Analytical and Bioanalytical Chemistry

Electronic Supplementary Material

Stem cell survival is severely compromised by the thymidine analog EdU (5-ethynyl-2′-deoxyuridine), an alternative to BrdU for proliferation assays and stem cell tracing

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Supplemental Methods

Animals

The C57Bl/6 mouse strain was originally purchased from Taconic Europe and bred as recommended whereas Sprague Dawley rats were obtained from Taconic Europe and used directly. All animal experiments were approved by, the Danish Council for Supervision with Experimental Animals (#2010/561-1792).

Isolation and culture of cells

Neonatal mouse EPCs were obtained and characterized as recently described [1] from 2-3 day old C57Bl/6 mice. Briefly, EPCs were dissociated from the surface of the heart by mild trypsin treatment, and harvested cells plated at low density and cultured for two passages as in general. The purity of EPCs were approximately 99% at the time of experiment as previously reported[1]. The mouse C2C12 myogenic precursor cell line and the embryonic human kidney derived cell line Hek293T were originally obtained from ATCC and kept in accordance with supplier's

recommendations. Adipose derived stem cells were isolated from gonadal fat pads of male Sprague Dawley rats (age: 3 months) more or less as previously described[2]. Briefly, tissue was minced extensively and digested with 0.86U/mL Collagenase NB 4 Standard Grade (Serva, Germany)/HBSS/2.265mM Ca2+ at 37°C for 60 min. Harvested cells were filtered, washed, and cultured in growth medium (DMEM/1.0g/L glucose/25mM HEPES supplemented with 4mM Ultraglutamine/10% FBS (all products from Lonza, UK)/1% PS).

For co-culture studies C2C12 and EPCs were cultured at a 2:1 ratio, and myogenic differentiation was accomplished by changing the medium to DMEM/2% Horse serum/1%PS as previously described[3,2].

Cells were labeled at high density with 4 μM CellTracker CM-DiI (Invitrogen, #C7000) as recommended by manufacturer, and then by 0-10μM EdU (Invitrogen) for 1h or 24h at 37 °C in humidified 5 % CO2. Cells were carefully washed to remove excess dye.

Coulter cell counting

Cultured cells were gently detached with 0.25% Trypsin-EDTA (Gibco), pelleted, and resuspended in Hank's Balanced Salt Solution (HBSS, Lonza)/10%CS/1%PS. Cell number and cell diameter were determined using a Beckman Coulter Counter Z2 (Ramcon) fitted with a 100 μm aperture. The size range of particles counted was set at 10-25 μm, and counting was performed in the indicated number of independent experiments each comprising triplicate measurements. Cell diameter was used to calculate cell volume.

Skeletal Muscle Regeneration model and transplantation

Skeletal muscle lesion was performed as previously described[3,2] in 12-week-old female C57BL/6 mice. Briefly, hind limb muscles of anesthetized mice were carefully exposed and one wound were introduced starting from the outer fascia to the widest part of m. gastrocnemius until reaching the calfbone. $1x10^5$ of labeled cells were injected directly into the lesion and in four sites around the lesion, after which the skin incision was sutured. The m. gastrocnemius was dissected out from mice 7 or 10 days following muscle damage. For immunohistochemistry, the tissues were snap frozen.

Immunofluorescence

Immunocytochemistry was performed as previously described[3,2]. Cultured cells or cryosections were gently fixed in 4% normal buffered formaldehyde (NBF) (10 min.), permeabilized for 10 min. in 0.3% triton X-100/TBS, blocked in 2 %BSA/TBS (10 min.), and incubated for 2 hours with primary antibodies (see below) diluted in 1%BSA/TBS. Secondary antibodies used were Alexa 647, 555 or 488 donkey α-IgG (1:200, Molecular Probes), and mounting medium contained DAPI (Vectashield, Vector Lab.).

Microscopic examinations were performed using a Leica DMI4000B Cool Fluo Package instrument equipped with a Leica DFC340 FX Digital Cam. In all experiments, exposure (camera settings) and picture processing (brief adjustment of contrast/brightness and color balance by using Photoshop) were applied equally to sample sections and controls (Isotype or no primary antibody present). Antibodies used included: goat anti-desmin (1:50, Santa Cruz Biotechnology), mouse anti-myogenin (1:100, DAKO), and rat anti-Laminin-2 (1:50, Abcam).

Statistical analysis

All analyses comprised at least three independent experiments. One-way ANOVA with a Tukey's Multiple Comparisons posttest was performed using GraphPad Prism to test significance ($p<0.05$).

Supplemental Figures

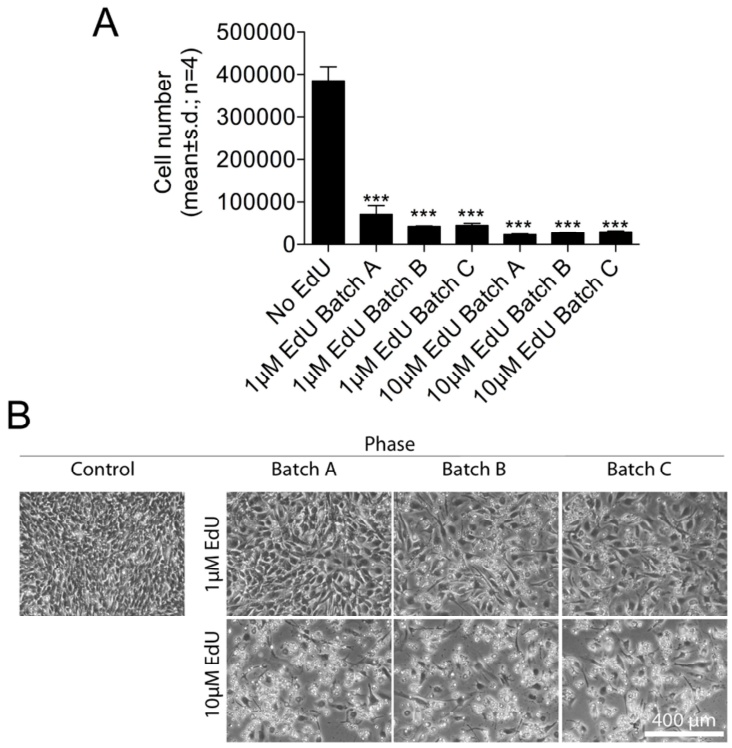

Fig S1. EdU reduces myogenic cell survival independent of batches

(A) Coulter-counting at 48h was used to determine the number of C2C12 myogenic cells labeled for 24h with the indicated concentrations and batches of EdU (mean±s.d.; n=4), whereas (B) phase microscopy was used to confirm cell morphology and number. Representative images are shown. Statistical significance as compared to 0µM EdU (tested by One-way ANOVA with a Tukey's Multiple Comparisons posttest) is indicated (p***<0.0001)

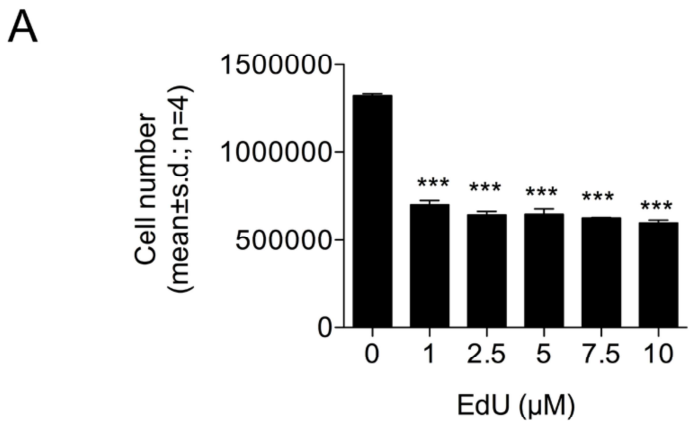

Fig S2. EdU reduces cell survival of human embryonic kidney cells

(A) Coulter-counting at 48h (proliferating) was used to determine the number of human embryonic kidney cells labelled for 24h with the indicated concentrations of EdU. (mean±s.d.; n=4). Statistical significance as compared to 0μM EdU or as indicated by a line (tested by One-way ANOVA with a Tukey's Multiple Comparisons posttest) is indicated (p***<0.0001)

References

1. Aagaard KS, Ganesalingam S, Jensen CH, Sheikh SP, Andersen DC (2013) Poor engraftment potential of epicardial progenitors upon intramyocardial transplantation into the neonatal mouse heart. International journal of cardiology. doi:10.1016/j.ijcard.2013.05.061

2. Andersen DC, Schroder HD, Jensen CH (2008) Non-cultured adipose-derived CD45- side population cells are enriched for progenitors that give rise to myofibres in vivo. Experimental cell research 314 (16):2951-2964. doi:10.1016/j.yexcr.2008.06.018

3. Andersen DC, Petersson SJ, Jorgensen LH, Bollen P, Jensen PB, Teisner B, Schroeder HD, Jensen CH (2009) Characterization of DLK1+ cells emerging during skeletal muscle remodeling in response to myositis, myopathies, and acute injury. Stem cells 27 (4):898-908. doi:10.1634/stemcells.2008-0826