Developmental Biology 365 (2012) 91–100

Contents lists available at SciVerse ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/developmentalbiology

Noggin recruits mesoderm progenitors from the dorsal aorta to a skeletal myogenic fate

Gonzalo Ugarte [a,1], Ornella Cappellari [a,b,2], Laura Perani [a,2], Anna Pistocchi [c], Giulio Cossu [a,b,c,*]

[a] Division of Regenerative Medicine, San Raffaele Scientific Institute, Milan
[b] Department of Cell and Developmental Biology, University College London, UK
[c] Department of Biology, University of Milan, Italy

ARTICLE INFO

Article history:
Received for publication 8 June 2011
Revised 8 February 2012
Accepted 10 February 2012
Available online 18 February 2012

Keywords:
Dorsal aorta
mesoderm progenitors
skeletal myogenesis BMP2/4
Noggin
NG2 proteoglycan

ABSTRACT

Embryonic mesoangioblasts are the *in vitro* counterpart of vessel-associated progenitors, able to differentiate into different mesoderm cell types. To investigate signals recruiting these progenitors to a skeletal myogenic fate, we developed an *in vitro* assay, based upon co-culture of E11.5 dorsal aorta (from MLC3 F-nLacZ transgenic embryos, expressing nuclear beta galactosidase only in striated muscle) with differentiating C2C12 or primary myoblasts. Under these conditions muscle differentiation from cells originating from the vessel can be quantified by counting the number of beta gal + nuclei. Results indicated that Noggin (but not Follistatin, Chordin or Gremlin) stimulates while BMP2/4 inhibits myogenesis from dorsal aorta progenitors; neutralizing antibodies and shRNA greatly reduce these effects. In contrast, TGF-β1, VEGF, Wnt7A, Wnt3A, bFGF, PDGF-BB and IGF1 have no effect. Sorting experiments indicated that the majority of these myogenic progenitors express the pericyte marker NG2. Moreover they are abundant in the thoracic segment at E10.5 and in the iliac bifurcation at E11.5 suggesting the occurrence of a cranio-caudal wave of competent cells along the aorta. BMP2 is expressed in the dorsal aorta and Noggin in newly formed muscle fibers suggesting that these two tissues compete to recruit mesoderm cells to a myogenic or to a perithelial fate in the developing fetal muscle.

Introduction

Classic transplantation experiments showed, and recent data confirmed that all skeletal myoblasts of the vertebrate body originate from somites (Buckingham et al., 2003; Christ and Ordahl, 1995), blocks of paraxial mesoderm that form and mature in a cranio-caudal sequence along the axial structures of the embryo, *i.e.* notochord and neural tube. Specifically, myogenic progenitors, identified by the expression of the Pair-ruled gene Pax3, are located in the dorsal dermo-myotome and are specified by Wnt and Shh signals emanating from the axial structures and from the dorsal ectoderm (Cossu and Borello, 1999; Reshef et al., 1998). The dorsal dermomyotome also contains progenitors for dermis, tendons, vascular endothelium and smooth muscle, some of which also express Pax3 and migrate towards the dorsal aorta (Brent and Tabin, 2002; Esner et al., 2006), while the anatomical location of skeletal myogenic progenitors is established, the exact lineage relationship among different cell types of the dorsal somite and their differentiated tissue progeny remains less defined. For example, retroviral lineage marking, unexpectedly but unequivocally demonstrated the existence of a common somitic progenitor for both endothelium and skeletal muscle (Kardon et al., 2002). Moreover, *in ovo* electroporation experiments have shown that BMP and Notch interfere with somitic cell fate diverting them from skeletal muscle and inducing endothelial and smooth muscle fate respectively (Ben-Yair and Kalcheim, 2008). Thus it appears that in mammalian mesoderm, cell fate is established in response to signaling molecules, locally produced by neighbor, differentiated cells. Interfering with the expression of one or more specific molecules thus results in altered proportion of competent cells undergoing a given differentiation pathway (Shin and O'Brien, 2009). While these reports focused on somites, much less is known on the subsequent phases of pre-natal skeletal muscle histogenesis. If multipotent progenitors exist in the somite and likely in other regions of the mesoderm, they should presumably undergo more than one differentiation pathways. In the last ten years a large number of progenitor cells have been clonally isolated and expanded *in vitro* from embryonic or adult mesoderm tissues, and shown to be multipotent (Asahara et al., 1997; Asakura and Rudnicki, 2002; De Bari et al., 2003; Minasi et al., 2002; Reyes and Verfaillie, 2001; Rodriguez et al., 2006; Tamaki et al., 2002; Toma et al., 2001; Torrente et al., 2004). With the possible exception of mesenchymal stem cells, little is known on the origin, lineage relationships and differentiation potency of these cells.

* Corresponding author at: Department of Cell and Developmental Biology, University College London, Rockefeller Bldg. 21 University Street, London WC1E 6BT.
E-mail addresses: gonzalo.ugarte@usach.cl (G. Ugarte), g.cossu@ucl.ac.uk (G. Cossu).
[1] Current address: Departamento de Biología, Facultad de Quìmica y Biologìa, Universidad de Santiago de Chile.
[2] These two authors contributed equally.

0012-1606/$ – see front matter © 2012 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2012.02.015

Mesoangioblasts were initially isolated from the embryonic dorsal aorta and partially characterized as cells expressing early endothelial and pericyte markers, and able to differentiate into different types of solid mesoderm, both *in vitro* and also when transplanted in chick embryos *in ovo* (Minasi et al., 2002) Embryonic mesoangioblasts undergo smooth muscle differentiation if exposed to TGF-β but do not spontaneously differentiate into skeletal muscle. However, if genetically labeled, mesoangioblasts, cultured together with unlabeled differentiating myoblasts undergo fusion and activate expression of muscle genes (Minasi et al., 2002). It is still currently unknown what are the signals released by differentiating muscle cells that activate myogenesis in mesoangioblasts. Here we show that muscle-derived Noggin – an antagonist of BMP-2/4 activity - recruits cells from the dorsal aorta to skeletal myogenesis and this activity is competed by endothelial-derived BMP that rather recruits these cells to a perithelial, smooth muscle fate.

Materials and Methods

Mice

MLC3F-nlacZ transgenic mice express nuclear β-gal under the transcriptional control of the myosin light chain 1/3 F promoter/enhancer (Kelly et al., 1995). In Myf5^{nlacZ} mice nuclear LacZ was targeted to the Myf5 locus (Tajbakhsh et al., 1996). EGFP mice have also been described (Hadjantonakis et al., 1998)

Co-culture of embryonic DA and C2C12 myoblasts

C2C12 myoblasts were plated at sub-confluence (10^4x ml) as a drop of 50 μl in a 0.5 cm area in the center of individual wells of a 24-well plate. After adhesion to the substrate, a single freshly isolated embryonic DA (dissected from the thoracic upper segment to the iliac bifurcation) from MLC3F-nlacZ embryo (Minasi et al., 2002) was added, and covered by a drop of Matrigel™ diluted 1:4. The co-culture was maintained in growth medium (DMEM + 10% FBS) for three days and then shifted to differentiation medium (DMEM + 5% horse serum). After three additional days the co-culture was fixed with paraformaldehyde 4% and then incubated with X-gal staining solution overnight at 37 °C. C2C12 myoblasts, 10 T1/2 fibroblasts, D16 mesoangioblasts and H5V endothelial cells were described before (Minasi et al., 2002). In some of these experiments, cells were labeled with BrdU (5 μM) in complete medium for 2 hours at different days of culture and in different experimental conditions.

DA-derived cells culture

Aorta-derived single cells were obtained by digestion of freshly isolated DA (E11.5) in PBS without calcium-magnesium containing 0.45 mg/ml of collagenase V (Sigma) and 0.15 mg/ml of dispase (Gibco) for 40 min at 37 °C. After recovery in 20% FBS containing growth medium, cells were either cultured for 12 hours and then stained with the antibodies described below, or separated by FACS sorting analysis (see below) and then co-cultured with C2C12 myoblasts as described above.

Growth Factors

Growth Factor Reduced Matrigel™ Matrix was purchased from BD Bioscience. Recombinant human Noggin/Fc chimera and neutralizing anti-human BMP-2/4 antibody from R&D Systems, Inc. BMP-2 and BMP-4 from Peprotech.

Cryosections

Mouse embryos were fixed in 4% PFA, washed in PBS, dehydrated by washing them in PBS containing increasing concentration of sucrose (10, 20 and 30%), included in O.C.T™ and frozen in liquid nitrogen-cooled isopentane. 7 μm-thick sections were cut with a Leica cryostat.

Immunofluorescence

Adherent cell cultures, co-cultures and cryosections were processed with the same standard protocol: after fixation with 4% PFA, samples were incubated with PBS 1% BSA 0.1% Triton X-100 (1 h at RT), blocked with donkey or goat serum 10% in PBS (30 min at RT), incubated with the primary antibody (1 h at RT), washed three times and incubated with the secondary antibody (Alexa Fluor® conjugated (Invitrogen), diluted 1:500, 1 h at RT). Then the samples were washed three times and incubated with Hoescht (1 mg/ml × 5 min at RT), washed and mounted. The primary antibodies used were: rabbit anti BMP-2 (1:250, Abcam), rat anti CD31 (1:2, Hybridoma Bank), mouse anti MyHC (1:2, MF20, Hybridoma Bank), goat anti Noggin (1:100, R&D Systems), mouse anti smooth muscle heavy chain (1:250, Abcam), rabbit anti NG2 (1:200, Chemicon), goat anti β-galactosidase (1:300, Biogenesis), rabbit anti Myf5 (1:150, Santa Cruz), mouse anti MyoD (1:200, Dako), anti-Phospho-Smad 1-5-8 (1:200 Cell Signaling Technology®), anti-BrdU (1:100 Amersham GE Healthcare).

In situ hybridization

Previously published probes were: Bmp4 (Furuta et al., 1997), and Noggin (Ybot-Gonzalez et al., 2007) In situ hybridization was carried out as described by (Henderson et al., 1999) with samples fixed for 2 h in 4% paraformaldehyde/phosphate buffered saline, then rinsed with PBS-Tween, dehydrated in 100% methanol and stored at −20 °C until processed for WISH. Antisense riboprobes were previously in vitro labelled with modified nucleotides (digoxigenin, Roche).

Semiquantitative RT-PCR

Total RNA was isolated from cells using TRIzol^r (Invitrogen) or RNeasy^r Micro Kit (Quiagen). cDNA was synthesized from total RNA previously treated with DNAse I, primed with random primers, and then reversed transcribed with Moloney murine leukemia virus reverse transcriptase. PCR protocols for amplify transcripts for BMP-2, Noggin and BMPR1A were performed as described in (Goulley et al., 2007; Hager-Theodorides et al., 2002; Van der Horst et al., 2002).

Primers

BMP-2 Fw	TGTGACCAGACTATTGGACACC	(454 bp)
BMP-2 Rv	AGTTCAGGTGGTCAGCAAGG	
GAPDH Fw	TTCACCACCATGGAGAAGGC	(238 bp)
GAPDH Rv	GGCATGGACTGTGGTCATGA	
Noggin Fw	TGAGCAAGAAGCTGAGGAGG	(334 bp)
Noggin Rv	GGATCGATCAAGTGTCTGGG	
BMPR1A Fw	GAAGTTGCTGTATTGCTGA	(216 bp)
BMPR1A Rv	GTAATACAACGACGAGCC	

Agarose beads

BMP-2 and BMP-4 were loaded in agarose beads (Affi-Gel^R Blue Gel, BioRad) incubating beads with growth factors at 3 μg/mL in PBS 0.1% BSA for 1 h at RT and then washed three times with PBS.

Transfection

C2C12 myoblasts were transfected with the shRNA for Noggin containing plasmid using Lipofectamine™ and PLUS™Reagent (Invitrogen) and further selectioned with puromycin (2 μg/ml) for three days.