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PAX3 MUTANT MESOANGIOBLASTS ARE DEFECTIVE IN MYOGENIC DIFFERENTIATION

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INTRODUCTION

MYOGENESIS

In vertebrates skeletal muscle derivates from embryo mesodermal sheet as demonstrated by quail-chick transplant experiments (Chevallier et al., 1977; Jacob et la., 1977).

Somites are little spheres composed of mesenchimal cells aggregated as epithelium around a central cavity. They derive from segmentation along paraxial mesoderm rostro-caudal axis, set besides the neural tube.

In the mouse somitogenesis occurs at seven day of embryonic life, proceeding in a rostro-caudal direction. Somites are delimitated by a basal membrane separating them from neural tube, notocord, dorsal ectoderm and lateral mesoderm. Soon after somite formation, the cells of the ventral region loose their epithelioid morphology and give rise to sclerotome, a pool of mesenchimal cells weakly connected, from which cartilage of vertebral column and ribs take rise. Simultaneously, the upper region of somites differentiate into the dermamyotome, a sort of column epithelium, that will be the source of muscle progenitor cells and precursors of other tissues.

Muscle differentiation in the embryo occurs in two main steps generating respectively primary and secondary fibres. In the mouse myogenesis begins at E8.5 (8.5 days prenatal life). Myoblasts committed to myogenic fate begin to proliferate before terminal differentiation. This cells begin to express MRF (Muscle Regulatory Factors), exit form cell cycle and place on self between G0 and G1 and then they differentiate into mononucleate myocytes then fusing into multinucleated myotubes. The beginning of differentiation is preceded by the expression of Miogenein, one of the MRFs and some members of the family of the MEF2 (Myocyte Enhancer Factor 2) transcription factors, that activate the expression of muscle-specific genes like MCK (Muscle creatine chinase), subunits of acetilcoline receptor and the sarcomeric proteins. During mouse embryogenesis, the pattern of expression of MRFs has the same cefalo-caudal profile of somitogenesis (Buckingham M., 1992). Myf-5 is expressed before the others, since 8 d.p.c. (days post coitum) in the myotome. Its expression reaches a peak at 10 d.p.c., beginning to decrease at 14 d.p.c.. In the same structure, following Myf-5 begins the expression of: Miogenein at 8,5 d.p.c., MRF-4 (Hinterberger T.J. et al., 1991) transiently since 9 d.p.c. and Myo-D starting from 10,5 days

d.p.c.. Myo-D and Miogenin expression is high during embryonic development, but it decreases in the post-natal life.

Every MRF (Myo-D, Myf-5, Miogenin and MRF-4) (Weintraub, H., 1993) in the vertebrates is capable, if ectopically expressed in non-myogenic cells, to induce a myogenic phenotype (Davis et al., 1987). It is normally accepted that Myf-5 and Myo-D act as myogenic determinating factors and Miogenin and MRF-4 act as differentiation factors (Cossu et al., 1996). In perinatal life, between sarcolemma and basal lamina of the myofibers, little round-shaped cells appear. This cells, called muscle satellite cells, don't express anyone of the typical myogenic markers and remain in a steady non-dividing state in normal conditions in adults. Upon muscle growth, damage or pathological conditions, satellite cells actively proliferate, express myogenic markers and differentiate into muscle (in normal regeneration conditions). The capacity of these cells to differentiate into skeletal muscle myofibres all along the lifespan, maintaining a pool still able to self-renew support the idea to consider the satellite cell as the real classically-defined skeletal muscle stem cell. Actually nowadays the fact is becoming complex, as reported in a number of publications (Seale et al., 2000; Seale et al., 2001; Zammitt, Beauchamp, 2001; Goldring et al., 2002; Partridge, 2002; Morgan, Partridge, 2003).

OSTEOGENESIS

During bone growth, osteogenic progenitors proliferate turning into osteogenic cells, the osteoblasts. These cells directly participate to bone morphogenesis producing organic components of protein matrix (collagene, proteoglicans, glicoproteins) also regulating mineral depositions. They are overall found on the expanding surface of bones and periostium (fibrous external coat) and endostium (cell coat into central cavity). They are delivered by blood vessels and begin to produce bone matrix on the cartilage (model for bone building) partially degraded. Their cytoplasm has high alkaline phosphatase activity.

The cells that are committed to the osteogenic lineage activate the transcription factor Cbfa-1, the master activator of osteogenesis. At the pre-osteoblast stage they express type I Collagen, followed by BSP (Bone Sialoprotein) and Osteopontin (two matrix proteins associated to bone formation) at the early-osteobalst stage, while the mature osteoblast stage is characterised by expression of Osteocalcin and terminal bone deposition as also described by studies on osteogenic cell cultures (de Oliveira et al., 2003; Kalajzic et al., 2003).

STEM CELLS

Stem cells are classically defined by the double characteristic of both retaining capacity of a long-term indifferentiate state and capacity to differentiate into one or more cell types. Generally this is possible because of their ability to undergo asymmetric division, in which one of the daughter cells is committed to differentiation fate (progenitor cell) but the other one retains the stem identity.

Actually, this kind of division is much frequent only in invertebrates. Two examples of that are bastomers of *Coenorabditis elegans* germ line and neuronal precursors of *Drosophila melanogaster*. In mammals there are more evidences of random asymmetric division giving one stem cell and one progenitor cell, or symmetric division, giving either two stem daughter cells or two committed progenitors.

Normally stem cells take part to tissue homeostasis replacing differentiated cells lost both because of physiological turnover and because of damage. Epidermis, small intestine and moreover haematopoietic system are examples of adult tissues in a dynamic state: even without any damage they continuously give rise to new daughter cells able to proliferate, differentiate and die.

Embryonic stem cells are almost totally totipotents because they are able to generate a great number of lineages. Cells of the inner cell mass (ICM) from mammal blastocyst can be indefinitely propagated in culture as pluripotent embryonic stem cells (Thomson et al, 1998), called ES. These cells, to remain in a indifferentiate and pluripotent state must express the transcription factor Oct-and have the LIF (Leukaemic Inhibitory Factor) (Nichols et al., 1998; Niva 1998). Stopping LIF administration ES spontaneously aggregates into embryoid bodies able to differentiate into many cell types (Bradley,1 990).

Blood is a very good example of self–renewal and haematopoietic stem cell (HSC) is the best characterized stem cell. All blood cells came from a common precursor, the pluripotent haematopoietic stem cell. The functional identification of HSC is overall based on surface marker expression. The earliest HSC show high expression of a transmembrane protein, CD34, and low expression of a related protein, CD38, while the most mature HSC highly express CD34 and lowly express CD38.

Many cell types reveal a multipotent capacity when in particular conditions. Progenitors normally found in bone are easily isolated from bone marrow stroma as adherent, clonogenic, fibroblastic cells (CFU-F, colony forming unit-fibriblastic) (Friedenstein., 1980; Friedenstein

et al., 1966; Owen & Friedenstein, 1988). After transplantation in vivo, these cells rebuild a portion for bone as an organ, also showing abnormal bone and marrow deriving from genetically defective tissue (Bianco et al., 1998). In low-oxygen conditions, cartilage differentiation has been found (Ashton et al., 1980).

Cells that are canonically not myogenic cells can form muscle in certain conditions (Cossu, 1997) and, on the contrary, canonically myogenic cells can adopt different fates in particular conditions. Derm fibroblasts are cells with unexpected myogenic potential. Has been demonstrated that fibroblasts are able to fuse with myogenic cells to form mosaic myotubes in culture (Chaudhari et al., 1989). Later Gibson et al. (1995) showed that dermal fibroblasts can form multinucleate dystrophin-expressing cells when injected into the muscles of dystrophin-negative mdx mice. Salvatori et al., (1995) also demonstrated that fibroblasts can fuse into muscle fibres *in vitro* and *in vivo*. Also the C2C12 mouse cell line as well as primary mouse cells, can be osteogenic upon treatment with bone morphogenetic protein 2 (BMP-2) (Katagiri et al., 1994). These cells can also became adipogenic when treated with long-chain fatty acids or thiazolidine-diones (Grimaldi et al., 1997). Moreover somite cells normally destined to express Myf-5 and became muscle cells, convert to dermis or cartilage cells when Myf-5 protein expression is prevented (Tajbakhsh et al., 1996).

MESOANGIOBLASTS

Our current appreciation of stem cells plasticity has changed dramatically in the last years, following the report that the adult bone marrow contains progenitors that can be incorporated into skeletal muscle (Ferrari et al., 1998; Gussoni et al., 1999), liver (Petersen et al., 1999; Lagasse et al., 2000) and the central nervous system (Kopen et al., 1999; Mezey et al., 2000; Brazelton et al., 2000). Conversely, the CNS contains stem cells that can differentiate into hematopoietic (Bjorson et al., 1999) or skeletal muscle (Galli et al., 2000). Also other progenitor stem cells have been described like endothelial progenitor cells (Kawamoto et al., 2001), mesenchimal stem cells (Bianco and Gehron Robey, 2000) and mesoderm adult progenitors (Jiang et al., 2002). Therefore, several adult tissues contain progenitors that, under specific conditions, may give rise to different embryologically unrelated derivatives; this multipotency is hard to reconcile with the fate decisions that are necessary to allocate cells irreversibly to a given tissue anlage during embryogenesis. Prompted by this apparent paradox, we investigated the origin of myogenic cells during fetal and perinatal development and reported the unexpected origin of a subset of myogenic progenitors from the dorsal aorta

and not from somites, canonical source of all skeletal muscle of the body (De Angelis et al., 1999). Interestingly, in the same year Jackson et al. (Jackson et al., 1999) reported that adult skeletal muscle contain progenitors that were capable of repopulating the haematopoietic system of a mouse. Thus, an unpredictable relationship appears to exist between progenitors of different systems, with an apparent common root into the emoangioblastic system. Based on these data, we proposed that during tissue histogenesis, when vessels penetrate into developing tissues, vessel-associated progenitors, which possibly originate from a common ancestor, would leave the vessel and adopt the fate of the tissue where the vessel has entered (Bianco and Cossu, 1999). This fate choice would depend upon local signals emanating from differentiating cells of that tissue and would be functionally different from fate choices dictated by embryonic signalling centers such as the notochord or Hensen's node, even though the same molecules may be involved. Some of the vessel-associated progenitors may remain undifferentiated and capable of differentiating later in life during postnatal growth or regeneration. Because of their origin, they would remain capable of differentiating into other tissue types when naturally or experimentally relocated into a different tissue.

To prove this hypothesis, we transplanted quail or mouse embryonic aorta into chick embryos and searched for donor cells in the chimera at the foetal stage. We now report that progenitor cells associated with the donor vessel are distributed in most mesoderm tissues that we have analysed, including tissues distant from the transplantation site such as the myocardium. This capacity was associated with both the endothelial and sub-endothelial component, although endothelial cells possibly contributed to mesoderm tissues to a greater extent. Furthermore we show that aorta-derived cells generate a clonal progeny that can be indefinitely expanded *in vitro*, while maintaining multipotency *in vitro* and *in vivo* upon transplantation into a chick embryo. These features define vessel-associated progenitors as true stem cells, that in the foetal stage of development are distributed to tissue anlagen through angiogenesis and through the circulation. They contribute to peri-natal tissue growth and may represent the ancestors of postnatal stem cells. These cells, being both coming from embryonic vessels and able to differentiate into most mesodermal cell types without loosing their self-renew capacity are hence called "meso-angioblasts".

Our in vitro studies reveal that mesoangioblasts express a number of different kind of markers detected by RT-PCR (shown in the Table 1) or mycroarrays. They express the haematopoietic markers CD34 and Sca-1 as well as VE-Caderin (endothelial), as expected, but only transiently they express the SCF receptor c-kit and the endothelial VEGF receptor Flk-1.

Very surprisingly, mesoangioblasts reveal to highly express the neurogenic and myogenic transcription factor Pax3 (Fig. 1), also involved in melanogenesis and cardiogenic neural crest

development. Recently we found that also the Pax3 protein is produced by mesoangioblasts, detected by anti Pax3 monoclonal antibody immunofluorescence.

Also they highly express the neural marker Emx2 (Fig. 1), recently found to be directly involved in proliferation of mammals adult neural stem cells (Galli et al., 2002).

	A1	A2	A4	A6	A14	B13
CD34	+	+	+	+	+	+
c-kit	-	-	-	-	-	-
Sca-1	+	+	+	+	+	+
Flk-1	-	-	-	-	-	-
Flt-1	-	-	-	-	-	-
Tie-2	+	+	+	+	-	-
Myf-5	-	-	-	-	-	-
Myo-D	-	-	-	-	-	-
Pax-3	+	+	+	+	+	+
Pax-7	-	-	-	-	-	-
Emx-2	+	+	+	+	+	+
VE-Cad	+	-	+	+	+	+
Mef-2D	+	+	+	+	+	+
Gata-4	+	+	+	+	+	+
NKX-2.5	-	-	-	-	-	-
Oct-4	-	-	-	-	-	-

Table 1: Gene expression profile of the mesoangioblasts of the first generation, showing the high expression conservation of the genes in the different clones.

PAX3

In mammals Pax3 is the homologue gene of the *paired box* gene family well characterized in Drosophila melanogaster. This is a transcription factor containing a DNA-binding paired domain regulating a number of downstream genes and it is involved in myogenesis, melanogenesis and neurogenesis. This is first expressed in all the somitic dorsal epithelium. Later its expression becomes restricted to the lateral region of dermamyotome, in migratory myogenic cells giving rise to the hypaxial musculature (Bober et al., 1994; Goulding et al., 1994; Williams & Ordhal., 1994). Pax3 is also necessary for the migration of myogenic cells to the limbs. Knock-out mice in Pax3 cannot form limb muscles because their myogenic cells

don't migrate from the somite, even if cells without Pax3 are able to differentiate into skeletal muscle (Datson et al., 1996).

"Splotch" is a typical mouse phenotype due to a loss-of-function muatation of Pax3 (Fig. 2). This is mainly characterized by myogenic and neurogenic defects. Splotch mice have normally spina bifida, exencephaly and lacking of myogenic precursors to the limbs. It is demonstrated that there are also deficiencies in cardiac neural crest development caused by the upregulation of Msx2. This gene is directly repressed by Pax3 in wild type mice (Kwang et al. Development 2002). The splotch embryos reveals that Pax3 has complementary functions in MyoD activation and inhibition of apoptosis in the somitic mesoderm (Borycki et al. 1999). Pax3 in the embryo represses his orthologue Pax7 during neural tube and somite development (Borycki et al., 1999). It is normally accepted that Pax3 marks myogenic identity in the embryo as well as Pax7 does it in the adult (Seale et al., 2000; Seale et al., 2001; Buckingham,2001; Relaix et al., 2004). Other studies on Pax3 mutant mice reveals that Pax3 activates the homeobox gene Lbx1, an early marker of migrating limb muscle precursor cells (Mennerich et al., 1998) and it is upstream c-Met, a gene coding the tyrosine kinase receptor of the ligand SF/HGF, directly involved in the migration of the somitic myogenic cells to the limb (Epstein et al., PNAS 1996; Birchmeier and Brohmann, 2000). Mutations in c-Met have similar phenotype to mutations in Pax3.

Furthermore, *Pax3* mutations interfere with development of the skeleton where multiple malformations were evident in the vertebral column, thoracic cage, and appendicular bones (Tremblay et al., 1998).

Pax3 is also necessary and sufficient to induce myogenesis in pluripotent stem cells (Ridgeway, Skerjanc, 2001) but its over-expression inhibits myogenic differentiation of C2C12 myogenic cell line (Epstein et al., 1995).

Nonetheless the loss of Pax3 expression in the embryo does not block completely myogenesis, so the exact role of Pax3 in muscle differentiation remains unclear.

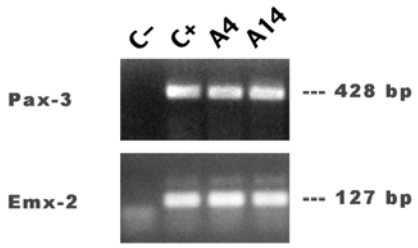

Fig 1. RT-PCR showing the expression of Pax3 and Emx2 in A4 and A14, two clones of mesoangioblasts of the first generation compared to positive (C+), total RNA of 9.5 days embryo, and negative (C-) control.

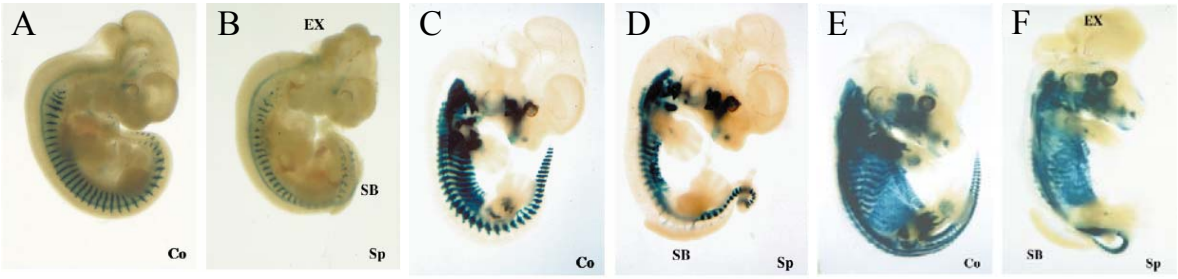

Fig. 2. Splotch (Sp) mouse with the construct Desmin-Lacz (the promoter of Desmin, an early myogenic marker leading the expression of the LacZ bacterial reporter gene) with respect to the wild type Desmin-Lacz control (Co). In the Sp animal the exencephaly (EX) and the Spina Bifida (SB) is evident and also the absence of expression of Lacz in the limb bud (Tremblay et al. 1998).

AIM

A number of more and less recent publications, as above reported, showing relationship of Pax3 with plasticity together with the observation that this gene is highly expressed in multipotent cells, the mesoangioblasts, lead us to consider Pax3 as a of "stem" gene. The aim of my thesis is to study the role of Pax3 in mesoangioblasts. We used the approach of isolation and characterization of mesoangioblasts coming from Pax3 knock-out mice as experimental model.

METHODS

MICE STRAIN USED (KNOCK-OUT MICE)

The females Pax3 knock-out mice were generated as previously described (fig.2, Relaix et al., 2003) (kindly provided by Margaret Buckingham and Frederick Relaix from the Pasteur Institute, Paris). They were crossed with C57 Black/6 mice to obtain the mouse generation we used. Homo and eterozygotes mice for the transgene were identified by the PCR analyses for the wild type and the knock-out Pax3 sequence. The oligonucleotides for the genotype analyses were: Pax3 Lacz UP 5' tag aca tca gtc cta ggt ctc cct cc 3', Pax3 WT DW 5' ttt aga acg cgc cca ctc tgg acc cgc 3', Pax3Lacz DW 5' aca aac gca cac cgg cct tat tcc aag c 3'. The knock-out omozygote phenotype was also detected by the identification of the "splotch" phenotype.

DNA EXTRACTIONS

DNA of the adult mice was extracted from the edge of the tail. The tissue was incubated with shaking over night at 55°C with the Digestion buffer (10 nM Tris-HCl, 10 mM EDTA, 100 mM NaCl, 0,5% SDS and 0,5 mg/ml Proteinase K). After centrifugation an equal volume of isopropanol was added to the supernatant, mixed and centrifuged, the pellet washed in 70% ethanol and incubated with TE (Tris 10 mM, EDTA 1 mM) buffer for 4 hours at 55° C. The pellet was resuspended and 3 µl were used for PCR. The amplification was performed according to the PCR Master Mix kit procedure (Promega).

DNA of the embryo was extracted with the appropriate Digestion buffer (100mM NaCl, 10 mM Tris-HCl pH 8, 25 mM EDTA pH 8, 0,5% SDS and 0,1 mg/ml Proteinase K) with shaking over night at 50°C, the supernatant extract with an equal volume of Phenol/Chloroform/isoamyl alcohol (25:24:1). ½ volume of 7.5 M ammonium acetate and 2 volumes of 100% ethanol to the aqueous phase was added. After centrifugation, the DNA pellet was washed with 70% ethanol and resuspended in TE buffer (see above) after air drying.

AORTA ISOLATION AND MESOANGIOBLAST GENERATION

Mouse dorsal aorta at the AGM (Aorta-Gonad-Mesonephros) region was isolated from E9.5 embryos by digesting the embryo body for 3 miniutes on ice with 0,02% Pancreatin, 0,5%

Tripsin (Sigma), and then was grown as an explant culture. After 5 days the explant was dissociated into a single cell suspension by gentle pipetting and by digestion with 1mg/ml of Collagenase/Dispase (Sigma) for 15 miniutes at 37° C. The cell suspension was plated at limiting dilution on a feeder layer of Mitomycin C-treated primary embryonic mouse STO fibroblast in 96 multiwell plates in complete medium with 20% FCS (Fetal Calf Serum). After 1-2 weeks, clones appeared in approximately 2-4 % of the wells. When the clones had grown to approximately 10^3 cells they were passed twice on feeder layers and thereafter on gelatin-coated dishes.

FLOW CYTOMETRY

FACS analyses were performed as follows: cells were dissociated form the dish floor by diluited Tripsin (0,05% Tripsin and 0,02% EDTA in CMF), blocked with 10 µl of FCS, incubated on ice with 1% BSA (Bovine Serum Albumine), washed twice and then incubated with 1µg of the appropriate anti murine monoclonal antibody for the investigated marker. The antibodies used were one for each of the following markers: CD45, CD34, Sca-1, Flk-1, c-Kit (all conjugated with Phyco-erythrin from Pharmingen), and CD31 (conjugated with Fluorescein). The cells were incubated at 4°C for 1 hour, washed three times and then separated on a FACS scan using a Consort-30 software. Forward and 90°C side-scatter were used to identify and gate positive and negative fractions. Background subtraction using an unrelated antibody was performed for each sample using IGg2a or IGg2b general isotypes.

DIFFERENTIATION INDUCTION

Cell lines were grown in DMEM (Dulbecco's modified medium) with 10% FCS (Fetal Calf Serum). Mesoangioblasts were treated with 100 ng/ml of BMP-2 in complete medium for 5 days (BMP2 was added every other days), then fixed and stained for alkaline phosphatase (ALP) or analysed for the expression of osteogenic markers by RT-PCR. In some experiments Ascorbic Acid 10^{-2} M, Dexamethasone 10^{-8} M and β-Glicerophosphate 10 mM were also added. Alternatively, cells were cultured at high density with 2% horse serum and treated with 10^{-7} M Dexamethasone, and then analysed for adipocyte morphology. For heart cell co-culture experiments, cells were transfected by Lipofectamine (Invitrogen) according to standard procedure with the plasmid (pcDNA3) delivering the CMV-Lacz construct, followed

by Neomycin selection of the integrated DNA. Cardiomyocyte differentiation was obtained by co-culture with primary rat neonatal cardiomyocytes (obtained as reported in De Luca et al., 2000) and skeletal myogenic differentiation was induced by co-culture with L6 myogenic cells without any previous genetic labelling.

APOPTOSIS DETECTION

Apoptosis were detected by the In Situ Cell Death Detection Kit (Boeringher Manheim): cells at early and/or late passages were fixed with Paraformaldehyde 4% for 30 minutes at 4 °C, washed twice, permeabilised for 2 minutes on ice (with 0.1% Triton-X in 0,1% sodium citrate) and incubated 1 hour at 37 °C in the dark with the TUNEL (TdT-mediated dUTP nick end labelling) reaction mixture (composed of 1 volume of Enzyme Solution and 9 volume of Label Solution), washed once, incubated with Hoechst 5 minutes at room temperature in the dark, washed once and analysed by fluorescence microscopy.

RNA EXTRACTION AND RT-PCR ANALYSES

Total RNA was extracted from the tissues or cells coltured in the different conditions using the SV Total RNA Isolation System (Promega): cell samples where harvested in a tube, lysated with the SV RNA lysis buffer and the RNA extraction was performed according to Promega instructions. RT-PCR was performed using the appropriate oligonucleotides directly in the same reaction tube for the reverse transcription and the following polimerization using the Access RT-PCR System kit (Promega): the AMV/tfl reaction buffer (diluited1:5), 0,2 mM dNTP mix, 1 mM $MgSO_4$, 50 pmol of each primer, 0.1 u/µl AMV Reverse Transcriptase, 0.1 u/µl Tfl DNA Polymerase and 0,3 µg of each RNA sample in nuclease free water up to 50 µl have been put in a single reaction tube. The RT-PCR steps were: 48 °C 45"; 94°C 2 min.; appropriate number of cycles of 94°C 30", T annealing 1 min and 68° for 2 min; 68 °C 7 min. The oligonucleotides used for reverse transcription and amplification were: VE-Cad (227 bp) (5′ gga tgc aga ggc tca cag ag 3′ UP and 5′ ctg gcg gtt cac gtt gga ct 3′ DW); Flk1 (270 bp) (5′ tct gtg gtt ctg cgt gga ga 3′ UP and 5′gta tca ttt cca acc acc ct3′ DW); CD34 (300 bp) (5′ ttg act tct gca acc acg ga 3′ UP and 5′ tag atg gca ggc tgg act tc 3' DW); c-Kit (400 bp) (5′ ggc tca taa atg gca tgc tc 3′ UP and 5′ ctt cca ttg tac ttc ata cat g 3′ DW); Cbfa1 (387 bp) (5′ ccg cac gac aac cgc acc at 3′ UP and 5′ cgc tcc ggc cca caa atc tc 3′ DW); BSP (563 bp) (5' gaa acg gtt tcc agt cca g 3' UP and 5' tga aac ccg ttc aga agg 3' DW); Coll1A2 (484 bp)

(5'gca atc ggg atc agt acg aa 3' UP and 5' ctt tca cgc ctt tga agc ca 3'); Osteocalcin (292 bp) (5' aag cag gag ggc aat aag gt 3' UP and 5' agc tgc tgt gac atc cat ac 3' DW); Osteopontin (437 bp) (5' tca cca ttc gga tga gtc tg 3' UP and 5' act tgt ggc tct gat gtt cc 3' DW); Osteonectin (499 bp) (5' cac ata act ctg agg cca ttg 3' UP and 5' gcc caa ttg cag ttg agt gat 3'DW)

β-GALACTOSIDASE STAINING

Cells were fixed in Paraformaldehyde 4%, washed twice in PBS, and stained for 4 h or overnight in 0.1% X-gal, 5 mM K4Fe(CN)6, 5 mM K3Fe(CN)6, 2 mM MgCl2, 0.2% NP-40, 0.1% sodium deoxycholate.

ALP AND VON KOSSA STAINING

The ALP assay were performed using the kit Sigma Alkaline. A Fast Violet B Salt pill was melted in distilled H_2O (A Solution). 2 ml Naphtol AS-MX Phosphatase Alkaline Solution in 48 ml H_2O (B solution) were added to A Solution. Cells were fixed with Acetone/Citrate for 30 seconds, washed with distilled H_2O for 45 seconds, added to B Solution 30 seconds in the dark, washed with H_2O for 2 minutes and incubated into Mayer's Ematossiline for 10 minutes.

Von Kossa staining were performed as follows: cells were covered with silver nitrate 5% at light for 15 minutes-1 hour, washed twice with distilled water and counterstained with blue methylene.

IN VIVO TRANSPLANTATION

For the in vivo experiments the α.Sarcoglican (SG) -/- mice, kindly provided from DIBIT-HSR S. Raffaele Institute of Milano, were used. In some cases the mesoangioblasts were marked before injection by the molecular markers DiI and/or DiO (Molecular Probes). DiI and DiO were suspended in DMSO (Sigma) at 2 mM (mother solution) diluited 1:100 with the cells and incubated for 5 minutes at 37 °C. The marking were assessed at epifluorescence microscope. The cells were injected in the Tibialis Anterior muscle of α –SG null mice 2 months old treated twice a week with 0.4 mg/ml of (intraperitonaeal) Ciclosporina A (Sigma).

Before injection mice were anaesthetized with the light form anaesthetic Zolentil 20 (Verbac).

At the end of the time course the limb muscles were cut and embedded in TISSUE-TECK OCT compound (Miles) on dry ice, cryostat sections of the embedded tissues were performed.

IMMUNOFLUORESCENCE

Cells or sections were fixed in 4% Paraformaldehyde (in PBS), washed twice in PBS, saturated in 1% BSA and 0,2% Triton-X, incubated for successive saturation with 10 % goat serum and then incubated with the primary antibody (monoclonal MF20, anti smooth α-actin, anti α-sarcoglican or polyclonal anti Myo-D) with 1,5% goat serum for 1 hour at room temperature, washed four times in 1% BSA and 0,2% Triton-X, incubated with the secondary antibody (anti mouse or anti rabbit rhodaminate or fluoresceinate) with Hoechst and with 1,5% goat serum, washed four times in 0,2% Triton-X and mounted with 60% Glycerol and 40 mM Tris-HCl pH 9,3. Then the samples were analysed on epifluorescence microscope.

RESULTS

MORPHOLOGY, PROLIFERATION AND VIABILITY OF PAX3 MESOANGIOBLASTS

In normal culture conditions, in DMEM 10% FCS on Collagene I coated dishes, without any specific treatment, both at early and at late passages Pax3 WT mesoangioblasts as well as KO cells have epithelioid morphology (Fig.3 A, B) also if compared to clones of the previous generation. As shown in the figure 4, there is no meaningful influence of Pax3 deletion on the proliferation capacity of mesoangioblasts related to Pax3 WT counterparts, when measured *in vitro* in normal growth conditions (Fig 4). Moreover all this mesoangioblast cell types have the same clonogenic efficiency and the same growth response when cultured on matrigel coated dishes (not shown).

As detected by the TUNEL assay, also the percentage of apoptosis in mesoangioblasts is not affected by Pax3 expression (Fig.5). This method identifies the apoptotic nuclei by the terminal deoxynucleotides transferase. This enzyme catalyses polymerization of fluorescein-labelled nucleotides to 3'–OH DNA ends of DNA fragments generated by apoptotic cells. Both at early and at late passages the apoptotic rate *in vitro* is less than 2% in the Pax3 KO as well as in the Pax3 WT cells in normal culture conditions.

GENE EXPRESSION PROFILE OF PAX3 MESOANGIOBLASTS

In a similar way of the first mesoangioblasts generated (Tab.1), RT-PCR on Pax3 mesoangioblasts was performed. No difference of gene expression was found, for the gene pool we analysed, between KO and WT mesoangioblasts (Fig. 6), as expected. Likewise the clones of mesoangioblasts of the previous generations, all the Pax3 mesoangioblasts are positive for the expression of the transcript of CD34, early marker of HSC (see also above), the adhesion molecule VE-Caderin, Sca-1 (Stem Cell Antigen-1) and the neural marker Emx2. Also we found that in absence of particular culture conditions *in vivo* as well as *in vitro* both of the mesoangioblasts show only a basic expression of the MyoD mRNA (not shown).

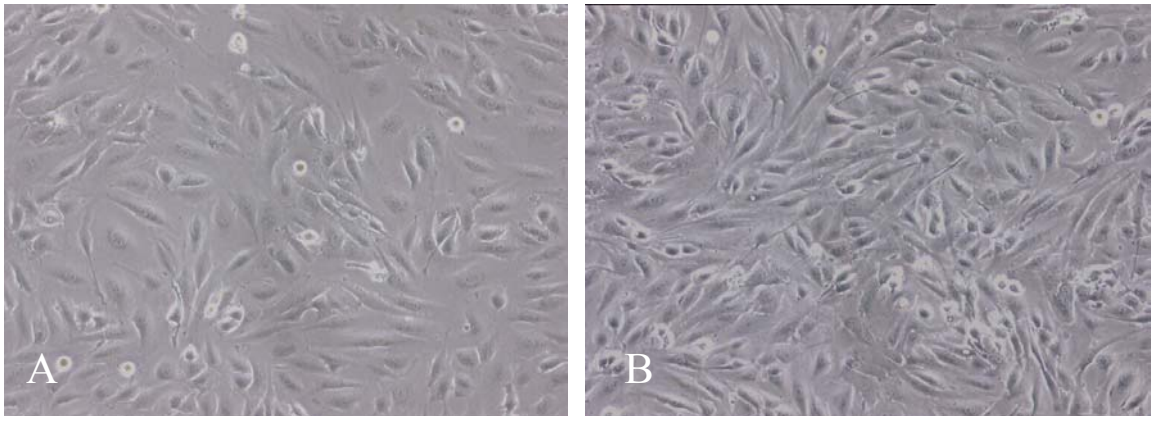

Fig 3. Morphology of Pax3 mesoangioblasts. Pax3 KO mesoangioblasts (A) show epithelioid morphology as well as the Pax3 WT counterparts (B).

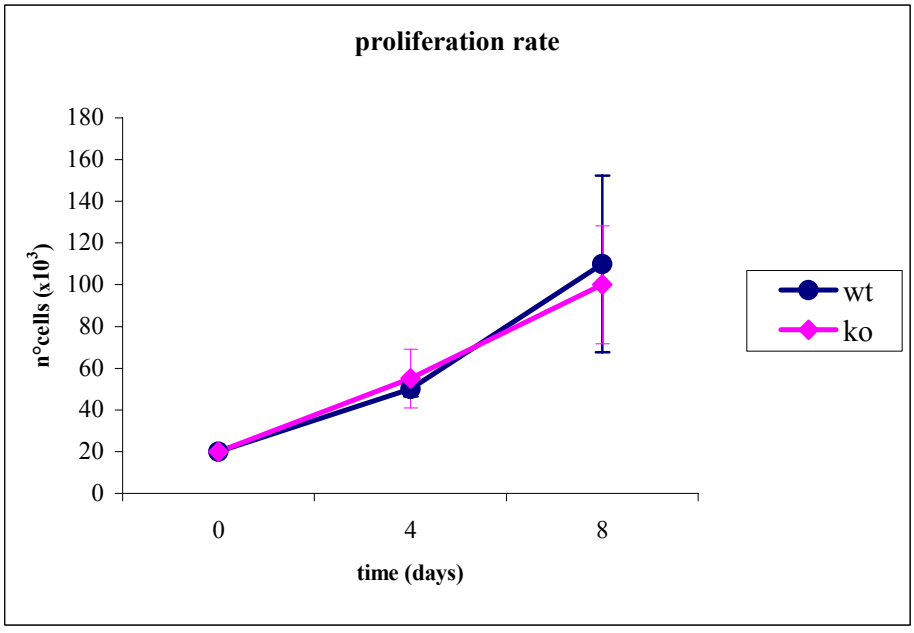

Fig. 4. Proliferation rate of Pax3 KO and Pax3 WT mesoangioblasts grown with DMEM and 10% FCS. Proliferation was measured counting, by the Thoma Chamber, the number of cells from the plate every 4 days.

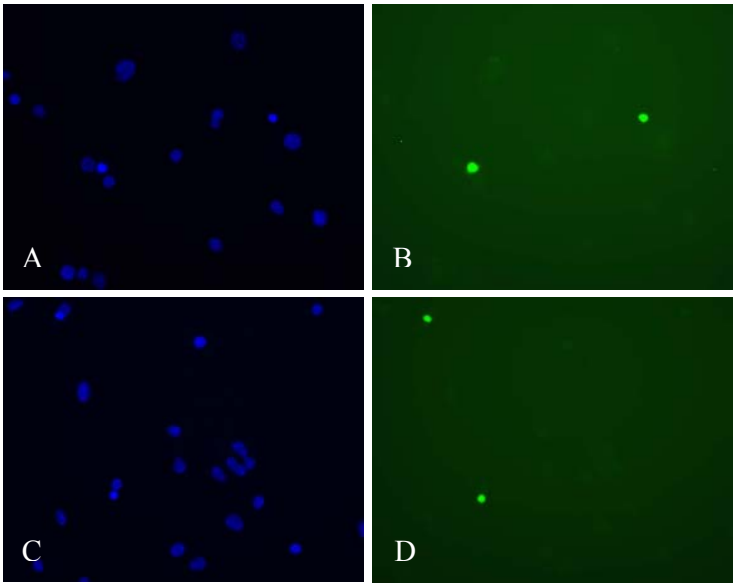

Fig. 5. Apoptotic TUNEL-positive nuclei of the Pax3 mesoangioblasts. Hoechst staining of Pax3 KO (A) and Pax3 WT (C) mesoangioblasts. Respectively in green (TUNEL fluorescein), the apoptotic nuclei of Pax3 KO (B) and Pax3 WT (D) mesoangioblasts. These are some of the few fields with apoptotic nuclei.

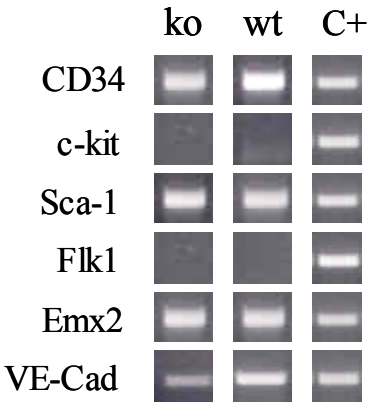

Fig. 6. RT-PCR of Pax3 KO and Pax3 WT mesoangioblasts in normal conditions. There is no difference between the Pax3 KO and the Pax3 WT mesoangioblast expression profile of the genes here reported. The positive control (C+) is the total RNA extraction of E9.5.

SURFACE MARKER EXPRESSION AND POPULATION HOMOGENEITY

FACS analyses were performed on Pax3 mesoangioblasts in order to observe the haematopoietic and endothelial surface marker expression and the identity profile of a single mesoangioblast cell line. We analysed the expression of the haematopoietic proteins Sca-1, c-kit (receptor of SCF, an haematopoietic growth factor) and CD34. We also analysed the expression of the endothelial marker Flk-1. Pax3 KO mesoangioblasts as well as Pax3 WT control are positive for Sca-1 and CD34, at around 100%. They are instead totally negative for Flk-1 and c-Kit (Fig. 7). Actually this marker is transiently expressed by RT-PCR. So, both of mesoangioblast cell types are totally homogeneous for the expression of everyone of these markers.

ADIPOCYTE, SMOOTH AND HEART MUSCLE DIFFERENTIATION

Culturing mesoangioblasts at high density in 2% HS (Horse Serum) with 10^{-7} M Dexamethasone, we observed adipogenic differentiation at around 10-20% with no difference between the differentiation rate of Pax3 KO and Pax3 WT mesoangioblasts (Fig. 4). Adipocytes differentiation is characterized by the accumulation of lipidic drops in the cytoplasm.

Upon treatment with TGFβ at the concentration of 5 ng/ml, in normal medium conditions, 20% of Pax3 KO mesoangioblasts are able to differentiate into smooth muscle fibres as well as Pax3 WT cells. This differentiation was identified by immunofluorescence with anti-α smooth actin monoclonal antibody (Fig. 8 A, B, D, E) likewise mesoangioblasts of the previous generation. Mesoangioblasts also show to differentiate spontaneously into smooth muscle cells al low rate (less than 1%) without giving any specific factor.

In order to investigate the cardiogenic capability of the Pax3 mesoangioblasts we used the co-culture method (see also further on), co-culturing Pax3 mesoangioblasts with neonatal rat cardiomyocytes. Before the co-culture mesoangioblasts were genetically marked with LacZ by transfection (see also Methods). Cardiac differentiation was detected by immunostaining of co-cultured mesoangioblasts with the anti-sarcomeric myosin monoclonal antibody (Fig. 9) and by the negative immunostaining of MyoD (not shown). The differentiation rate was detected counting up the number of Lacz-positive nuclei into myosin-positive cardiomyocytes. Both Pax3 WT and Pax3 KO mesoangioblasts reveal to differentiate into

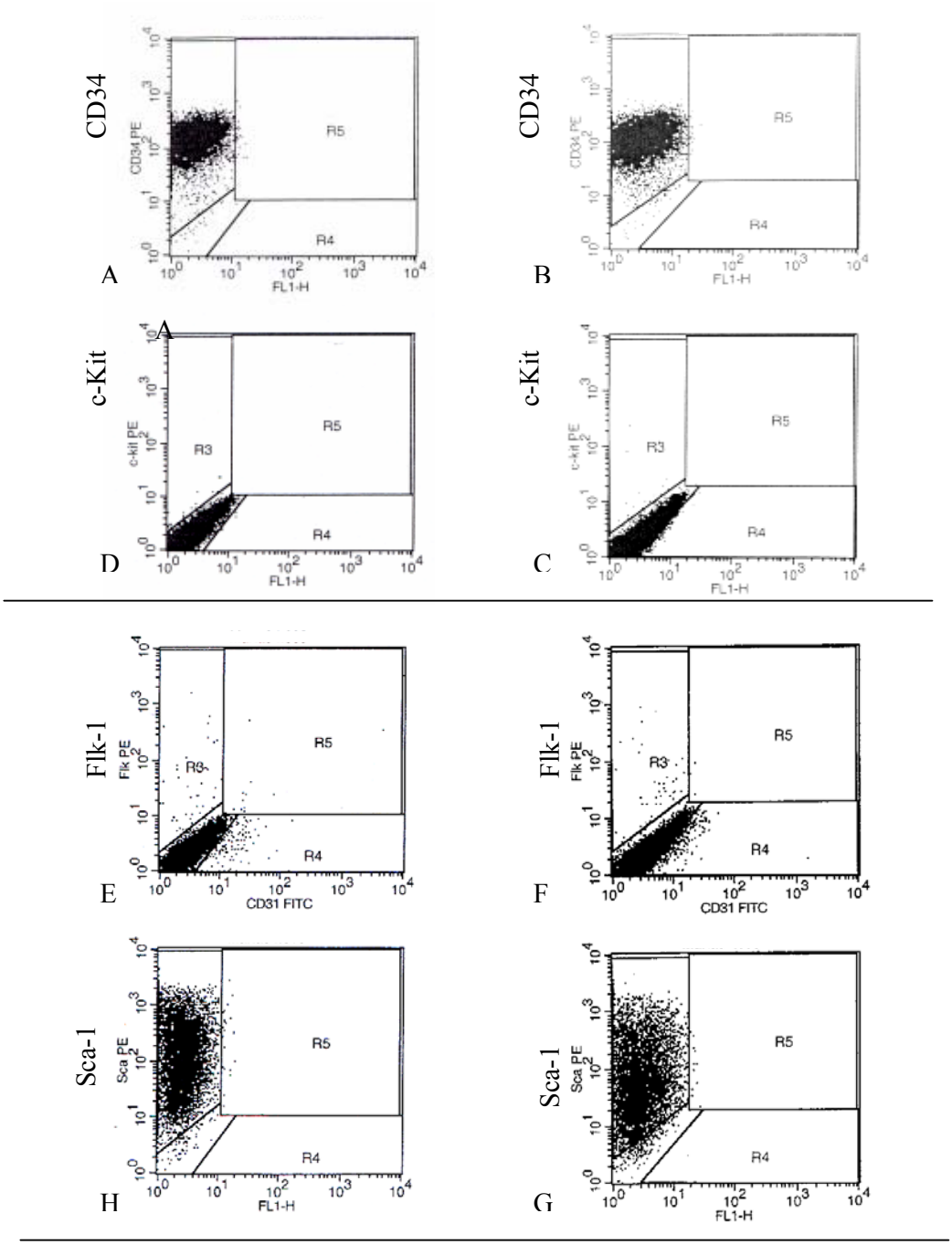

Fig 7. The Pax3 mesoangioblasts are homogeneous for the expression of surface markers, detected by FACS analyses. Both for Pax3 KO (A,D,E and H) and for Pax3 WT (B,C,F and G), all the cells of each clone express CD34 and Sca-1, but they do not express c-kit and Flk-1 at all.

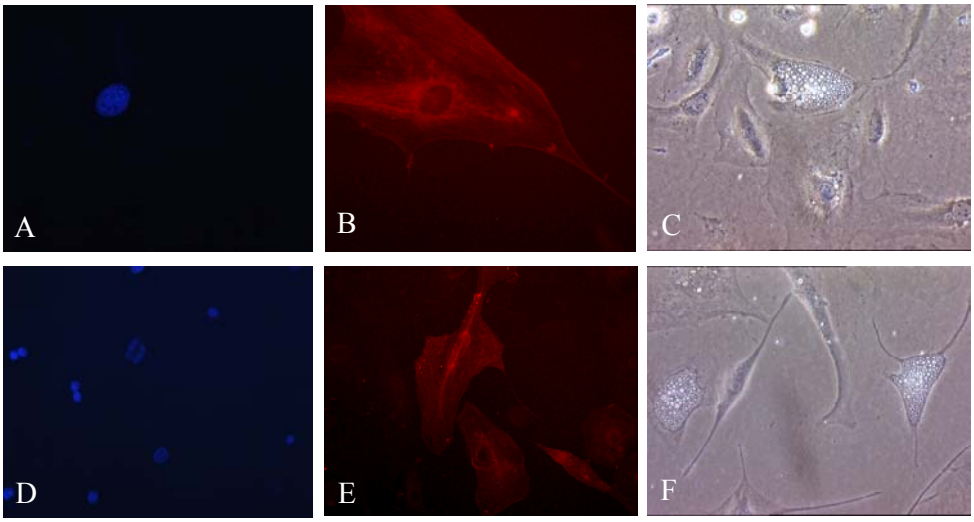

Fig. 8. Pax3 KO mesoangioblasts treated with 5 ng/ml TGF-β stained with Hoechst (A) and with monoclonal antibody anti α-smooth actin (B) result to differentiate at similar rate of the Pax3 WT control (D, Hoechst and E, α-smooth actin). Upon treatment with Dexamethasone, also adipogenesis of Pax3 KO mesoangioblasts (C) show similar efficiency of the WT counterparts (F).

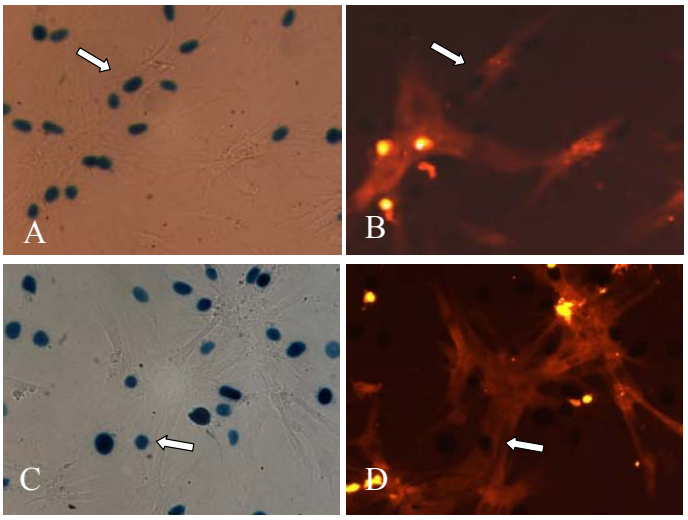

Fig 9. Pax3 KO clones marked with LacZ (A) by transfection express sarcomeric myosin (B, white arrow) if in co-culture with neonatal rat cardiomyocytes as the WT counterpart (C, D).

cardiomyocytes at around 10% (alternatively it was detected by immunostaining with the cardiac specific anti-troponin I antibody).

OSTEOGENIC DIFFERENTIATION AND OSTEOGENIC MARKER EXPRESSION

Upon treatment with 50-100 ng/ml of BMP-2 (added every two days for 1 week) we observed a striking improvement of ALP (alkalin phosphatase) protein expression, by the ALP assay *in vitro*, of the Pax3 KO mesoangioblasts. With respect to the WT control, in which the ALP-positive cells are about 30%, the Pax3 KO mesoangioblasts are positive only at 2-3% (Fig. 10 A, B). The response resulted to be dependent on the dose of BMP-2, added twice a week (Table 2), without altering the proportions and it reached saturation at the concentration of 500 ng/ml. Giving also Dexamethasone, Ascorbic acid and ß-Glicerophosphate together with BMP-2 there is an increase of the ALP protein expression but without a rescue if compared to the WT cells (Table 3). Osteogenic marker expression analyses shows that in the Pax3 WT mesoangioblasts the transcription of ALP is already activated in normal conditions, even without treatment with BMP-2, while in the KO cells treatment with BMP-2 is necessary to detect the transcript (Fig. 11), although both of the cell types have normal expression of Cbfa1 (Fig. 11 A). They also have equal basic expression of Collagene1 (Fig. 11 B) and short term osteogenic induction only with BMP-2 is sufficient to activate the expression of BSP, Osteopontin, Osteonectin and Osteocalcin without any detectable difference with respect to long-term treatment even with Dexamethasone, Ascorbic acid and ß-Glicerophosphate (Fig. 11 C). In long-term culture conditions, giving BMP-2 for 1 week and also Dexamethasone, Ascorbic acid and ß-Glicerophosphate for the following two weeks, the KO mesoangioblasts restore osteogenic differentiation forming calcified nodules at similar percentage of the WT counterparts, detected by the von Kossa method (Fig. 12).

SKELETAL MUSCLE DIFFERENTIATION

The induction of differentiation *in vitro* in skeletal muscle of a not yet committed cell type to myogenic lineage is not possible only with giving specific inducing molecules in culture as for other lineages like above reported. In order to obtain differentiation into skeletal muscle *in vitro*, we put the mesoangioblast cells together in culture with a committed cell type to myogenesis, realising a co-culture (the same basic principle used for the induction of

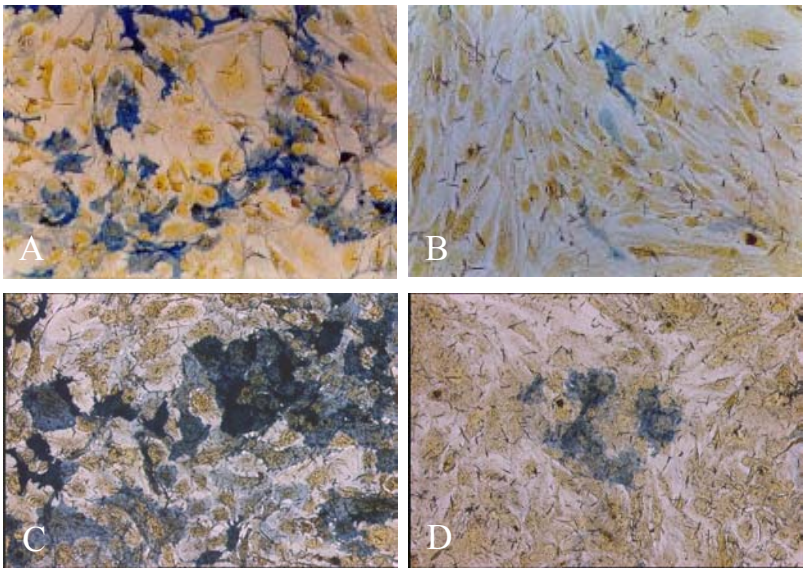

Table 2

ng/ml	100	500	1000	
KO	2±0.4	6.5±2.4	6.5±2.1	%
WT	15±3	29±6	29±1.4	%

Table 3

	BMP-2	B, Asc, De., β-G	
KO	2±0.4	12±4.4	%
WT	15±3	63±10.2	%

Fig 10. ALP (Alkaline Phosphatase) assay. ALP activity impairment of Pax3 KO mesoangioblasts (B) respect to Pax3 WT cells (A), when treated with BMP-2. Dose dependence of the response with saturation at 500 ng/ml of BMP-2 (Table 2). Long-term treatment with BMP-2 and other osteogenic factors (Ascorbic Acid + Dexamethasone + β-Glicerophosphate) as shown in C (WT) and D (KO), triggers an up-regulation of ALP activity in both of the cell types (Table 3).

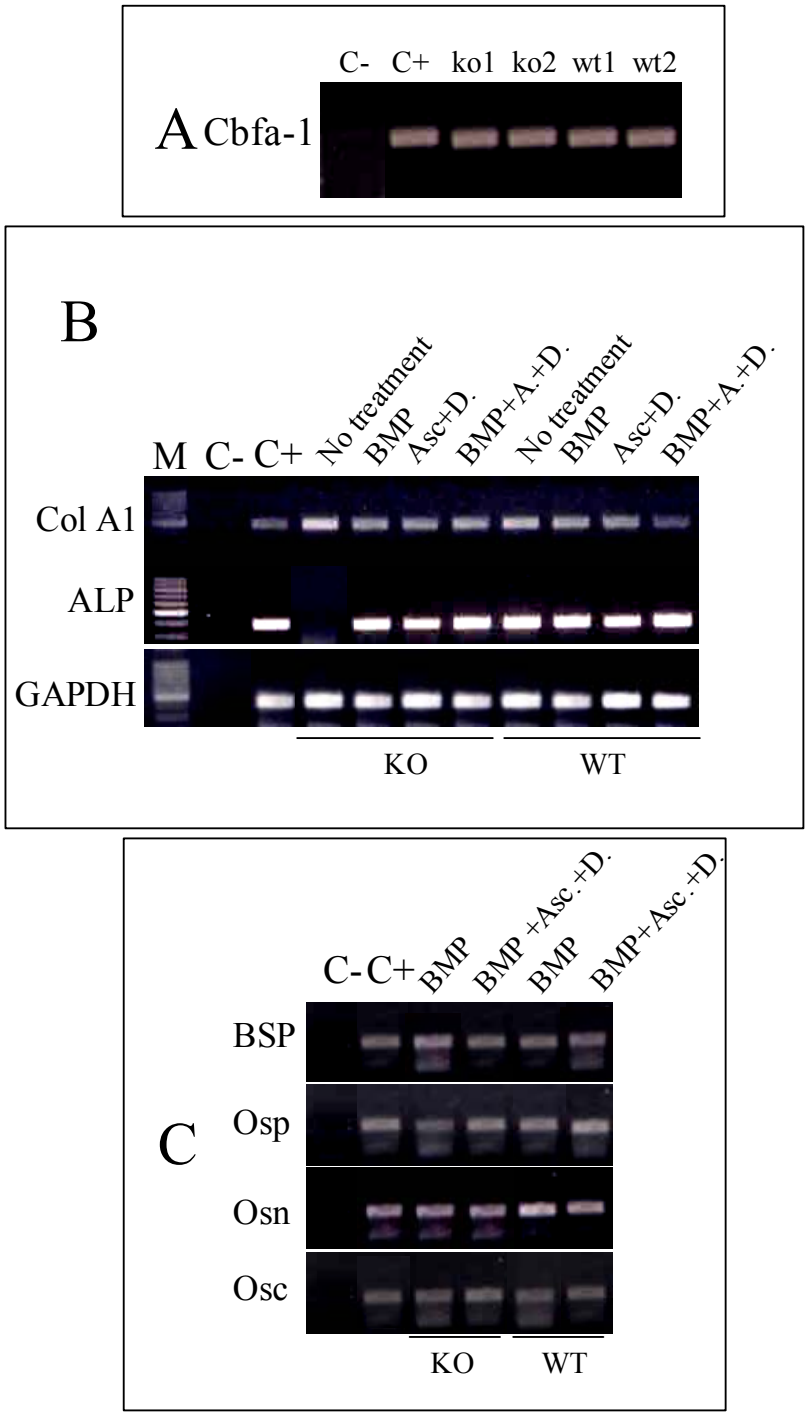

Fig. 11. Detection of osteogenic markers by RT-PCR. Cbfa1 is expressed also without any specific treatment (A). Here two lines of every Pax3 genotype (ko1, ko2, wt1 and wt2) are reported (A). Despite normal expression of Type I Collagene (ColA1), the ALP transcript is not expressed in the Pax3 KO mesoangioblasts in basic growth conditions differently from the WT counterparts (B). During osteogenic induction later osteoblast markers are normally activated (C).
(BMP: BMP-2. Asc: Ascorbic Acid. D.: Dexamethasone. C+: Osteoblast RNA. BSP: Bone Sialoprotein. Osp: Ostopontin. Osn: Osteonectin. Osc. Osteocalcin).

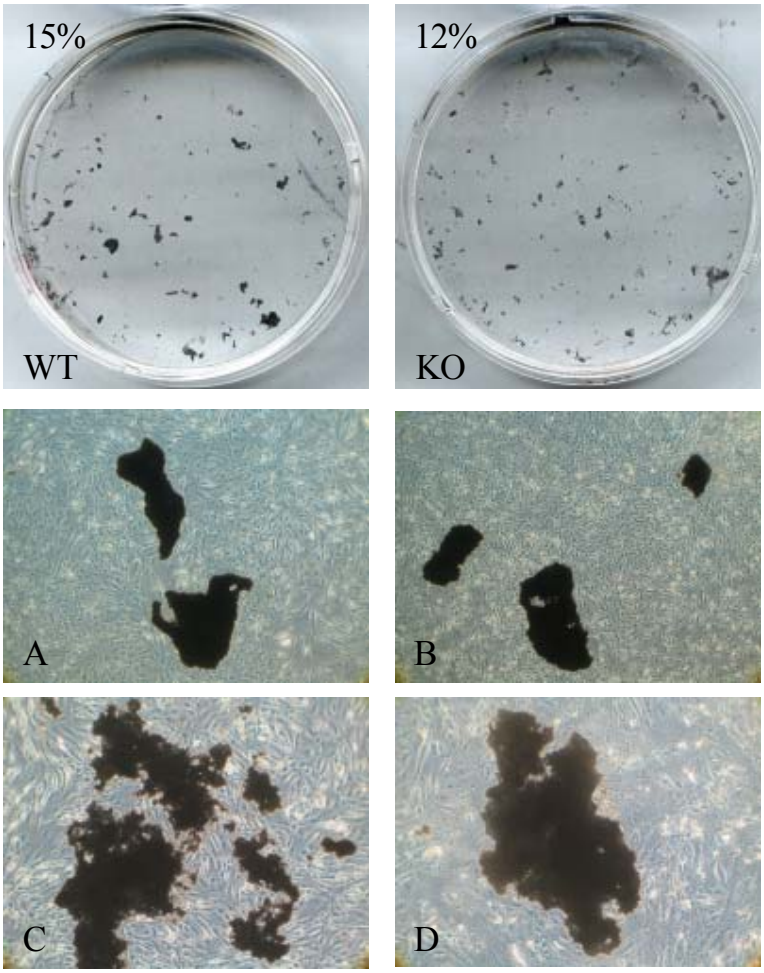

Fig. 12. Von Kossa assay. The percentage of mineralised nodules is about 15% in Pax3 WT mesoangioblasts and about 12% in Pax3 KO mesoangioblasts as indicated in the upper pictures. In the lower panels are shown details of calcified nodules (A and C: Pax3 WT mesoangioblasts. B and D: Pax3 KO mesoangioblasts).

mesoangioblasts to differentiate into cardiomyocytes). In this case we didn't mark mesoangioblasts before the co-culture because we adopted the method of the co-culture between rat and mouse cells. This procedure is possible without any previous labelling of the cells because we can take advantage of the morphological difference between rat and mouse nucleus well shown by the Hoechst staining. For this reason in this set of experiments Pax3 mesoangioblasts were put in co-culture with the L6 rat myogenic line. The Pax3 WT mesoangioblasts show to be able to differentiate into skeletal muscle fibers at around 5%, with similar efficiency of mesoangioblasts of the previous generation (shown in Minasi et al., 2002) but mesoangioblasts coming from Pax3 knock out mouse were not able to undergo skeletal myogenic differentiation at all. Nonetheless the two mesoangioblast cell types appear to express the same low rate of MyoD transcript by semiquantitative RT-PCR (not shown). The differentiation rate was measured by the detection of the number of mouse nuclei into myosin-positive myofibres by immunofluorescence for the anti-myosin MF-20 monoclonal antibody. One (Fig. 13 A, D and G) or more (Fig. 13 B, E and H) mouse (mesoangioblast) nuclei were found in single myofibre.

The lacking of skeletal muscle differentiation *in vitro* was confirmed by *in vivo* experiments. The same number of cells of the two mesoangioblast types were injected in the tibialis anterior muscle of the α-SG null mouse, a particular type of knock out mouse model with dystrophic phenotype (characterised by the loss of the α-sarcoglycan). DiI and DiO labelling of Pax3 WT and Pax3 KO mesoangioblasts respectively (Fig. 14 A, B, C, D, E, F) showed that the two cell types survive at the same rate into the host muscle (Fig. 15 G, H), but only after injection of Pax3 WT mesoangioblasts areas of sarcoglycan-positive fibres were found (Fig. 15 E, F), detected by anti-α-sarcoglycan immunofluorescence. Here we also reported sarcoglycan restoration after injection of WT cells without previous marking (Fig. and 16).

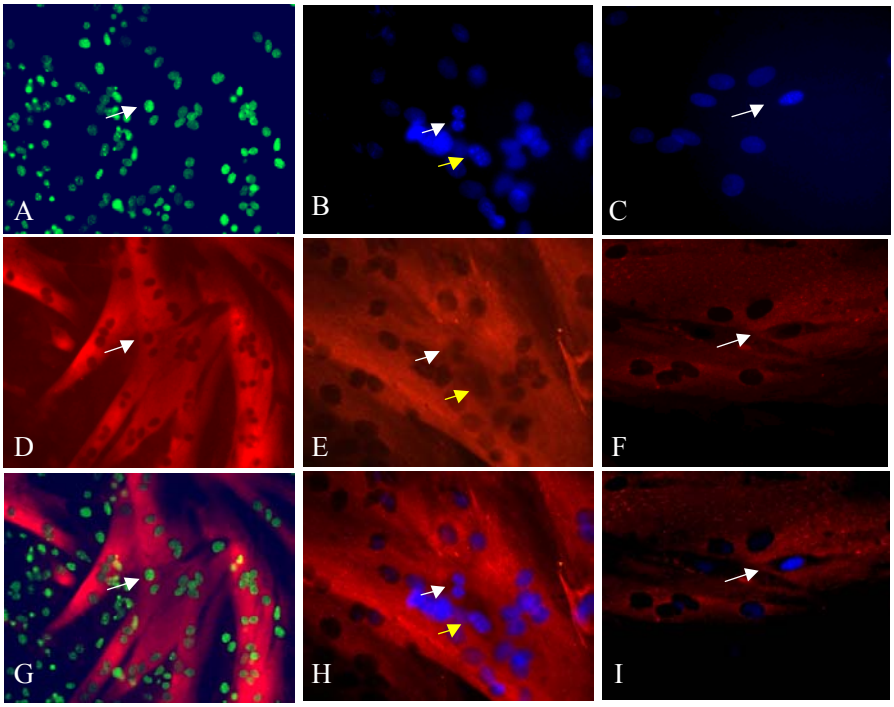

Fig. 13. Co-cultures with the L6 rat myogenic cell line show that there is no differentiation of the Pax3 KO mesoangioblasts into myosin-positive myofibres (C, F and I -merge-, white arrows) as there is a percentage (A,D and G -merge- as B,E and H-merge-, white arrows) of nuclei of the Pax3 WT clones into myosin-positive myofibres (Myosin–negative cells of the Pax3 WT cells are shown by yellow arrows).

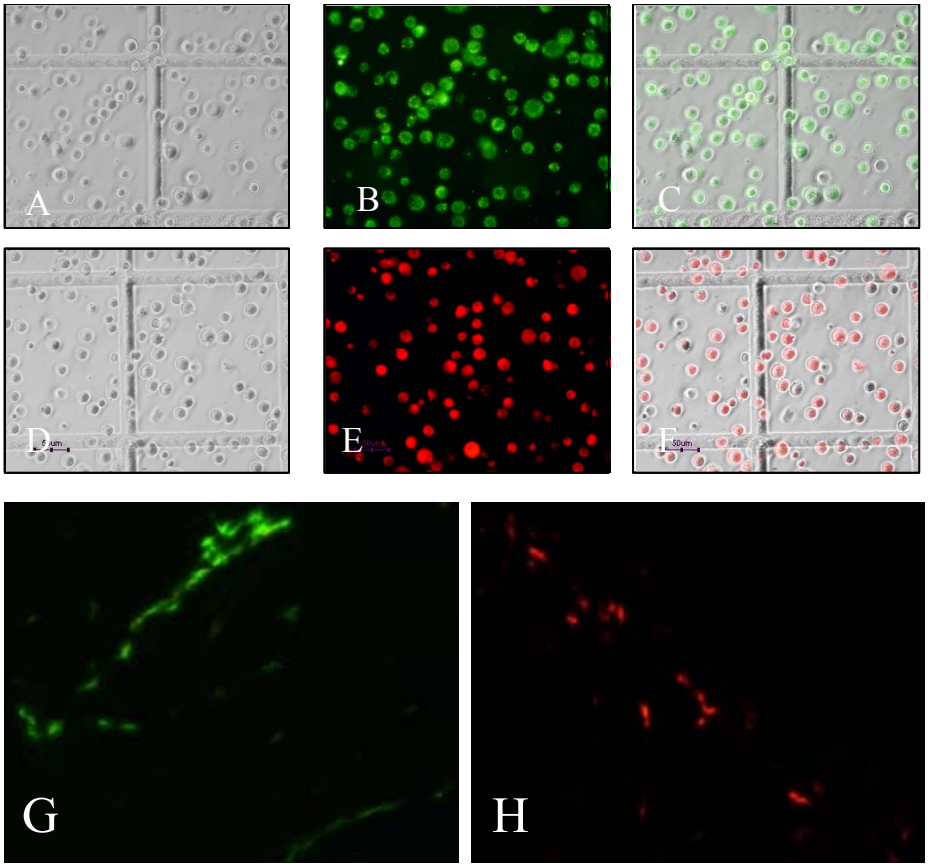

Fig. 14. Pax3 KO mesoangioblasts (A) were labelled with DiO (B) with around 100% efficiency (C). Pax3 WT mesoangioblasts (D) were labelled with DiI (E) at the same efficiency (F) of the former. 14 days after the injection in the tibialis anterior muscle of a 1 mounth old α-SG null mouse, the number of Pax3 KO mesoangioblasts incorporated (G) is equal to the number of Pax3 WT mesoangioblasts (H).

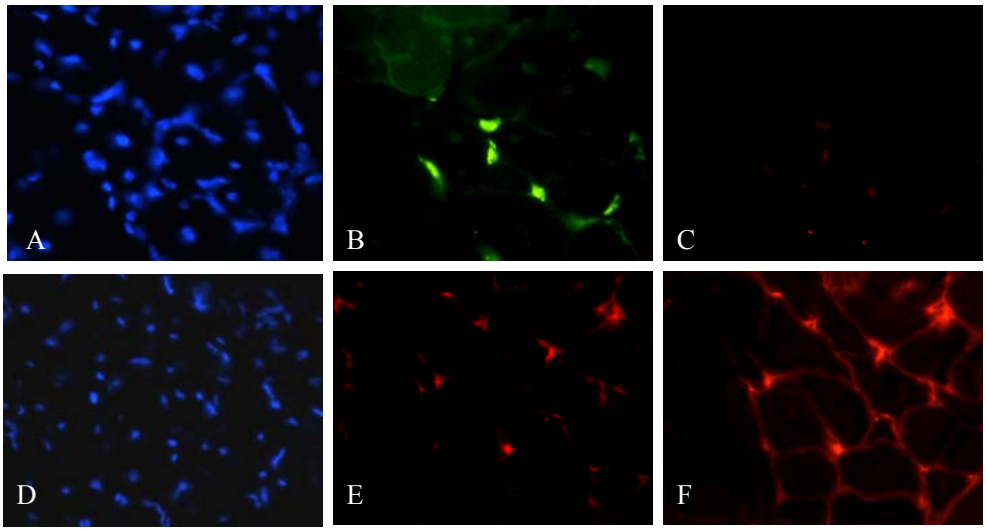

Fig. 15. Pax3 KO mesoangioblasts marked with DiO and injected into the tibialis anterior muscle of α-SG null mouse are found in the muscle (A: Hoechst, B: DiO) 14 days after but they are negative for α-SG protein (C). Pax3 WT mesoangioblasts (D:Hoechst), marked with DiI (E), are found into α-SG-positive areas (F).

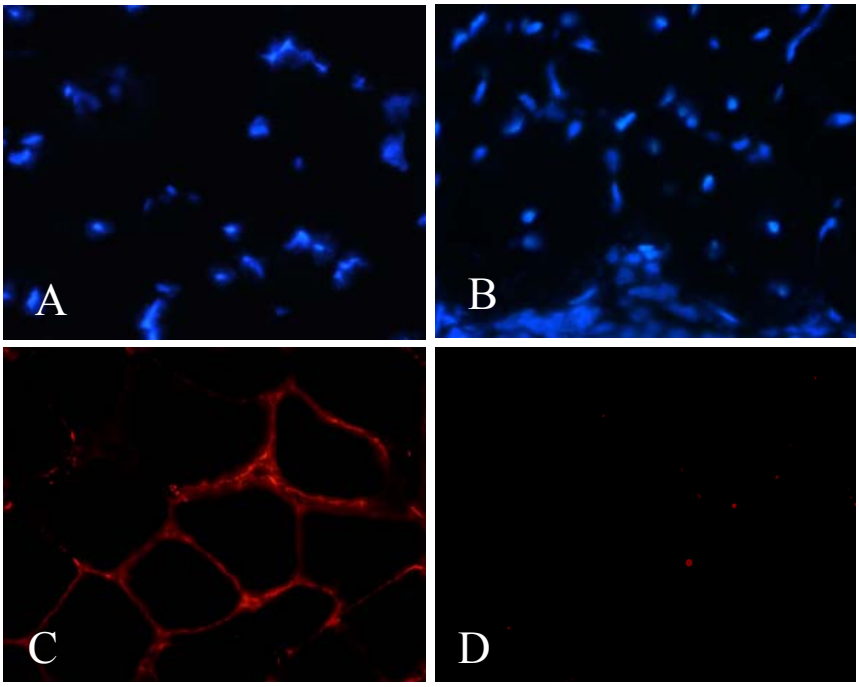

Fig. 16. After injection of not previously marked Pax3 WT mesoangioblasts are found positive areas for α-SG (C) of α-SG null mice as after injection of not previously marked Pax3 KO mesoangioblasts no α-SG-positive area was found (D) (A: Hoechst staining of the field C; B: Hoechst staining of the field D).

DISCUSSION

PAX3 IS EXPRESSED IN MESOANGIOBLASTS

Embryonic dorsal aorta, isolated from E9.5 embryos, does not contain levels of Pax3 message detectable by RT-PCR (De Angelis et al., 1999) since Pax3 expressing neural crests have not yet migrated towards it. In contrast mesoangioblasts isolated from the same aorta show robust expression of Pax3 even at early passages. This supports the hypothesis that *in vivo* acquisition of a mesoangioblast phenotype, likely mimicked by *in vitro* growth conditions, triggers Pax3 activation in these cells. To test the possible role of Pax3 in mesoangioblast self-renewal, survival and differentiation potency, we studied these parameters in Pax3 knock out embryos, which display the same phenotype of *"Splotch"* embryos, natural Pax3 mutants. All the analyses on Pax3 knock out mesoangioblasts were made in comparison with wt siblings, used as positive control. We initially analysed by RT-PCR a number of marker genes that define the mesoangioblast phenotype in both wt and Pax3 null isolates. Consistent with previous results (Minasi et al., 2002) both wt and Pax3 null mesoangioblasts were found to express early emoangioblast genes such as CD34, Sca1, and VE Caderin; in addition we confirmed that these cells highly express neural genes like Emx2, more characterized in neural stem cells (Galli et al., 2002), and Pax3. A number of evidence (see Introduction) reveal that this gene is involved in multipotency for several types of stem-progenitor cells.

Our first results indicated that the basic features of mesoangioblasts are not influenced by Pax3. Pax3 null mesoangioblasts show a similar epithelioid morphology, similar proliferation rate in normal growth conditions, clonal efficiency and expression of mesoangioblast markers when compared to mesoangioblasts from wild type embryos. FACS analysis demonstrated that both wt and Pax3 null mesoangioblasts are highly homogeneous for the expression of surface markers. In agreement with the RT-PCR data, both Pax3 null and wt cells are equally positive for CD34, Sca-1, and totally negative for Flk-1 and c-kit.

PAX3 IS REQUIRED FOR ALKALINE PHOSPHATASE (ALP) EXPRESSION IN MESOANGIOBLASTS

We then tested whether absence of Pax3 would interfere with the different differentiation pathways that can be induced in these cells. Results showed that, even in the absence of Pax3 expression, mesoangioblasts are able to differentiate *in vitro* into adipocytes, smooth muscle cells and cardiomyocytes as efficiently as their normal counterparts.

In order to induce osteogenic differentiation we treated mesoangioblasts with BMP2 *in vitro* and observed that Pax3 null cells show a dramatic decrease in the expression of ALP, an early but not univocal marker of osteogenic differentiation. In comparison with wt cells, 30% of which express ALP, only 3% of Pax3 null cells expressed this marker. This finding suggested impaired osteogenic differentiation and indeed the Pax3 null embryo shows, besides neurogenic and myogenic deficits, impairment if skeleton development, especially in face bone, ribs, knee and pelvis (Tremblay et al., 1998).

However when we analysed expression of other osteogenic genes we observed that Cbfa1, the master gene of osteogenesis, even in absence of any treatment is equally expressed in Pax3 null as well as in wt cells. The same pattern was observed for Col-1 (Collagene 1) and even only by short-term treatment with BMP-2 was observed for expression of Bone Sialo-protein (BSP), Osteopontin, Osteonectin and Osteocalcin, other early and late osteogenic markers. This indicates that mesoangioblasts are basically able to be committed to the osteogenic program. The surprising fact was that unlike the other early genes ALP is not produced in null cells, suggesting that it may be a direct target of Pax3, independently from the whole osteogenic program that indeed occurs in the Pax3 null embryo although it is abnormal.

PAX3 IS REQUIRED FOR MESOANGIOBLAST MYOGENIC DIFFERENTIATION

Since skeletal myogenesis is induced in mesoangioblasts only in co-culture with myogenic cells, mouse mesoangioblasts were co-cultured with rat myogenic cells (L6 line) so that mouse nuclei inside myosin positive myotubes or mono-nucleated myocytes could be detected by Hoechst staining (mouse nuclei show gross particles while rat nuclei are homogenous). In these co-cultures we observed that while wt mesoangioblasts differentiate into myotubes with a frequency of 5% of the population, no Pax3 null mesoangioblast nuclei were detected inside muscle cells indicating that they have completely lost skeletal muscle differentiation capacity. This agrees with the model introduced by Tajbakhsh et al. (1998)

who studied the phenotype of a Pax3-Myf5 double knock-out mouse and observed no muscle formation in the body of the embryo. These data support the hypothesis that Pax3 and Myf5 acts upstream and converge on MyoD activation. Consistently we observed by immunofluorescence that Pax3 null mesoangioblasts are totally negative for the expression of MyoD protein. These data show for the first time that one of the genes regulating myogenesis in embryonic myogenic progenitors from the paraxial mesoderm is also required to regulate myogenesis in mesoangioblasts that rather derive from lateral mesoderm.

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