TGFβ/BMP activate the smooth muscle/bone differentiation programs in mesoangioblasts

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Summary

Mesoangioblasts are vessel-derived stem cells that can be induced to differentiate into different cell types of the mesoderm such as muscle and bone. The gene expression profile of four clonal derived lines of mesoangioblasts was determined by DNA micro-array analysis: it was similar in the four lines but different from 10T1/2 embryonic fibroblasts, used as comparison. Many known genes expressed by mesoangioblasts belong to response pathways to developmental signalling molecules, such as Wnt or TGFβ/BMP. Interestingly, mesoangioblasts express receptors of the TGFβ/BMP family and several Smads and, accordingly, differentiate very efficiently into smooth muscle cells in response to TGFβ and into osteoblasts in response to BMP. In addition, insulin signalling promotes adipogenic differentiation, possibly through the activation of IGF-R. Several Wnts and Frizzled, Dishevelled and Tcfs are expressed, suggesting the existence of an autocrine loop for proliferation and indeed, forced expression of Frzb-1 inhibits cell division. Mesoangioblasts also express many neuro-ectodermal genes and yet undergo only abortive neurogenesis, even after forced expression of neurogenin 1 or 2, MASH or NeuroD. Finally, mesoangioblasts express several pro-inflammatory genes, cytokines and cytokine receptors, which may explain their ability to be recruited by tissue inflammation. Our data define a unique phenotype for mesoangioblasts, explain several of their biological features and set the basis for future functional studies on the role of these cells in tissue histogenesis and repair.

Supplemental data available online at http://jcs.biologists.org/cgi/content/full/117/19/4377/DC1

Key words: Stem cells, DNA microarray, Differentiation

Introduction

Many different types of stem/progenitor cells have been described to be somehow associated with the vasculature: hematopoietic stem cells (HSC) (Orkin, 2000), hemangioblasts (Jaffredo et al., 1998), endothelial progenitor cells (EPC) (Kawamoto et al., 2001), mesenchymal stem cells (MSC) (Bianco and Gehron Robey, 2000) and, more recently, mesoderm adult progenitors (MAP) (Jiang et al., 2002) and mesoangioblasts (Cossu and Bianco, 2003). How many types of stem cells exist in this compartment and their lineage relationships are currently unresolved issues, given the difficulty in proper identification and characterization of a specific cell-type in a given tissue niche at a given developmental stage. For example, it is unknown whether hemangioblasts persist in post-embryonic life and may be the ancestors of the EPC that derive from bone marrow and participate in physiological and pathological angiogenesis. Similarly unknown are the lineage relationships of mesoangioblasts and MSC that share many biological features (perivascular location and differentiation potency) but differ for antigen expression (CD34 is expressed in the former cells only) and for the fact that mesoangioblasts probably derive from a primitive luminal angioblast (transient expression of early endothelial markers such as Flk1) whereas this has not been proposed for MSC. Finally, MAP are also present in the same niche and express similar markers but, at variance with the MSC and mesoangioblasts, appear to express a much broader developmental potency and can also differentiate into ectodermal and endodermal derivatives. In principle gene array technology may help to define more precisely the phenotype of these cells; in practice technical problems still hamper this effort. Prospective, acute isolation on the basis of expression of two or even three markers may result in heterogeneous cell populations that only share the expression of the markers used for purification; in vitro expansion of the progeny of a single cell induces changes in gene expression that probably reflect adaptation to culture conditions and may mask or at least modify the original program of gene expression. However, until micro-array analysis is possible on a single cell, we are left with these approaches, that, although biased by the problem mentioned above, are still very powerful in producing information on new or known genes. This information may then be used to improve purification, and possibly to better understand the biology of these families of stem cells.

We recently reported the isolation, characterization and in vitro expansion of a new type of mesoderm stem cell, termed 'mesoangioblast' that derives from embryonic vessels and is capable of differentiating in many cell types of the mesoderm (Minasi et al., 2002). Mesoangioblasts are isolated from explants of dorsal aorta or other embryonic or juvenile post-natal vessels. Although the explant contains different cell types, such as endothelial cells, pericytes, mesenchymal cells and possibly hematopoietic cells, the only cells that continue to grow and become immortal under the conditions employed have all the same cobblestone, epitheliod morphology and all the clonal isolates express the same set of genes such as CD34, Thy-1 and Sca1 (Minasi et al., 2002). In vitro mesoangioblasts differentiate into many mesoderm cell types, such as smooth and striated muscle, bone and endothelium, when treated with the appropriate signalling molecules or co-cultured with differentiating cells of the same tissue. When transplanted into a 2-day-old chick embryo, mesoangioblasts colonize most mesoderm tissues and differentiate into many cell types of the mesoderm, but fail to colonize ectoderm or entoderm. When delivered through the arterial circulation, mesoangioblasts significantly restore skeletal muscle structure and function in a mouse model of muscular dystrophy (Sampaolesi et al., 2003). To gain information on the pattern of genes expressed by mesoangioblasts, we established transcriptional profiles for four different mesoangioblast lines, all derived from the same embryonic aorta, and compared these profiles with that obtained from the 10T1/2 line of embryonic fibroblasts. Here we report the results of this analysis and correlate the information on expressed genes with the biological features and the developmental potency of mesoangioblasts.

Materials and Methods

RNA extraction

Total cellular RNA was isolated from 10^7 proliferating cells of three independent cultures of 10T1/2 cells and from four clones of mesangioblasts (A2, A4, A6 and A14) using an RNeasy RNA isolation kit (Qiagen, Valencia, CA, USA) following manufacturer's recommendations. Disposable RNA chips (Agilent RNA 6000 Nano LabChip kit) were used to determine the concentration and purity/integrity of RNA samples using an Agilent 2100 bioanalyzer.

Microarray analysis

Target synthesis, GeneChip hybridization and data acquisition

Biotin-labelled target synthesis was performed, starting from 5 μg of total cellular RNA, according to the protocol supplied by the manufacturer (Affymetrix, Santa Clara, CA, USA). Labelled cRNA was purified using RNeasy spin columns (Qiagen) and fragmented (15 μg) as described in the Affymetrix GeneChip protocol. Disposable RNA chips (Agilent RNA 6000 Nano LabChip kit) and an Agilent 2100 bioanalyzer were used to determine the concentration and quality of cRNAs as well as to optimize the fragmentation. The fragmented cRNAs were then hybridized to an identical lot of Affymetrix MGU74Av2, MGU74Bv2, and MGU74Cv2 GeneChip arrays for 16 hours. GeneChips were washed and stained using the instrument's standard Eukaryotic GE WS2 protocol, using antibody-mediated signal amplification.

The amount of a transcript mRNA (signal) was determined, using the MAS 5.0 absolute analysis algorithm, as the average fluorescence intensity among the intensities obtained from the probe set (i.e. 10 to 15 paired, perfectly matched and single nucleotide-mismatched probes consisting of 25-base oligonucleotides). The signal of a probe set is calculated as the one-step biweight estimate of combined differences of all the probe pairs (perfectly matched and mismatched) in the probe set. A one-sided Wilcoxon's signed rank test was used to calculate a *P* value that reflects the significance of differences between perfectly matched and mismatched probe pairs. The *P* value was used to make the absolute call for probe sets. A call 'present' (P) was assigned to transcripts for *P* value between zero and 0.04, a call 'marginal' (M) was assigned to transcripts for *P* value between 0.04 and 0.06, and a call 'absent' (A) was assigned to transcripts for *P* value between 0.06 and 0.5. The MAS 5.0 comparison analysis algorithm was used for comparing gene expression levels between two samples. To determine the *P* values, a signed rank analysis was performed on the perfectly matched and mismatched differences for each probe pair in a probe set from the two arrays in the comparison. The *P* value was used to assign a change call, 'increased' (I), 'decreased (D) or 'not changed' (NC). In addition, a fold-change calculation was performed to indicate the relative change of each transcript represented on the probe array. The signal log ratio calculation is an extension of the signal calculation. The one-step discrimination of the $\log_2$ ratio was used to correct the outlier probes. The one-step biweight method was used to compute the average $\log_2$ ratio of the probe set. All expression values for the genes in the absolute and comparison analyses were determined using the MAS 5.0 global scaling option that allows several experiments to be normalized to one target intensity.

Data filtering and clustering analysis

As a first approach, differentially expressed genes were selected, employing the Affymetrix MAS 5.0 software, because the sequences showed a 'change call' 'I' or 'D' in at least 90% of the pair-wise comparisons between the four mesoangioblast clones and between the mesoangioblast clones and the three $10T\frac{1}{2}$ replicates. Genes showing a detection call 'A' (absent) in both mesoangioblast clones and $10T\frac{1}{2}$ were obviously excluded. The generated list and, independently, the MAS 5.0-generated absolute analysis data were uploaded onto GeneSpring™ software version 6.0 (Silicon Genetics, Redwood City, CA, USA). Data normalization was achieved by two methods: (1) Each signal was divided by the 50th percentile of all signals in a specific hybridization experiment; (2) Each signal was divided by the median of its values in all samples. A more reliable list of 'changing' genes was obtained by applying the filtering options of GeneSpring. Specifically, a restriction, which passes genes with a normalized intensity above 2.0 and below 0.5, was applied. Then, using all the mesoangioblast clones as replicates, we performed a one-way ANOVA test between the mesoangioblast group and the $10T\frac{1}{2}$ group. In particular a parametric test, with variances assumed equal (Student's *t*-test, *P* value cut-off 0.05, multiple testing correction: Benjamini and Hochberg False Discovery Rate) was applied. The gene list passing all these filters and tests was selected as 'changing genes' and subjected to clustering. Hierarchical clustering was performed using the analysis options (gene trees and condition trees) included in the GeneSpring™ package, applying the standard correlation equation.

Frzb1 protein production

Frzb1 protein was produced as previously described (Lyens et al., 1997). Briefly, 293T cells were transiently transfected with p*Frzb1*-HA plasmid, which encodes HA-tagged Frzb1, by standard lipofectamine technique (Invitrogen). Frzb1-conditioned medium (Frzb1-CM) was obtained by culturing transfected cells in D-MEM plus 5% FCS for 48 hours. The presence of Frzb1-HA protein in the conditioned medium was assessed and quantified by western blot using an anti-HA antibody (BabCo), as previously described (Borello et al., 1999). Fresh FCS (5%) was added to the conditioned medium

to provide growth factors during the proliferation assay. Control conditioned medium was obtained by an identical procedure from 293T cells transiently transfected with the empty vector.

Cell proliferation assay

Mesangioblast cells (Minasi et al., 2002) were seeded at a density of 10^4 cells/ml in Frzb1-CM or in control CM. We used two different preparations of conditioned medium at two different concentrations, undiluted (Frzb1 100%) or diluted 1:2 with D-MEM plus 10% FCS (Frzb1 50%). Cells were cultivated for three days and counted every 24 hours. At each timepoint, viable cells (assessed by trypan blue exclusion) were counted in triplicate in a hemocytometer.

Neural differentiation

For co-culture experiments cells were previously infected with a third-generation lentiviral vector pRRLsin.PPT-PGK.GFP expressing GFP, as described elsewhere (Minasi et al., 2002). Mash1, NeuroD1, Ngn1 and Ngn2 cDNA (kind gift of F. Guillemot) were cloned in the eukaryotic expression vectors pCDNA3 (Invitrogen) and pIRES2EGFP (Clontech). Mesoangioblasts, transfected with various combinations of expression vectors encoding bHLH proneural genes, were grown alone or in co-culture with embryonic neuroblast [from 12.5 days post-coitum (dpc) embryos] in neural stem cell differentiation medium (Gritti et al., 1999) on matrigel-coated chamberslides for 7 to 10 days. At the end of the culture period cells were fixed and analysed for the expression of βIII-tubulin or Glial Fibrillar Acidic Protein (GFAP).

In ovo transplantation of mesoangioblast cells

GFP-labelled mesoangioblasts (see above) were injected, as clumps of cells (containing approximately 10^5 cells), in the neural tube and brain vesicles of H10-12 chick embryos. Embryos were allowed to develop in ovo for 1 to 7 days. Chick embryos were then harvested, fixed, embedded in Tissue-Tek® OCT compound (Sakura Finetek USA, Torrance, CA) and cryostat-sectioned as described (Minasi et al., 2002). Sections containing GFP-positive cells were stained with antibodies directed against β III tubulin or GFAP and scored for the presence of GFP$^+$/β III tubulin$^+$ or GFP$^+$/GFAP$^+$ double-labelled cells.

In vitro differentiation of mesoangioblasts

Cells were treated with 5 ng/ml TGFβ1, with or without pretreatment with the neutralizing antibody anti-TGFβRII (250 μg/ml; R&D Systems) (Tsang et al., 1995), in medium supplemented with 2% horse serum for 5 to 7 days (TGFβ1 was added every other day), then fixed and stained with an antibody recognizing αSMA antibody. Alternatively, mesoangioblasts transiently transfected with Noggin or Chordin expression vectors (human noggin pMT21, *Xenopus* chordin pCS2, kindly provided by N. Itasaki), or mock transfected, were treated with 100 ng/ml BMP2 in complete medium for 5 days (BMP2 was added every other day), then fixed and stained for alkaline phosphatase. For mineralized nodule assays, mesoangioblasts were maintained in growth medium supplemented with 50 μg/ml ascorbic acid, 10 mM β-glycerol phosphate, dexamethazone 10^{-8} M and 100 ng/ml BMP2 for 25 days (zur Nieden et al., 2003). At the end of the culture period, cultures were stained for alkaline phosphatase and mineral deposition was assessed using the von Kossa method, according to standard procedures. For adipocyte differentiation, cells were first grown in DMEM, 10% fetal calf serum, 1 mM glutamine, 500 μM isobutylmethylxanthine (IBMX), 1 μM dexamethasone, and 1 μg/ml insulin. After 2 days, cells were transferred to adipocyte growth medium (DMEM containing 10% fetal calf serum, 1 mM glutamine, 25 mM glucose, 0.5 mM glutamine and 1 μg/ml insulin) and re-fed every 2 days. Differentiation of mesangioblasts into mature adipocytes was confirmed by Red Oil staining.

The proportion of cells differentiating in each different cell type was calculated by counting the cells expressing the appropriate differentiation markers against the total number of cells. An average value was determined by counting cells in at least ten microscopic fields, in at least three different experiments.

Immunofluorescence

Immunofluorescence on cell culture was performed as previously reported (Minasi et al., 2002). The antibodies used in this study and their working dilution were the following: alpha Smooth Muscle Actin monoclonal antibody (mAb) (1:350; SIGMA), calponin mAb (1:500; Sigma), SM22 mAb (1:300) (Chiavegato et al., 1999), SM-MyHC 1 and 2 mAb (1:400) (Borrione et al., 1989), MF20 mAb (1:2; DSHB-Hybridoma Bank), TuJ1 mAb (1:400; BabCo), anti-GFAP polyclonal antibody (1:200; DAKO).

Primary antibodies were reacted with the appropriate secondary antibodies (Molecular Probes and Jackson ImmunoResearch), conjugated with either TRITC, Alexa 594, or Alexa 488, for fluorescence detection.

Western blotting

Western blotting on cell lysates (40 μg proteins/lane) was performed as previously reported (Sampaolesi et al., 2003), using the following antibodies: TGFβRI (V-22), TGFβRII (c-16), BMPR1A, Smad1 (H-465), Smad2/3 (N-19), Frizzled (H-300), β-catenin (c-18), IGF1Rβ (c-20), Wnt5a. All antibodies were from Santa Cruz and used at 1:500 final dilution, with the exception of anti-Wnt5a (1:1000; R&D Systems).

Results

Gene profile of mesoangioblasts

We used the Affymetrix MG-U74 set of high-density oligonucleotide arrays, exploring 6000 characterized mouse genes and 30,000 expressed sequence tags (ESTs), to identify specific genes, or classes of genes, representing the molecular phenotype of mesoangioblasts. The analysis was based on three main background points. First, we were assisted by the availability of independent mesoangioblast clones (Minasi et al., 2002), which eliminated a problem frequently met in stem cell phenotyping, i.e. contamination by other cell types. Second, we analysed four independent clones (A clones: A2, A4, A6, A14) and focused on gene lists satisfying the analysis criteria in all clones. This allowed to limit the inclusion of genes representing clonal phenotypic variations and provided a methodological approach more stringent than simple statistical analysis of different screenings on the same cell population. Third, the gene expression profile of the mouse fibroblast cell line 10T1/2 was determined in parallel with individual A clones. Similar to mesoangioblasts, 10T1/2 are mouse embryonic, fibroblast-like cells, which maintain a high proliferation capacity. However, 10T1/2 cells are not capable of differentiating into any defined phenotype, unless they are treated with 5′-azacytidine, a potent demethylating agent: in this case they differentiate into myoblasts and, to a lesser extent, adipoblasts or chondroblasts. Therefore, they do not meet the criteria defining stem cells, but can rather be considered as ‘conditional’ multipotent cells. In this respect, the subtraction of genes expressed by both 10T1/2 and mesoangioblasts should facilitate the construction of a highly

selective list of genes peculiar to the mesoangioblast stem cell phenotype.

The overall number, or complexity, of RNA sequences expressed by individual mesoangioblast clones and 10T1/2 cells is relatively similar, ranging between 10662 and 12745 sequences, which are called 'present' by the Affymetrix MAS 5.0 absolute analysis algorithm. The complete set of data is available as supplementary material (A clones data file), so that others can design alternative criteria for analyzing the data. The Venn diagram shown in Fig. 1 provides a summary of the obtained results: 9233 sequences are expressed by all mesoangioblast clones, whereas 11861 sequences are expressed by all 10T1/2 replicates; 8674 sequences are expressed by both 10T1/2 cells and all A clones; and 203 sequences are specifically expressed in all A clones, but not in 10T1/2 and, conversely, 580 sequences are specifically expressed in 10T1/2. In order to identify differentially expressed genes, we employed the Affymetrix MAS 5.0 comparison algorithm to perform pair-wise comparisons between all cell lines. A first consistent list of 1842 'changing' genes (decreased or increased in mesoangioblasts vs 10T1/2) was obtained. 'Poorly changed' genes (i.e. those showing a normalized intensity between 0.5 and 2) were filtered out, thus restricting the list to 1665 sequences. Among these sequences, the ANOVA test passed 1412 sequences. This list was then used for clustering analysis. A combination of a 'gene tree' and a 'condition tree', computed using the standard correlation equation available in the GeneSpring software package, is provided as supplementary material (Fig. S1). In brief, the 'condition tree' clustering shows that individual cell populations display characteristic expression profiles and different degrees of similarity. The best correlation was found between A14 and A2 and, in hierarchically descending order A6 and A14. The expression profile of 10T1/2 cells is clearly distant from any of the mesoangioblast clones. The 'gene tree' shows clusters of genes more abundant in all A clones or in a single A clone as compared with 10T1/2 cells, as well as genes specifically more expressed in 10T1/2. We focused further analysis on the 554 genes present in the cluster defined as 'Aall', i.e. genes satisfying all the criteria described above and significantly more expressed in all the mesoangioblast clones in comparison with 10T1/2 cells.

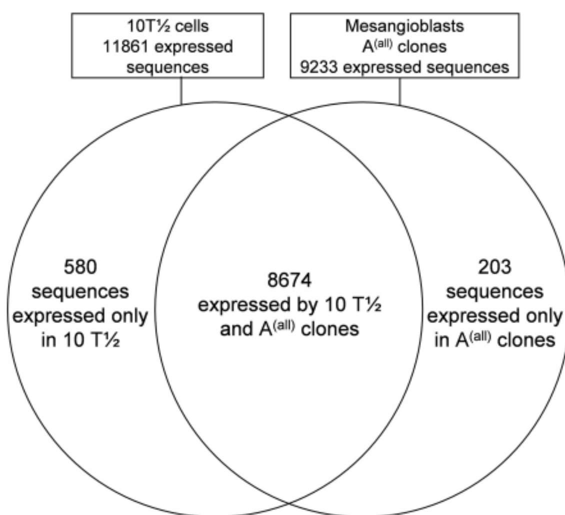

Fig. 1. Absolute analysis of RNA transcripts in mesoangioblasts and 10T1/2 cells. The Venn diagram shows the overall number of sequences expressed by all mesoangioblast clones (9233) and 10T1/2 cells (11861), as well as the number of sequences expressed by both mesoangioblasts and 10T1/2 cells (8674), and specifically by mesoangioblasts (203) or 10T1/2 cells (580).

This gene list was uploaded onto GO Mining Tool (Affymetrix, Santa Clara, USA) to identify prevalent categories in the families' 'biological process', 'molecular function' and 'cellular component'. As it is shown in Fig. S2A (see supplementary material), the prevalent GO categories in 'biological process' are 'development', 'morphogenesis', 'organogenesis', 'defence response', and 'transcription'; in 'molecular function' (Fig. S2B) we could detect as statistically relevant 'DNA binding', 'nucleotide binding', 'protein binding', 'transcription factor' and 'transcription regulator'; in 'cellular component' (Fig. S2C) the most prevalent category is 'nucleus'. To facilitate the identification of specific and functionally relevant genes, which might represent the molecular signature of mesoangioblasts, we further filtered the 'Aall' gene list for genes showing at least a twofold increased expression in all mesoangioblast clones as compared with 10T1/2. We came up with 428 sequences: 99 of them, based on the results obtained from querying GO categories, can be grouped into one of the following families (Fig. 2): (A) DNA binding/transcription factors/transcriptional regulators; (B) integral membrane proteins/membrane receptors; (C) cytoskeleton/cell adhesion and/or motility; (D) defence response/immune response/inflammatory response.

DNA binding/transcription factors/transcriptional regulators

In this category we found genes known to be involved in the genetic programs at the basis of maintenance and/or differentiation of different types of progenitor cells. These include Runx2/Cbfa1, a crucial regulator of osteoblast development and differentiation (Komori et al., 1997), and C/EBP delta, required in the early phase of adipogenic differentiation (Cao et al., 1991) and also involved in osteogenesis (McCarthy et al., 2000). This finding might be sufficient to explain the osteogenic and adipogenic potential of mesoangioblasts (see below). In addition, mesoangioblasts express several genes regulating different phases of lymphoid or myeloid differentiation (such as SOX4, Irf-1, Bcl6, c-fos, Notch1, RelB and NF-kappaB2) or involved in neural specification/differentiation (such as Dlx1 and Dlx2, Nr2f1/COUP-TFI, Ap-2 alpha, Irx1). Other transcription factors found in the cluster were recognized as being functionally involved in several complex developmental processes. This is the case for NFI/A, which is expressed in a wide variety of embryonic and adult tissues (Chaudhry et al., 1997); NFI/B, which is implicated in normal lung development (Grunder et al., 2002); Hic-1, a candidate tumor suppressor gene, whose ablation produces the developmental defects observed in the Miller-Dieker syndrome (acrania, exencephaly, cleft palate, limb abnormalities and omphalocele) (Carter et al., 2000); Peg3/Pw1, a zinc finger containing protein, shown to be a necessary participant of p53-mediated apoptosis in fibroblasts (Relaix et al., 2000) and neurons (Deng and Wu,

2000); Nfkbia, whose ablation in mice produces severe runting, skin defects, and extensive granulopoiesis postnatally (Beg et al., 1995); Hoxa5, a homeotic gene involved in the regionalization and specification of the stomach (Aubin et al., 2002); Hoxa3, which was found to be essential for the formation of the carotid body in the mouse embryo (Kameda et al., 2002); Klf5/BTEB2, shown to be a target for angiotensin II signalling and an essential regulator of cardiovascular remodeling (Shindo et al., 2002); and Jun, which was recently shown to be required for axial skeletogenesis by regulating notochord survival and intervertebral disc formation (Behrens et al., 2003).

Integral membrane proteins/membrane receptors

In this group we identified two well-known stem cell markers: CD34, a cell surface sialomucin-like adhesion molecule expressed in hematopoietic stem cells (Civin et al., 1985) and Thy-1, a 25 kDa glycosylphosphatidylinositol-linked membrane glycoprotein, which is expressed in hematopoietic stem cells, T lymphocytes, neural cells and, during angiogenesis, in endothelial cells, pericytes and smooth muscle cells (Risau, 1998). Mesoangioblasts also express Sca-1, another stem cell marker (Miles et al., 1997), which, however, is also present in 10T1/2. Many of the genes identified in this group encode proteins that have been involved in the development of the vasculature. For example: Efnb2, a marker of arterial endothelial cells at very early stages of development (Adams and Klein, 2000) and Edg3, a member of the family of receptors that bind sphingosine 1-phosphate (S1P) (Panetti, 2002). Among cell surface receptors, mesoangioblasts express Tgfbr2, whose ablation is embryonic lethal around 10.5 days of gestation, because of defects in the yolk sac hematopoiesis and vasculogenesis (Oshima et al., 1996); Pdgfra, which has well-recognized roles in the proliferation and migration of mesenchymal cell types, particularly smooth muscle (Betsholtz et al., 2001); Ogfr, which mediates the action of the native opioid growth factor (OGF), a tonic inhibitory peptide that plays a role in cell proliferation and tissue organization during angiogenesis, development, cancer, cellular renewal and wound healing (Zagon et al., 2002); Osmr, a membrane receptor which binds the pleiotropic cytokine oncostatin M, a member of the IL-6 family, which was shown to have a role in blood and vascular cell development, liver development, bone formation and remodelling (Gomez-Lechon, 1999).

Cytoskeleton/cell adhesion and/or motility

Mesoangioblasts are migrating cells; it is therefore not surprising that they express a variety of genes involved in cell adhesion, motility and remodelling of cell morphology. Ncam1 was shown to be essential for the proper migration of neuronal precursors to the olfactory bulb (Chazal et al., 2000). Embigin

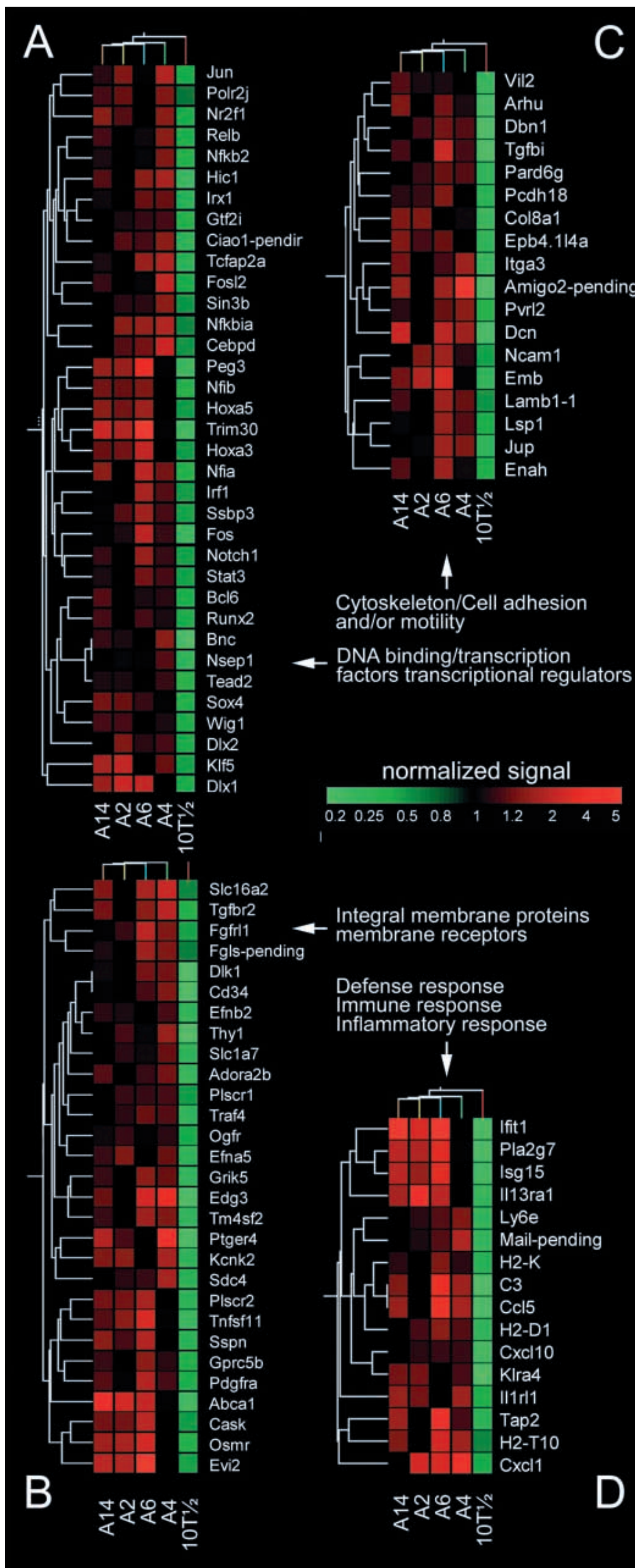

Fig. 2. Clustering analysis of functionally selected genes specifically expressed by mesoangioblasts. The figure shows a combination of two hierarchical clustering analyses (gene tree and condition tree) performed on functionally selected genes, grouped in the following categories: (A) DNA binding/transcription factors/transcriptional regulators; (B) Integral membrane proteins/membrane receptors; (C) Cytoskeleton/cell adhesion and/or motility; (D) Defence response/immune response/inflammatory response. The gene tree is shown on the left, the condition tree on the top of each panel. Gene colouring was based on normalized signals as shown in the colour bar.

is a *trans*-membrane glycoprotein belonging to the immunoglobulin superfamily, which enhances integrin-mediated cell-substratum adhesion (Huang et al., 1993). Arhu/Wrch1 is a recently identified member of the Rho gene family, which could mediate the effects of Wnt-1 signalling in the regulation of cell morphology, cytoskeletal organization and cell proliferation (Tao et al., 2001). Lsp1 is an intracellular, filamentous actin-binding protein, which negatively regulates fMLP-induced polarization and chemotaxis of neutrophils through its function on adhesion via specific integrins (Wang et al., 2002). Enah/Mena is involved in organizing actin polymerization particularly at adherens junctions (Vasioukhin and Fuchs, 2001). Decorin has recently been proposed to be involved in the establishment and/or coordination of a critical myoblast density, through inhibition of migration, a mechanism that might be relevant in allowing normal muscle differentiation during embryonic myogenesis (Olguin et al., 2003).

Defence response/immune response/inflammatory response

Finally, mesoangioblasts express a variety of genes that are implicated in diverse defence response processes. The IL-13 receptor mediates inflammatory responses and is expressed on human B cells (Hershey, 2003). C3 is secreted by epithelial and vascular tissues at local sites of inflammation and is involved in T-cell responses in vivo (Pratt et al., 2002). The CXC subfamily of chemokines, among which CXCL1, CXCL10, CCL5/RANTES and receptors CCR1, 3, 4 and 5 are also expressed in mesoangioblasts (Rossi and Zlotnik, 2000; Payne and Cornelius, 2002).

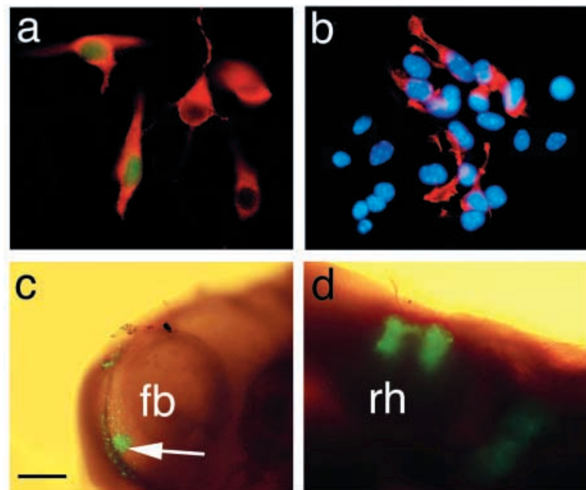

Fig. 3. Abortive neural differentiation of mesoangioblasts. (a) Immunofluorescence analysis of a co-culture of GFP expressing mesoangioblasts with differentiating cells from E10 embryonic neural tube, under neural stem cell differentiation conditions. Cultures were stained with TuJ1, an antibody against β III-tubulin (red). GFP-positive cells appear green mainly in the nucleus where strong staining for β III-tubulin is not present. (b) Immunofluorescence analysis of mesoangioblasts, cultured under neural stem cell differentiation conditions and stained with anti-GFAP (red); nuclei were stained with DAPI (blue). (c,d) Whole-mount fluorescence analysis of an E5 chick embryo brain injected with GFP-labelled mesoangioblasts. Clusters of GFP-positive cells can be detected both in forebrain (fb) and hindbrain where they appear to be restricted to specific rhombomeres (rh). Scale bar in c: 50 μm for a; 75 μm for b; 600 μm for c and d.

Expression of neural-specific genes in mesoangioblasts does not relate to a neurogenic potential

Because mesoangioblasts are able to differentiate only into mesodermal cell types, we predicted that the majority of genes expressed by these cells would also be expressed in the mesoderm of the developing embryo. A screening of the literature for the embryonic expression pattern of genes preferentially expressed in mesoangioblasts confirmed this prediction (Fig. S3 in supplementary material). Surprisingly, however, many genes appeared to be expressed in neural tissue or in both neural and mesoderm tissues; very few are expressed in non-neural ectoderm or in endoderm.

Neural genes preferentially expressed by mesoangioblasts appear to be involved in the development of the nervous system. This is the case for GPRC5B, which is expressed in brain and spinal cord (Robbins et al., 2002); Tm4sf2, which has been implicated in activity-dependent brain plasticity (Boda et al., 2002); TRAF4, which is required for the closure of the neural tube (Regnier et al., 2002); ABCA1, phospholipid scramblase 1 and 2 (for a review, see Amon et al., 2002); Efna5, which acts as a guidance cue that restricts limbic thalamic axons from inappropriate neocortical regions (Uziel et al., 2002).

The expression of many neural-specific genes (both receptors and transcription factors) was puzzling in spite of the mesodermal origin of mesoangioblasts. Therefore we performed a series of experiments designed to test the possible existence of a neurogenic differentiation potential in mesoangioblasts, not revealed by previous transplantation experiments in the chick mesoderm (Minasi et al., 2002).

GFP-labelled mesoangioblasts were cultured in neural-promoting media (Gritti et al., 1999) alone or in co-culture with embryonic neuroblasts or neural stem cells, and at different times scored for co-expression of GFP and neural markers (Fig. 3a,b). With the exception of rare cells co-expressing GFP and βIII-tubulin or GFAP, no other differentiation markers nor morphological changes associated with neurogenesis or gliogenesis were observed. Furthermore, forced expression of pro-neural genes such as Mash-1, neurogenin 1 and 2 and Neuro D, alone or in combination, failed to induce the expression of neural markers (not shown). Finally, transplantation of GFP-labelled mesoangiobioblasts into embryonic chick neural tube (HH10) resulted in survival and proliferation of grafted cells, none of which, however, expressed neural markers (Fig. 3c,d). Thus, it appears that mesoangioblasts are essentially incapable of neural differentiation (at least under the various conditions employed here) and only rare and abortive neurogenesis can be experimentally induced.

Signal transduction pathways in A clones and their relation to differentiation potency

Mesoangioblasts are bona fide stem cells, capable of self-

renewal as well as of differentiation into several cell types. We were prompted to investigate the presence of pathways that regulate these processes. Screening of NetAffx, OMIM, LocusLink, MGI and PubMed databases, as well as of Science STKE and Transpath databases, allowed us to generate pathway-oriented gene lists.

Fig. 4a shows that A clones express several members of the TGFβ/BMP receptor family pathway, particularly Tgfbr2 and Tgfbr1 and also Acvr1 and Bmpr1a. Madh family members are also expressed, particularly Madh4, Madh1 and Madh2. The presence of many of these proteins was also confirmed by western blot (Fig. 4c). Remarkably, the relative amount of the two TGFβ receptor proteins reflects the RNA content as determined by microarray analysis. Mesoangioblasts readily respond to TGFβ1 by activating transcription of smooth muscle-specific genes, such as Calponin 1 and Smooth Muscle Myosin Heavy Chain (SM-MyoHC) (Simper et al., 2002) (Fig. 4e-h). This differentiation pathway was partially inhibited when the availability of one of the receptors, Tgfbr2, was decreased by pre-treatment with a specific neutralizing antibody (Tsang et al., 1995) (Fig. 5a-d). Mesoangioblasts also respond to BMP2 by differentiating first into Alkaline Phosphatase (AP)-positive cells and then into Von Kossa-positive osteogenic nodules (Fig. 4i). When we transfected mesoangioblasts with the BMP antagonists chordin or noggin (Balemans and Van Hul, 2002; Nagano et al., 2000), prior to

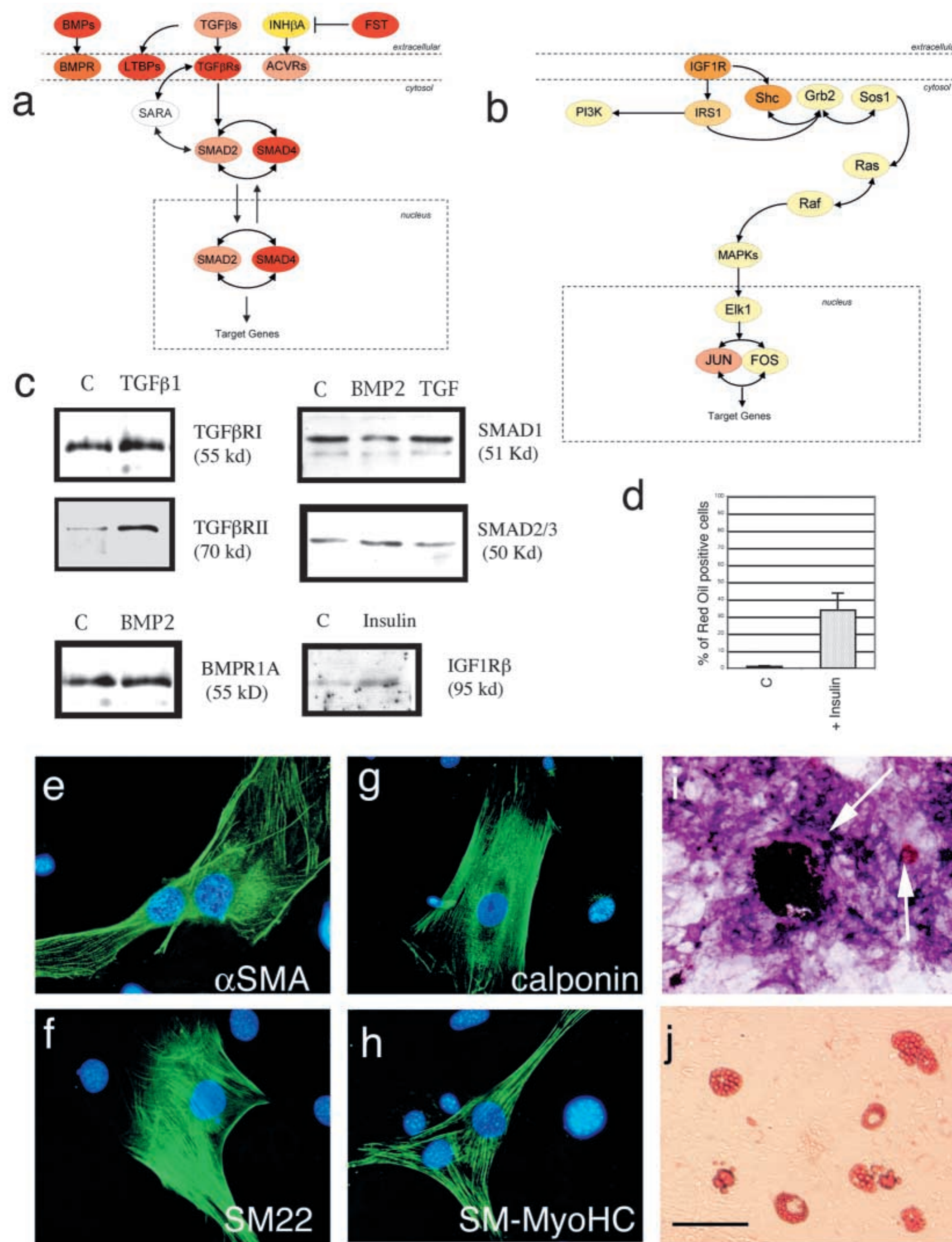

Fig. 4. TGFβ, BMP and insulin mediated differentiation of mesoangioblasts. (a,b) Schematic representation of the TGFβ/BMP (a) and insulin (b) signalling pathways in mesoangioblasts: the colour codes indicate the abundance of the different pathway components, ranging from red (highly expressed) to pale red to orange to yellow (poorly expressed); white: not in the array. (c) Western blot analysis of cell lysates from mesoangioblasts treated or untreated (C) with TGFβ1, BMP2, or insulin; antibodies against TGFβRI, TGFβRII, BMPR1A, Smad1, Smad2/3, or IGF1Rβ were used. (d) Histogram representing the percentage of mesoangioblasts staining Red Oil positive, after treatment with insulin-containing medium. (e-g) Immunofluorescence analysis of mesoangioblasts treated with TGFβ1 and stained with antibodies against smooth muscle α-actin (αSMA; e), SM22 (f), calponin 1 (g) and smooth muscle myosin (SM-MyoHC; h) (all in green), merged with DAPI-stained nuclei (blue). (i) AP and Von Kossa staining of mesoangioblasts treated with BMP2 under osteogenic-promoting condition. Arrows point to calcified nodules. (j) Red Oil staining of mesoangioblasts treated with insulin under adipogenic-promoting condition. Scale bar in j: 50 μm for e-h; 200 μm for i; 150 μm for j.

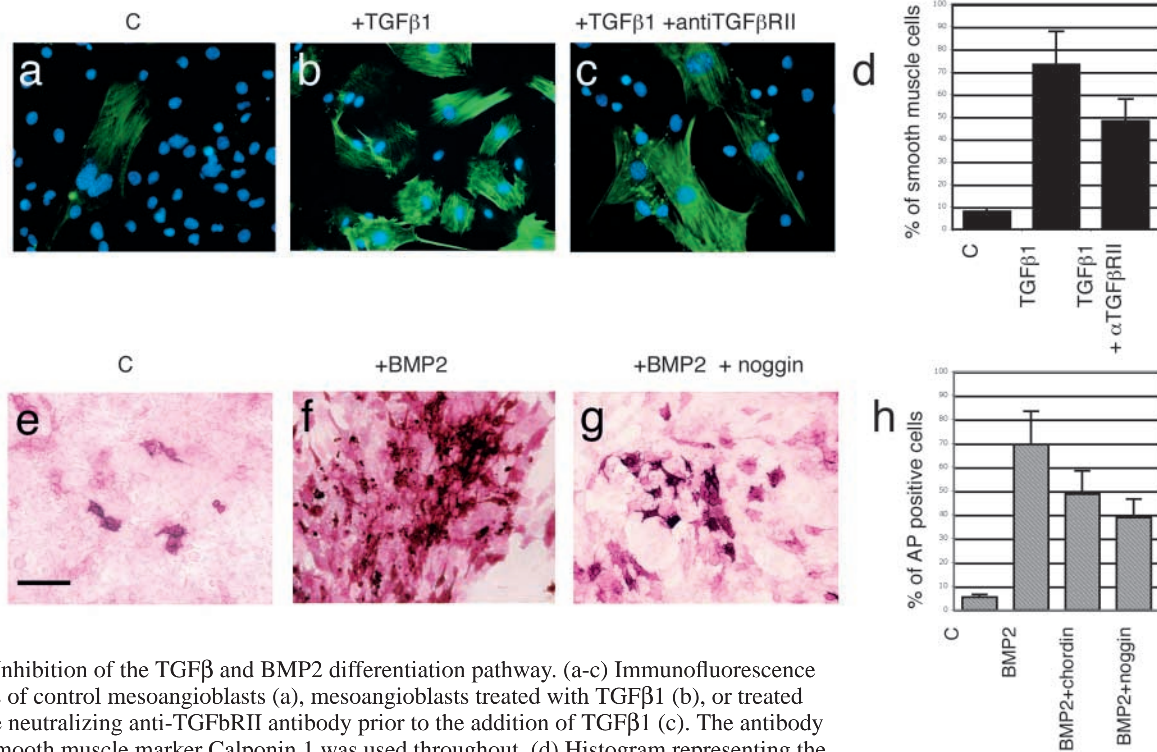

Fig. 5. Inhibition of the TGFβ and BMP2 differentiation pathway. (a-c) Immunofluorescence analysis of control mesoangioblasts (a), mesoangioblasts treated with TGFβ1 (b), or treated with the neutralizing anti-TGFbRII antibody prior to the addition of TGFβ1 (c). The antibody to the smooth muscle marker Calponin 1 was used throughout. (d) Histogram representing the percentage of mesoangioblasts expressing Calponin 1 in the indicated culture conditions. (e-g) AP staining of control mesoangioblasts (e), mesoangioblasts treated with BMP2 (f), or transfected with noggin expression vector prior to the addition of BMP2 (g). (h) Histogram representing the percentage of AP-positive mesoangioblasts in the indicated culture conditions. Scale bar in e: 150 μm for a-c; 200 μm for d-f.

the treatment with BMP2, we observed a reduced osteoblast differentiation, as shown by AP staining (Fig. 5e-h). The majority of the population responds to these molecules, suggesting that smooth muscle and bone are indeed the preferred differentiation pathways in mesoangioblasts.

In addition, mesoangioblasts differentiate into adipocytes in response to insulin (Fig. 4d,j), a signal that can be mediated by the membrane receptors of the IGF family, which are expressed in mesoangioblasts (Fig. 4c). Other signal transduction pathways might be active in mesoangioblasts, based on the expression of specific membrane receptors such as Fgfr1, Fgfr2 and Pdgfra, Pdgfrb (not shown).

As shown in Fig. 6a, all mesoangioblast clones express Wnt receptors, particularly Fzd4 and 8, and other cell membrane components involved in Wnt binding, such as Lrp1, Sdc2, Gpc1 and 4. Signal transduction pathway components are also expressed at a high level: Dvl, Dvl2, βcatenin, Gsk3b, Axin, as well as many protein kinases (Csnk1e, Csnk1a1, Csnk2a2) and phosphatases (Ppp2cb, Ppp2r1a, Ppp2r5c), which are known to regulate the activity of the Wnt pathway. We also confirmed, by western blot analysis, the presence of some of the protein products corresponding to key pathway components, such as Frizzled and β-catenin (Fig. 6b and data not shown). Wnt-dependent transcription might be accomplished through Tcf3 and, to a lesser extent, Tcf4 and 7; other mediators of Wnt-dependent transcription are represented by Cited2, Ruvbl1, Madh4 and Ctbp1. Many Wnt target genes are also expressed, particularly follistatin, Wirch1 and Kruppel-like factor 5, which are not present in 10T1/2. Finally, mesoangioblasts express Wnt family members, in particular we detected the presence of Wnt5a protein (Fig. 6b), suggesting the existence of a functional Wnt autocrine loop, which might contribute to mesoangioblast maintenance. Because Wnt genes control proliferation and differentiation in several mesodermal cell types, we verified whether mesoangioblasts have biological activity related to the expression of the Wnt pathway. To this purpose we exposed mesoangioblasts to medium conditioned by cells expressing the Wnt antagonist, Frzb-1 (Borello et al., 1999). Figure 6c,d shows that Frzb-1 inhibits the growth of mesoangioblasts in a concentration-dependent fashion.

Discussion

Gene profile of mesoangioblasts

The aim of this work was to understand the biology of mesoangioblasts, a novel class of stem cells, by identifying classes of genes, or specific genes, representing their molecular phenotype. The results obtained revealed a complex and specific molecular phenotype, marginally overlapping with that expressed by other classes of stem cells but clearly different from that expressed by the embryonic fibroblasts. In fact, a comparison of the gene list representing the cluster 'Aall' with a previously published gene list representing putative 'stemness' genes (Ramalho-Santos et al., 2002) has revealed that only five genes responding to our selection criteria are shared by the two lists [transcriptional enhancer factor (TEA) domain family member 2, lysosomal-associated protein

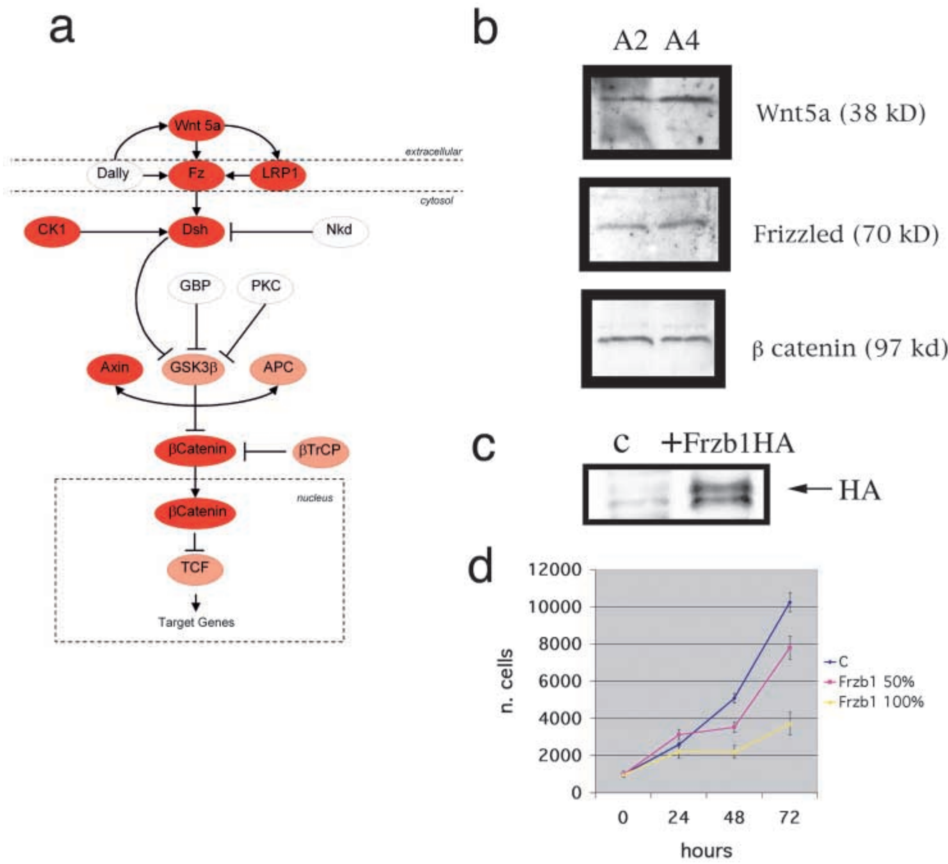

Fig. 6. Wnt pathway and proliferation in mesoangioblasts. (a) Schematic representation of the Wnt signalling pathway in mesoangioblasts; colour codes as in Fig. 4. (b) Western blot analysis of cell lysates from the mesoangioblast clones A2 and A4, using antibodies against Wnt5a, Frizzled and β-catenin. (c) Western blot analysis of concentrated culture medium from Frzb1-HA transfected 293T cells, using anti-HA antibody. (d) Growth curve of mesoangioblasts exposed to different concentrations of Frzb1 (pink and yellow lines, respectively, representing 50% and 100% of added medium from Frzb1-HA transfected 293T cells) and in control culture conditions (blue line).

transmembrane 4B, aldehyde dehydrogenase-family seven-member A1, wild-type p53-induced gene 1 and a gene of unknown function]. This finding is not surprising because it has been recently pointed out (Fortunel et al., 2003; Evsikov and Solter, 2003) that a unique 'stemness' phenotype (if existing) is unlikely to be defined by a transcriptional profiling approach. Hybridization to DNA microchips only provides clues to mechanisms that need a successive biological proof before they can be considered as functionally significant. For example, as it will be discussed in the following section, the expression of neural-specific functions does not ensure, per se, that mesoangioblasts are capable of differentiating into neural cells.

Lack of neural potential in mesoangioblasts

Mesoangioblasts are derived from the vasculature and have been shown to differentiate only into mesoderm cell types both in vitro and in vivo, upon transplantation into chick embryos (Minasi et al., 2002) or dystrophic mouse muscle (Sampaolesi et al., 2003). In agreement with their mesodermal origin, an analysis of embryonic tissue distribution of known genes (for which in situ hybridization data are available) revealed that the majority of genes preferentially expressed by mesoangioblasts are indeed expressed in the mesoderm. Surprisingly, however, many genes expressed by mesoangioblasts are expressed in neural tissues or in both neural tissues and mesoderm, whereas very few genes were expressed in non-neural ectoderm or entoderm. This observation, later confirmed by a detailed analysis of several transcription factors and surface receptors preferentially expressed during neurogenesis, suggested the possibility that mesoangioblasts may have a cryptic neurogenic potential not apparent upon the experimental conditions previously employed. To address this possibility we performed a series of experiments designed to demonstrate this potential. However, culture conditions that promote neural differentiation, co-culture with embryonic neuroblasts and even expression of proneural genes, such as Mash1, Neurogenin 1 and 2 and NeuroD (alone or in couples), failed to induce neural or glial differentiation. The expression of βIII-tubulin or GFAP was observed in a minority of the cell population, which never progressed to express more mature markers or acquire a mature neural or glial morphology. These results can be explained by an intrinsic refractory state of mesoangioblasts to induced neurogenesis, much as it happens in entodermal cells that fail to activate myogenesis in response to forced expression of MyoD (Schafer et al., 1990). Alternatively, the right conditions to promote neurogenesis may not have been used; however, considering the available data on other stem or progenitor cells, this appears a less likely possibility.

Myogenic (smooth) and osteogenic potency of mesoangioblasts

Among the family of membrane receptors, signal transducers and transcription factors, mesoangioblasts express at high level many members of the TGFβ/BMP pathway, including several

receptors and SMADs. In all cases examined by western blot analysis, we found a qualitative and quantitative correspondence with the hybridization signal obtained from the microarray analysis (see, for example, TGFβ receptors I and II). In accordance with this, more than 50% and up to 80% of the mesoangioblasts promptly differentiate into smooth muscle cells in response to TGFβ1 and into osteoblasts in response to BMP2. Furthermore, a different mesoangioblast clone, which spontaneously expresses an immature smooth muscle phenotype, has been instrumental in the identification of two transcription factors, necdin and msx2, which are induced by TGFβ and, when expressed in mesoangioblasts, activate a smooth muscle program (Brunelli et al., 2004). The strong propensity to differentiate into smooth muscle and bone, together with their location in the perivascular niche suggest a close relationship with mesenchymal stem cells, of which mesoangioblasts may be progenitors (Cossu and Bianco, 2003). However, the two cell types differ for the expression of CD34, strongly expressed in the former but not in the latter and for the developmental options: mesoangioblasts differentiate into cardiac and skeletal muscle far more efficiently than mesenchymal stem cells, but fail to differentiate into cartilage, at least under the conditions that promote chondrogenesis in the latter cells.

Wnt pathway and skeletal myogenesis in mesoangioblasts

Many members of the Wnt signalling pathway are expressed at high level in mesoangioblasts, including several Frizzled and co-receptors, axin, GSK-3, beta catenin and Tcfs. The pathway is probably active through an autocrine loop in mesoangioblasts because: (1) Many Wnt target genes, such as c-Myc or CDK4 are indeed expressed; and (2) Exposure of mesoangioblasts to medium conditioned by cells expressing Frzb-1, a soluble Wnt antagonist, reduces their growth rate in a dose-dependent fashion. Besides their role in cell growth and transformation, different Wnts have a positive role in the activation of differentiation in many cell types. However, simple withdrawal of mitogens did not induce spontaneous differentiation into any recognizable cell type, such as neurons or skeletal muscle. Indeed, mesoangioblasts mainly express Wnt5a that is involved in early angiogenesis and this is compatible with an angioblast phenotype. Wnt5a has been shown to induce skeletal myogenesis in somites, in combination with Sonic hedgehog (Münsterberg et al., 1995). However, attempts to induce myogenesis by co-culture of mesoangioblasts with Wnt1 or Wnt7a expressing cells failed, even when activated Gli1 (to complement the Shh pathway) and the Notch inhibitor Numb were expressed in these cells. In contrast, C2C12 myogenic cells over-expressing Frzb-1, a soluble Wnt antagonist, recruited mesoangioblasts to myogenesis as efficiently as control C2C12, suggesting that myogenesis is induced in mesoangioblasts by molecules different from Wnts. We conclude that activation of skeletal myogenesis in mesoangioblasts is mediated by signals released by differentiating cells and probably different from those released by the neural tube and dorsal ectoderm during embryogenesis. Not in agreement with these observations are recent data showing that side population (SP) cells, notably a bone marrow-derived progenitor population, can be induced to skeletal myogenesis by Wnts present in regenerating muscle (Polleskaya et al., 2003). Clearly, further experiments will be necessary to clarify this issue.

Cytokine, cytokine receptors and the migratory ability of mesoangioblasts

Among genes selectively expressed by mesoangioblasts are many cytokines, chemokines and their receptors. This is consistent with a role of these cells in tissue regeneration and first inflammatory response to damage. In this context it is interesting to note that, when injected into the femoral artery, mesoangioblasts can adhere to the endothelium and extravasate but only in the presence of inflammation, as it occurs in muscular dystrophy or after local injection of a myotoxic agent. Indeed, mesoangioblasts express many but not all of the proteins that leukocytes use to this purpose, and this may explain a lower efficiency (only 30% of injected mesoangioblasts end up in downstream skeletal muscle), opening perspectives to optimize this process for future cell therapy protocols in larger animals or in patients.

Furthermore, we have recently observed that mesoangioblasts respond to HMGB1, a nuclear protein released by necrotic and by inflammatory cells (Scaffidi et al., 2002). They proliferate, migrate through the endothelial layer and accumulate in vivo around beads soaked with HMGB1 and implanted into skeletal muscle (Palumbo et al., 2004). Indeed, dystrophic muscle contains a high amount of HMGB-1, as it happens in the regenerating muscle.

It is indeed tempting to speculate that tissue regeneration may have adapted signals and pathways from fetal histogenesis. At variance with histogenesis, regeneration is usually associated with inflammation, and thus it probably requires more molecules and cell types to complete tissue repair. Like other types of stem cells, mesoangioblasts may have been selected for both processes (either of which depends on active angiogenesis) and equipped with receptors and pathways needed for participation in both processes.

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