MATERIALS AND METHODS

**Differentiation protocols**

Cells were plated onto 35 mm dishes (2 X 104 cells/cm2) and cultured in adipogenic medium (Cambrex Biosciences Inc., Verviers, Belgium), consisting of DMEM, 10% FBS, 1 µM dexamethasone, 0.2 mM indometacin, 10 µg/ml insulin, 0.5 mM 3-isobutyl-1-methilxantin) or osteogenic medium (DMEM, FBS 10%, 50 µg/ml ascorbic acid, 10 mM β-glicerophosphate, 10 nM dexamethasone) which was changed every 3 days. After 14 days, cells were washed in cold PBS, fixed with 4% PFA in PBS and stained with Adipored (Cambrex Biosciences) or 40 mM Alizarin Red S, pH 4.1 (Yamakawa et al., 2003). The presence of lipid vacuoles was visualized under fluorescence microscope, while the production of calcium deposits was examined in light microscopy. For chondrogenic differentiation cells (2.5x105) were cultured as "pellet" for 30-40 days in 15 ml centrifuge tubes in Chondrogenic Differentiation Medium (Cambrex Biosciences, consisting of DMEM, 100 µg/ml sodium piruvate, 10 ng/ml TGFβ3, 100 nM dexamethasone, 25 µg/ml 2-phospho ascorbate) (Lee et al., 2004). Medium was changed every second day. Cells were then washed, fixed in 4% PAF for 20 min at 4°, included in OCT medium (Fisher, Hampton, NH) and frozen at -80° C. Five µm sections were cut, fixed again as before, washed, stained with 1% Alcian Blue in 3% acetic acid, pH 2.5 for 30 min and observed at the light microscope.

**Experiments of cell proliferation and differentiation on titanium matrices**

m17.ASCs (about 8 x 103) were seeded on titanium grade 2 disks in 50 µl medium for 3 hours before adding the appropriate medium volume. For proliferation and viability assays cells were detached at sequential times (1, 2, 5 days) and counted in an inverted microscope, after being resuspended in a Trypan blue solution. MTT assay was performed on identical samples by adding 100µl/ml of a 0.5 mg/ml MTT solution in PBS for 4 hours. Samples were then washed, 100µl DMSO was added, and sample adsorbance was read at 520 nm at the Microplate Reader (Model 3550, Bio-Rad). Three experiments were performed in triplicates. For test Cells plated three days before were used for experiments of osteogenic differentiation with the protocol described above.

RESULTS

**The m17.ASC line displays multipotency**

This spontaneously immortalized cell line displayed the multilineage potential of MSC. Indeed, when cultured in appropriate differentiation media, m17.ASCs acquired features of the osteogenic, adipogenic, and chondrogenic phenotypes, as they were specifically stained with alizarin red, adipo-red and Alcian blue respectively (Fig. 2).

**The m17.ASC line can be induced to produce bone proto-tissues, when plated on titanium matrices**

m17.ASCs (8x103) were seeded onto Titanium grade 2 scaffolds and their adhesion, proliferation and osteogenic differentiation potential on this scaffold analyzed and compared to those of the same cells plated in conventional polystyrene culture plates. On titanium scaffolds cells were able to adhere and proliferate, although at a lower grade, as shown both on the basis of direct cell counting and in a MTT assay (Fig. 8a). When induced to osteogenic differentiation, they expressed the early osteogenic marker osteocalcin, beside the more ubiquitous pro-collagen 1, both of which were detected by RT-PCR (Fig.8b). Moreover, osteogenic differentiation of m17.ASC on titanium scaffolds was confirmed by the presence of extracellular calcium deposits, detectable by calcein] staining (late marker) (Fig. 8c). The osteoblastic cell line MC3T3 was used as positive control in these experiments.