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Cell sorting of various cell types from mouse and human skeletal muscle

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ABSTRACT

Muscle stem cells or satellite cells are required for skeletal muscle regeneration. It has been shown that the satellite cell microenvironment, including neighboring cells such as endothelial cells, macrophages or fibroblasts are essential for complete and efficient regeneration. A deficient behavior of these cells compromises regeneration. Therefore, there is a strong interest in understanding the cellular and molecular interactions at work between these cell types during muscle regeneration. Fluorescence-activated cell sorting allows to isolate these four cell types at different time points of regeneration, for further high throughput or behavioral experiments. We present here a method for the concomitant isolation of 4 cell types present in the regenerating skeletal muscle: muscle stem cells, endothelial cells, fibro-adipogenic precursor cells and macrophages.

1. Introduction

Normal adult skeletal muscle regenerates ad integrum after injury. In experimental injury models in mouse, muscle tissue undergoes necrosis followed by rapid infiltration of immune cells. Few days later (depending on the type and extent of the injury), new myofibers are formed. After about one month, regenerating muscle is similar to uninjured muscle except that the myofibers exhibit central nuclei. Satellite cells (SCs) are the principal contributors for the regeneration of skeletal muscle. Upon myofiber damage, quiescent SCs become activated, divide, and give rise to a population of transient amplifying myogenic precursor cells (skeletal myoblasts). Later on, most of these myogenic precursors exit the cell cycle, lose Pax7 expression, differentiate and fuse with the host fibers or between them to form new myofibers. A subset of myogenic precursor does not differentiate and self-renews to replenish the pool of SCs [1].

While SCs are indispensable for skeletal muscle regeneration [2,3], other cell types, such as fibroblasts, endothelial cells and immune cells, among which macrophages, have been shown to play important roles in this process. Indeed, these cells establish specific interactions with SCs to ensure efficient muscle regeneration. However, the cell dynamics are highly complex and occur with specific temporal and spatial kinetics.

Immune cells invade the muscle early after injury, first as neutrophils, then as macrophages. Macrophages serve as key effectors in the muscle stem cell niche to guide SCs through the regeneration process through direct interactions with myogenic cells. They first invade the muscle as inflammatory macrophages (coming from inflammatory circulating monocytes) and specifically stimulate myogenic precursor proliferation and trigger Fibro-Adipogenic Precursor (FAP) apoptosis (see below). Then, upon the phagocytosis of necrotic muscle debris, they switch their inflammatory status into anti-inflammatory cells, that stimulate myogenic differentiation and promote myofiber growth [4–6].

Skeletal muscle is laced with a dense microvasculature and most quiescent SCs are located close to capillaries. Vascular cells also coordinate both the acute SC response and the late stages of muscle regeneration when the tissue returns to homeostasis. Endothelial cells (ECs) predominantly exhibit pro-myogenic effects on SCs, through a strong stimulating effect on their differentiation, while peri-endothelial cells, such as smooth muscle cells and fibrogenic cell types, are crucial for the re-entry into quiescence on completion of regeneration. Inversely, SCs and myogenic precursors have been shown to exhibit pro-angiogenic activity [6–8].

Finally, fibroblasts and FAPs are the main source of matrix proteins during muscle regeneration and are required for muscle regeneration [9]. Upon muscle injury, FAPs are activated, rapidly expand and control matrix remodeling, while providing an environment favoring myogenic differentiation [10,11].

While these observations show the existence of specific interactions between SCs and the neighboring cells during muscle

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regeneration, the molecular mechanisms involved in this complex network of communication are still poorly known. In this study, we describe a protocol for isolating pure populations of SCs, macrophages, ECs and FAPs from regenerating mouse skeletal muscle and for isolating SCs and ECS from human muscle samples.

2. Isolation of murine muscle cells

2.1. Induction of muscle regeneration

Muscle injury was performed by the injection of cardiotoxin (CTX), a kinase C inhibitor extracted from *Anja nigricollis* snake venom, that induces depolarization and contraction of muscle cells and destroys the cell membrane structure [12]. CTX injection provides an homogeneous damage in the whole muscle, and the following sequential steps of the regeneration process are well described [13]. Intramuscular injection of CTX (12 μM, 50 μl, Latoxan, France) was performed in the *Tibialis Anterior* muscle of adult male mice [14]. Adult 6–8 week-old male C57Bl/6 mice were bred and used in compliance with French and European regulations. Principal investigator is licensed for these experiments and the protocols were approved by local Animal Care and Use Committee and the French Ministry of Agriculture. Usually, the time points studied are days 1, 2, 4 and 8 after injury. At day 1 after injury, myofibers are necrotic, while immune cells (neutrophils and circulating monocytes) enter into the damaged muscle. At day 2, macrophages phagocyte the dead myofibers and FAPs are highly expanding while SCs are also in the amplification phase. At day 4 after injury, the regeneration process is visible with the appearance of the new regenerating myofibers indicative of myogenic differentiation. Macrophages are still numerous in supporting this process, while FAPs are remodeling the matrix and angiogenesis takes place. At day 8, muscle has almost recovered its original appearance, with nanofibers characterized by the central location of their nuclei, microvasculature maturing and the almost disappearance of macrophages and FAPs.

2.2. Flow cytometry

All materials and reagents used are listed in Tables 1 and 2 respectively.

2.2.1. Cell preparation

1. Prior dissection, prepare the digestion medium (collagenase B 10 mg/ml – dispase II 2,4U/ml) in DMEM/F12 serum-free (1 ml/muscle). Heat the medium at 37 °C and filtrate through a 0.22 μm filter.
2. Dissect *Tibialis Anterior* of both hind limbs. In a Petri dish on ice, roughly discard visible fat, tendons and fascia.

Table 2
Reagents.

Reagent	Cat. No	Manufacturer
Murine muscle cell isolation		
DMEM/F12	31331-028	Gibco
Collagenase B 10 mg/ml	11 088 831 001	Roche
Dispase II 2.4 U/ml	04 942 078 001	Roche
Fetal Bovine Serum	10270-106	Gibco
ACK Buffer	10-548E	Lonza
1X PBS	14190-094	Gibco
Fc block	130-059-901	Miltenyi
Anti α7 integrin-647	AB0000538	AB lab
Anti CD34-FITC	11-0341	eBioscience
Anti CD45-PE	12-0451	eBioscience
Anti CD31-eFluor450	48-0311	eBioscience
Anti Sca1-PerCP-Cy5.5	45-5981	eBioscience
Anti F4/80 APC-eFluor780	47-4801	eBioscience
Anti CD140a PE-Cy7	25-1401	eBioscience
Rat IgG2a Isotype control FITC	11-4321	eBioscience
Rat IgG2b Isotype control PE	12-4031	eBioscience
Rat IgG2b Isotype control eFluor 450	48-4031	eBioscience
Rat IgG2a Isotype control PerCP-Cy5-5	45-4321	eBioscience
Rat IgG2a Isotype control APC-eFluor 780	47-4321	eBioscience
Rat IgG2a Isotype control PE-Cy7	25-4321	eBioscience
Human muscle cell isolation		
ECGMV2	C-22022	Promocell
Collagenase B 1 mg/ml	11 088 831 001	Roche
Dispase II 2.4 U/ml	04 942 078 001	Roche
Fetal Bovine Serum	10270-106	Gibco
1X PBS	14190-094	Gibco
Anti CD31-FITC	11-0319	eBioscience
Anti CD56-APC	555518	BD Pharmingen
Mouse IgG1 Isotype Control FITC	11-4714	eBioscience
Mouse IgG1 Isotype Control APC	555751	BD Pharmingen

Table 1
Material.

Material	Cat. No	Manufacturer
Murine muscle cell isolation		
Material for mouse dissection (thin forceps, rasor blade or thin scissors, thin sharp scissors)		
Petri dishes	11815275	Thermo Fisher Scientific
0.22 μm filters	16532	Minisart
Polypropylene Round Bottom Tube	352063	BD Falcon
Polystyrene Round Bottom Tubes	352054	BD Falcon
70 μm cell strainers	130-098-463	Miltenyi
30 μm celltrics strainers	04-004-2326	Sysmex
50 ml Polypropylene conical tubes	352070	BD Falcon
15 ml Polypropylene conical tubes	352097	BD Falcon
Human muscle cell isolation		
30 ml Polystyrene Sterilin tube	080005	Dominique Dutscher
0.22 μm filters	16532	Minisart
70 μm cell strainers	130-098-463	Miltenyi
100 μm cell strainers	130-098-463	Miltenyi
50 ml Polypropylene conical tubes	352070	BD Falcon
15 ml Polypropylene conical tubes	352097	BD Falcon
FACS ARIA III		BD Biosciences

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