the onset of the vascular network.

We recently reported the ectopic formation of bone made by cells of recipient's origin following the implantation of porous ceramic cubes seeded with exogenous mouse mesenchymal stem cells (MSC) into immunocompetent syngenic mice. The implanted MSC appeared pivotal at the early stages of the tissue development. No bone formation was observed when the porous ceramic cubes were implanted without prior seeding with MSC. To investigate the nature of stem/progenitor cells, including endothelial progenitors, locally present or recruited in the bone regenerative niche and to have a better comprehension of the cross talk and interactions among these cells, we implanted porous ceramic cubes seeded with Green Fluorescent Protein (GFP)-positive MSC in syngenic, GFP-negative recipients. Implants were harvested at different times and enzymatically digested to generate single-cell suspensions. Recovered cells were sorted to separate GFP+ seeded MSC and GFP- cells recruited into the scaffold.

By this approach, we isolated and characterized two "waves" of cells of different nature actively participating in the formation of the newly formed bone and specifically migrating from the host to the implanted graft only when the ceramic was seeded with exogenous MSC. The first specific population, recovered 7 days after implantation, was enriched in CD31+ endothelial cells, and the second one, recruited at day 11, was enriched in CD146+ pericyte-like cells. The cells recovered 7 days after implantation formed vascular structures *in vitro* when specifically tested, whereas the cells recovered after 11 days formed a tissue engineered bone when re-implanted in immunodeficient mice in combination with bioceramic scaffolds. The mobilization of the second wave was tightly dependent on the presence of the first population. Furthermore, we showed that the populations enriched in CD146+ elements retained some fundamental properties distinctive of stem cells, such as the capacity of performing an extraordinarily high number of *in vitro* cell divisions, and the maintenance of a differentiation potential.

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