1. **Aim 1. To characterize the vascular process, i.e. activation and resolution phases, within skeletal muscle regeneration**

Hypothesis

*The patterns of vascular activation and maturation and FAP expansion and clearance are coupled during muscle regeneration.*

FAPs contribute to skeletal muscle regeneration through an expansion and clearance phase that occurs at 3-4 days and between 4 and 7 days after damage, respectively [18](#_ENREF_18). Angiogenesis also begins early (3 days) during muscle regeneration, a notion supported by the reported endogenous pattern of angiogenesis-related factors [31](#_ENREF_31), [59](#_ENREF_59). The acute inflammation resulting after tissue damage destabilizes endothelial junctions increasing vessel permeability that allows plasma components and inflammatory cells to exit the bloodstream, and leads to endothelial cell-activation [42](#_ENREF_42). Subsequent vessel maturation involving adjacent pericytes and smooth muscle cells alters microcirculatory properties such as permeability [60](#_ENREF_60). However, the sequence of changes in endothelium-gene expression, morphology, and functionality (vascular perfusion and permeability) of newly induced vessels during skeletal muscle regeneration have not been fully described yet. Such descriptive work is key to establish correlations between the various phases of angiogenesis and the activity of other cellular components of the regenerative process such as FAPs, myogenic cells, and inflammatory cells, and will lead to the formulation of hypotheses on the molecular mechanisms underlying such correlations, which will be functionally tested in the next aim.

Experimental procedure:

The activation and maturation/stabilization phases of angiogenesis will be defined by ex-vivo gene expression analysis on endothelial cells freshly isolated from tibialis anterior (TA) and gastrocnemius (GC) muscles collected from healthy mice treated with saline solution or the myotoxin NTX [18](#_ENREF_18). This analysis will take advantage of a core service for transcriptome analysis by next generation sequencing recently established in the Rossi laboratory, which has optimized techniques to obtain high quality libraries from FACS-sorted cells. Pathway analysis (Ingenuity) will be used to interpret these results, focusing on the expression of genes known to be part of the activation signature of signaling pathways involved in angiogenesis. In addition, a candidate gene approach will be taken, in which particular attention will be given to: Ang-2, VEGF, Dll4, MPC-1and the receptors Tie2, VEGF-R, and Notch-1, for the activation phase, and PDGF-B, EphrinB2, TGF-β1, Sema3A and their receptors PDGFRβ, EphB4, TGFβ-R, and NP-1 for the maturation/stabilization one. These analyses will be performed at 1, 3, 5, 7, 10, 14, and 28 days after the tissue damage.

The morphology of vascular structures newly induced during muscle regeneration will be assessed by immunofluorescence staining of fresh-frozen sections of TA and GC muscles collected from healthy mice treated with saline solution or NTX, and analyzed by standard or confocal microscopy [18](#_ENREF_18), [55](#_ENREF_55). Antibodies that specifically recognize EC (PECAM), smooth muscle cells (α-smooth muscle actin, α-SMA), pericytes (NG2) and vascular basement membranes (laminin and collagen type IV) will be used. The maturation index of the induced vessels will be calculated as the percentage of endothelial structures associated with pericytes [61](#_ENREF_61). Notably, pericytes will be unequivocally defined as perivascular cells displaying a typical dendritic morphology, expressing NG2, but not α-SMA, and embedded in the vessel basal lamina [55](#_ENREF_55).

The establishment of functional blood flow in newly induced vascular structures will be assessed by intravenous injection of fluorescently labeled tomato lectin, which binds to the luminal endothelial surface of vessels only if they are connected to the systemic circulation. An amount of 50 μg of fluorescein isothiocyanate (FITC)-labeled *Lycopersicon esculentum* lectin in 50 μl (Vector Laboratories) will be injected into the femoral vein and allowed to circulate for 4 minutes before perfusion of 1% paraformaldehyde and collection of TA and GC leg muscles, which will be processed as previously described [62](#_ENREF_62).

The vascular leakage will be quantified by injecting intravenously the Evans Blue dye, which binds to plasma proteins and therefore is used as a tracer for their extravasation [63](#_ENREF_63). After 4 hours, the animals will be sacrificed by fixative perfusion to clear intravascular dye, the tissue will be harvested and weighed and the extravasated dye will be extracted with formamide and quantified with a spectrophotometer as previously reported [54](#_ENREF_54). *In aggregate, these analyses will allow us to establish the precise temporal sequence of changes taking place in the vascular compartment during muscle regeneration, and to start building temporal correlations with the behavior of other cell types involved, the mapping of which has been a focus of the Rossi lab for years.*