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REGULAR ARTICLE

Isolation, characterization and osteogenic differentiation of adipose-derived stem cells: from small to large animal models

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Abstract One of the most important issues in orthopaedic surgery is the loss of bone resulting from trauma, infections, tumours or congenital deficiency. In view of the hypothetical future application of mesenchymal stem cells isolated from human adipose tissue in regenerative medicine, we have analysed and characterized adipose-derived stem cells (ASCs) isolated from adipose tissue of rat, rabbit and pig. We have compared their in vitro osteogenic differentiation abilities for exploitation in the repair of critical osteochondral defects in autologous pre-clinical models. The number of pluripotent cells per millilitre of adipose tissue is variable and the yield of rabbit ASCs is lower than that in rat and pig. However, all ASCs populations show both a stable doubling time during culture and a marked clonogenic ability. After exposure to osteogenic stimuli, ASCs from rat, rabbit and pig exhibit a significant increase in the expression of osteogenic markers such as alkaline phosphatase, extracellular calcium deposition, osteocalcin and osteonectin. However, differences have been observed depending on the animal species and/or differentiation period. Rabbit and porcine ASCs have been differentiated on granules of clinical grade hydroxyapatite (HA) towards osteoblast-like cells. These cells grow and adhere to the scaffold, with no inhibitory effect of HA during osteo-differentiation. Such in vitro studies are necessary in order to select suitable pre-clinical models to validate the use of autologous ASCs, alone or in association with proper biomaterials, for the repair of critical bone defects.

Keywords Adipose-derived stem cells · Osteogenic differentiation · Biomaterials · Hydroxyapatite · Tissue engineering · Rat · Pig · Rabbit

Introduction

Research on mesenchymal stem cells has greatly advanced during the recent past. These adult pluripotent stem cells play a key role in maintaining the homeostasis of their resident tissue and are able to differentiate into cellular lineages belonging to the mesodermic layer (Caplan 1994) and to transdifferentiate into neurons, hepatocytes and β-islet cells (Jiang et al. 2002; Baksh et al. 2004; Chen et al. 2004).

Autologous mesenchymal stem cells isolated from bone marrow (BMSCs, bone marrow stromal cells) have previously been successfully used for osteo-regeneration in patients with critical bone defects (Quarto et al. 2001; Kitoh et al. 2004; Funk et al. 2007; Jäger et al. 2009), whereas the use of autologous adipose-derived stem cells (ASCs) to promote bone healing and regeneration has been reported in just a few cases. For instance, Lendeckel et al. (2004) have shown that these cells repair calvarial bone defects in a 7-year-old child

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and, recently, Mesimäki et al. (2009) have reported the first case of a microvascular custom-made ectopic bone flap developed by using autologous ASCs and reimplanted in a patient with a hemi-maxillectomy. ASCs with their high multi-differentiative potential (Zuk et al. 2002; De Ugarte et al. 2003; Rigotti et al. 2007; de Girolamo et al. 2007, 2008; Bunnell et al. 2008) and their reduced immunogenicity (Le Blanc et al. 2003; Barry 2003; Majumdar et al. 2003) can be considered excellent candidates for applications of regenerative medicine. Furthermore, ASCs can be easily obtained from adipose tissue with a more abundant cellular yield than BMSCs (Nöth et al. 2002).

Autologous human ASCs (hASCs) have previously been used with success to improve the lipotransfer technique (Matsumoto et al. 2006), to heal ischaemic lesions in patients who have undergone radiotherapy (Rigotti et al. 2007), to treat facial lipoatrophy (Yoshimura et al. 2008a) and for cosmetic breast augmentation (Yoshimura et al. 2008b). This improvement might be explained on the basis that ASCs are able either to differentiate or to recruit resident cells, when implanted in the physiological microenvironment. Recently, Cai et al. (2009) have demonstrated that hASCs are able to preserve heart function and augment local angiogenesis following their administration in the context of myocardial infarction in athymic rats.

We have previously characterized hASCs and shown their in vitro differentiative potential into osteogenic, chondrogenic and adipogenic lineages (de Girolamo et al. 2007, 2008). We have loaded hASCs onto various types of scaffold to test their osteoinductive features and have observed that these cells are sensitive to the effect of the three-dimensional (3D) environment. Other authors have demonstrated that ASCs can be isolated from the adipose tissue of various animals such as the rat (Tholpady et al. 2003; Kingham et al. 2007; Yoshimura et al. 2007), rabbit (Peptan et al. 2006; Torres et al. 2007), pig (Qu et al. 2007) and horse (Vidal et al. 2007).

Moreover, various ASCs-based pre-clinical models have been reported in bone tissue engineering. Osteoinduced ASCs loaded on specific scaffolds exhibit a great healing potential when implanted in rabbit and rat calvarial defects (Dudas et al. 2006; Yoon et al. 2007) and, recently, Tobita et al. (2008) have demonstrated, in rats, that ASCs mixed with platelet-rich plasma induce periodontal ligament-like and alveolar bone regeneration.

In this work, we have isolated ASCs from small, medium and large animals, such as rats (rASCs), rabbits (rbASCs) and pigs (pASCs). We have compared the features of these cells in the undifferentiated state and their ability to differentiate in vitro into cells of the osteogenic lineage. The behavior of ASCs seeded on hydroxyapatite (HA), a biomaterial used for some orthopaedic applications, has also been evaluated.

Materials and methods

Harvesting of adipose tissue and isolation and culture of ASCs

ASCs were isolated from adipose tissues harvested from various areas: inguinal and perirenal regions from rat, interscapular site of rabbit and caudal area from pig.

rbASCs were purified from interscapular adipose tissue of adult New Zealand White rabbits (Torres et al. 2007) killed by intramuscular administration of an overdose (5 mg/kg) of a solution of 50 mg/ml sodium pentothal. Primary cultures of the stromal vascular fraction (SVF) were established as previously described with minor modifications (Peptan et al. 2006). Adipose tissue was finely minced and washed with phosphate-buffered saline (PBS) containing 3× P/S (50 U/ml penicillin and 50 μg/ml streptomycin; Sigma-Aldrich, Milan, Italy) and centrifuged at 1200*g* for 2 min to remove erythrocytes and cellular debris. Samples were then digested in a shaking water bath for 60 min at 37°C by 0.1% type I collagenase (Worthington, Lakewood, N.J., USA) in PBS supplemented with 3× P/S. After digestion, the collagenase I was neutralized by adding an equal volume of DMEM (Sigma-Aldrich) supplemented with 10% FBS (fetal bovine serum; Sigma-Aldrich), 50 U/ml penicillin, 50 μg/ml streptomycin and 2 mM L-glutamine (Sigma-Aldrich). The samples were centrifuged for 10 min at 1200*g* to separate the SVF from adipocytes, cellular debris and undigested tissue. The SVF was filtered through sterile medicinal lint and the pellet was resuspended in control medium (CTRL). Cell number and viability were determined by trypan-blue exclusion.

The same protocol was used to isolate mesenchymal stem cells from pig caudal adipose tissue (Qu et al. 2007).

ASCs from Sprague-Dawley rats were isolated as previously described with minor modifications (Tholpady et al. 2003; Kingham et al. 2007; Yoshimura et al. 2007). Adipose tissue was collected from both inguinal and perirenal regions. The minced tissue was digested with 0.075% collagenase type I at 37°C for 45 min and then centrifuged for 10 min at 350*g*.

ASCs derived from the SVF were plated in control medium at a density of 10^5cells/cm^2 and maintained at 37°C in a humidified atmosphere with 5% CO_2. After 48-72 h, non-adherent cells were discarded by washing with PBS. During all the period of culture, growth medium was changed three times a week.

After reaching 80%-90% of confluence, cells were detached by incubation with 0.5% trypsin/0.2% EDTA

(Sigma-Aldrich). ASCs were plated at a density of 5×10^3 cells/cm^2 for further expansion and experiments.

MTT cell proliferation assay

From passage 1 to 4 in culture, ASCs were trypsinized and plated at a density of 5×10^3 cells/cm^2 in control medium. ASCs viability was monitored at various time points (1, 6, 10 or 14 days) by addition, to the culture medium, of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Sigma-Aldrich) at a final concentration of 0.5 mg/ml and further incubation for 4 h at 37°C. The resulting formazan precipitate was then solubilized by using 100% dimethylsulphoxide and the absorbance was read at 570 nm with a Wallac Victor II plate reader (Kingham et al. 2007).

Fibroblast-colony-forming unit assay

The fibroblast-colony-forming unit (CFU-F) assay was performed as previously reported with minor modifications (Wu et al. 2000; Vidal et al. 2007). Starting from a suspension containing 10^3 cells/ml, ASCs were plated by serial dilution in 6-well plates (from 48 cells/cm^2 to 1 cell/cm^2) and cultured in control medium supplemented with 20% FBS. At day 6, the medium was changed and, at day 10, cells were fixed with 100% methanol and stained with 0.5% crystal violet (Fluka, Buchs, Switzerland). The frequency of the CFU-F was established by scoring individual colonies (consisting of at least 20-30 cells) with respect to the number of seeded cells.

Osteogenic differentiation and evaluation of differentiation markers

ASCs were differentiated towards the osteogenic lineage by using a specific inductive medium (OSTEO) supplemented with 10 nM dexamethasone (Sigma-Aldrich), 10 mM glycerol-2-phosphate (Sigma-Aldrich), 150 μM L-ascorbic acid-2-phosphate (Sigma-Aldrich) and 10 nM cholecalciferol (Sigma-Aldrich; de Girolamo et al. 2007, 2008). Differentiation was performed in monolayer, cells being seeded at a density of 5×10^3 cells/cm^2. Cells were cultured at 37°C in a humidified atmosphere (5% CO_2) for various periods, the medium being changed three times a week. Osteogenic differentiation of ASCs was assessed by evaluating morphological changes, alkaline phosphatase (ALP) activity, calcified extracellular matrix deposition and specific bone protein expression.

ALP activity

In order to determine ALP activity, cells cultured for 7 and 14 days were washed with PBS and lysed in non-denaturant conditions by using 0.1% Triton X-100 (Sigma-Aldrich) in double-distilled H_2O. ALP activity was determined by incubating cellular lysates at 37°C in the presence of 1 mM p-nitrophenylphosphate (Sigma-Aldrich) in alkaline buffer (100 mM diethanolamine and 0.5 mM $MgCl_2$, pH 10.5; Bodo et al. 2002). The reaction was stopped with 1 N NaOH and the absorbance of each sample was read at 410 nm with a Wallac Victor II plate reader. ALP activity was then normalized to total protein content as determined by BCA protein assay (Pierce Biotechnology, Rockford, Ill., USA).

Calcified extracellular matrix deposition

After 7, 14 and 21 days of culture, extracellular matrix mineral deposition was assessed by evaluating the presence of red nodules stained by 40 mM alizarin red-S (AR-S, pH 4.1; Fluka) after fixation in 70% ice-cold ethanol. The quantification of mineral deposition was performed by incubating the stained sample with 10% w/v cetylpyridinium chloride (CPC; Sigma-Aldrich) in 0.1 M phosphate buffer (pH 7.0) for 15 min to extract AR-S. Absorbance was read at 550 nm with a Wallac Victor II plate reader (Halvorsen et al. 2001).

Expression of bone-specific proteins

Osteocalcin and osteonectin protein expression was evaluated by Western blot analysis in osteogenic-differentiated ASCs. Cells incubated in CTRL or OSTEO medium were detached from the dish, collected by centrifugation and resuspended in lysis buffer (50 mM TRIS pH 8, 150 mM NaCl, 1% Nonidet P40, 0.1% SDS) supplemented with proteinase inhibitors. The protein concentration of cell lysates was analysed by a Pierce protein assay kit (Pierce). Proteins (20 μg) were loaded onto 10% SDS-polyacrylamide gels and electrotransferred onto Hybond-ECL extra nitrocellulose membrane (Amersham Bioscience). After being blocked in 5% nonfat dry milk in TBS-T (TRIS-buffered saline and 0.1% Tween-20) for 1 h at room temperature, membranes were incubated at 4°C overnight with either rabbit anti-osteocalcin or mouse anti-osteonectin (1:200 dilution; Santa Cruz Biotecnology, Santa Cruz, Calif., USA) and mouse anti-β-actin (1:3000 dilution; Sigma-Aldrich). Specific proteins were revealed by horseradish peroxidase (HRP)-conjugated secondary antibodies (GE Healthcare) and the ECL Western Blotting Analysis System Kit (GE Healthcare) according to the manufacturer's protocol. Osteocalcin and osteonectin expression was then quantified by using ImageJ software. The osteo-specific proteins content was normalized to that of β-actin.

The expression of osteocalcin and osteonectin was also determined by immunofluorescence. ASCs differen-

tiated for 28 days and cultured on coverslips were washed with PBS^+ (PBS supplemented with 0.1 mM $CaCl_2$ and 0.1 mM $MgCl_2$) and fixed with 3% paraformaldehyde for 20 min. Autofluorescence was blocked with 50 mM NH_4Cl in PBS^+. The cells were permeabilized for 4 min in PBS^+ containing 0.1% Triton X-100 and then incubated with rabbit anti-osteocalcin antibodies, mouse anti-osteonectin antibodies, mouse anti-β-actin antibodies or rabbit anti-β-tubulin antibodies (Santa Cruz Biotecnology) for 60 min at room temperature. Specific proteins were revealed by Alexa-Fluor-488 goat anti-mouse antibodies and Alexa-Fluor-568 goat anti-rabbit antibodies (Invitrogen, Camarillo, Calif., USA) for 30 min at room temperature. Cover-slips were analysed by confocal fluorescence microscopy (Bio-Rad MRC 1024; Bio-Rad Laboratories, Hercules, Calif., USA).

ASCs-scaffold constructs

Undifferentiated rbASCs (5×10^4 cells/scaffold) were loaded onto clinical-grade porous (60%) HA granules (Permedica, Merate, Italy and Biomaterials Research Center, University of Milan, Italy) to evaluate their ability to grow and differentiate in a 3D environment. Cells were allowed to adhere overnight to scaffolds in microcentrifuge tubes and, the following day, the seeded scaffolds were transferred to a 24-well plate and cultured for 14 and 21 days, either in CTRL or OSTEO medium. ALP and calcium deposition were analysed as described above.

Statistical analysis

Data are expressed as means±SD and statistical analysis (two-way analysis of variance) was performed by using GraphPad Prism v5.00 (GraphPad Software, San Diego, Calif., USA): for OSTEO vs CTRL: $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$; for HA vs PA: $^{\$}P<0.05$, $^{\$\$}P<0.01$, $^{\$\$\$}P<0.001$.

Results

Isolation and characterization of undifferentiated ASCs

Mesenchymal stem cells were isolated from adipose tissues derived from three animal species and from various anatomical sites: inguinal and perirenal adipose tissue from rats, interscapular region from rabbits and caudal region from pigs. We purified a consistent number of cells with an average of $6.2\times10^5\pm3.8\times10^5$ and $8.9\times10^5\pm8.4\times10^5$ progenitor cells/ml raw adipose tissue from inguinal and perirenal regions, respectively, of rats and $3.5\times10^4\pm2.8\times10^4$ rbASC cells were purified from each millilitre of rabbit interscapular adipose tissue, whereas we isolated an average of $1.8\times10^5\pm4.7\times10^4$ cells/ml adipose tissue from porcine caudal area (Fig. 1a). One week after isolation, ASCs started to proliferate rapidly and, at passage 1, the variability of ASC cell numbers, among the same different sites and species, was reduced (Fig. 1a). All ASC cells showed a homogeneous fibroblast-like shape that was maintained for many

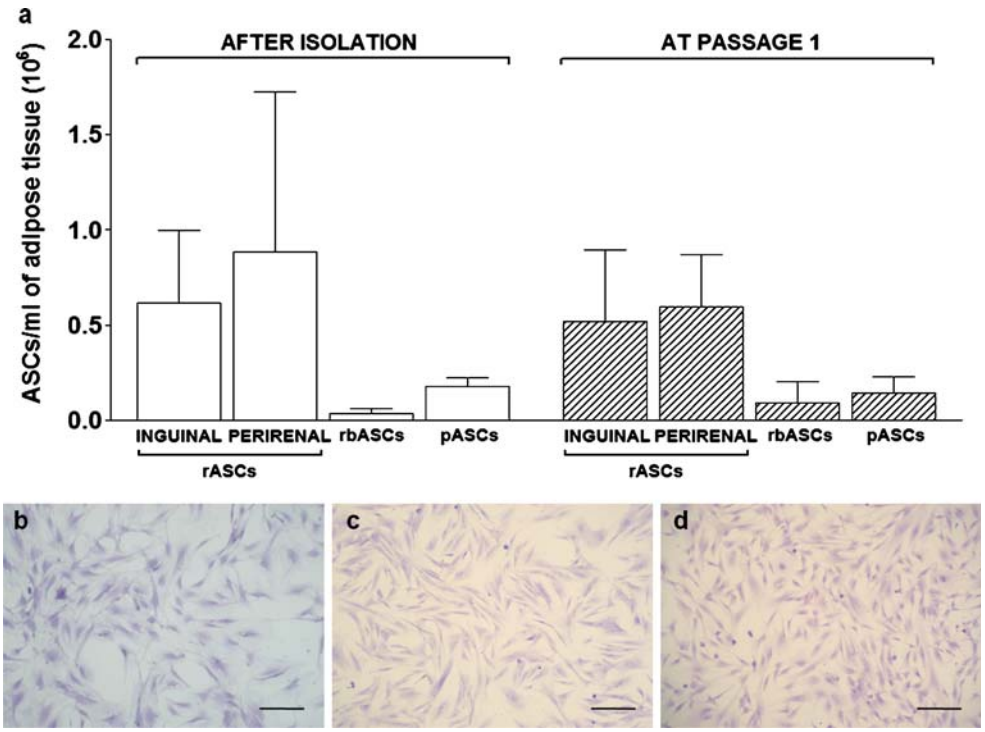

Fig. 1 **a** Cellular yield of ASCs after isolation and at passage 1. ASCs were isolated from adipose tissue harvested from various anatomical sites in rat (*rASCs*), rabbit (*rbASCs*) and pig (*pASCs*); *n*=3. **b–d** Microphotographs of undifferentiated inguinal rASCs (**b**), rbASCs (**c**) and pASCs (**d**) fixed at passage 6 and stained with haematoxylin-eosin (light microscopy). *Bar* 100 μm

passages in culture, without any signs of cellular aging (Fig. 1b–d). These cells reached 80%-90% confluence every 2-3 days: the doubling time (DT) was 74±19 h for rASCs from inguinal adipose tissue and 93±27 h for rASCs from the perirenal region (Fig. 2a). rbASCs grew more rapidly with respect to rASCs and pASCs with a DT of 46±12 h (Fig. 2b), whereas the DT of pASCs was 54±19 h (Fig. 2c). MTT assay confirmed that rbASCs and pASCs possessed a greater ability for self-renewal than rASCs (data not shown).

These ASCs showed a marked clonogenic ability. Indeed, about 9.7% of inguinal rASCs and 7.4% of perirenal rASCs were able to produce CFU-F at passage 1; this frequency gradually decreased during passages (Fig. 3a). Surprisingly, at passage 1, rbASCs presented a stronger clonogenic ability (about 23%), which slowly decreased during culture, reaching a CFU-F value of 7% at passage 4 (Fig. 3b). Interestingly, pASCs maintained a high clonogenic potential without any significant difference between early and late passages (12%±6% for all passages; Fig. 3b).

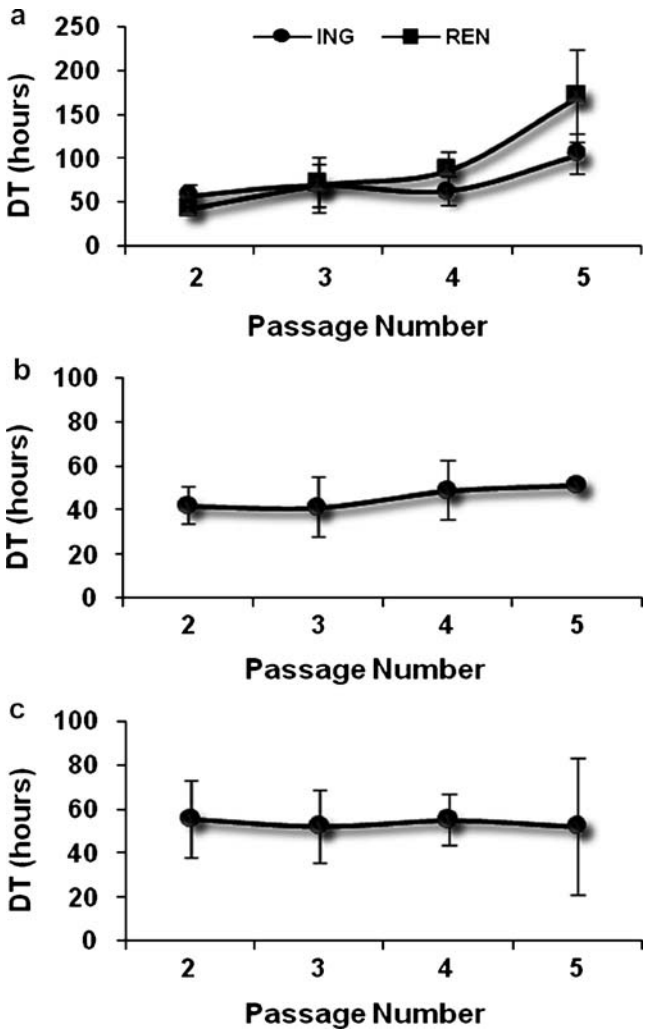

Fig. 2 Doubling time (*DT*) of rASCs (**a**), rbASCs (**b**) and pASCs (**c**) at various passages in culture (n=3); $DT=t\times\ln(2)/\ln(N/N_0)$, where t is the time in culture (in hours), N is the final number of cells and N_0 is the initial number of cells (*ING* inguinal adipose tissue, *REN* perirenal adipose tissue)

Osteogenic differentiation of ASCs

We next tested the osteogenic potential of ASCs derived from the three different animal tissues by culturing rASCs, rbASCs and pASCS in osteogenic medium for different times. As early as 7 days in culture, all of the cells exhibited some morphological modifications; indeed, cell appearance changed from an elongated fibroblastic to a more cuboidal shape (Arrigoni et al. 2009).

When the cells were induced to osteo-differentiate over 7 days, both inguinal and perirenal rASCs showed abundant ALP activity compared with undifferentiated cells, with an increase of about 56% for inguinal and 83% for perirenal rASCs; this activity significantly increased for both cellular populations after 14 days of osteogenic differentiation (about +235% with respect to the undifferentiated cells; Fig. 4a). Furthermore, both inguinal (Fig. 4b, c) and perirenal differentiated cells produce a greater amount of calcified extracellular matrix compared with cells cultured in control medium. rASCs of both origins, when differentiated for 7–21 days, were able to produce high levels of calcium depots; however, these increases were not statistically significant, since the variability among samples was large (Fig. 4d).

In rbASCs, as early as 7 days of osteogenic differentiation, ALP activity was significantly abundant in osteo-differentiated cells in comparison with undifferentiated cells, with an increase of about 148%. Surprisingly, after 14 days in culture, ALP activity, for both undifferentiated and differentiated cells, was higher than that detected after 7 days (Fig. 4e). To confirm the osteogenic differentiation ability of rbASCs, we quantified the calcified extracellular matrix deposition after 7, 14 and 21 days of culture (Fig. 4f, g): osteo-induced cells produced an abundant amount of calcified extracellular matrix compared with undifferentiated cells, with an increase of 22% and 50% after 7 and 14 days, respectively. This increase was also maintained after 21 days of differentiation (+43%; Fig. 4h).

pASCs that differentiated towards osteoblastic-like cells showed an upregulation of ALP levels of 50% and 180% in comparison with undifferentiated cells both after 7 and 14 days (Fig. 4i). Analysis of calcified extracellular matrix deposition in differentiated pASCs for the same periods of time showed increases in terms of calcified matrix deposition of 45% and 30%, respectively, with a further

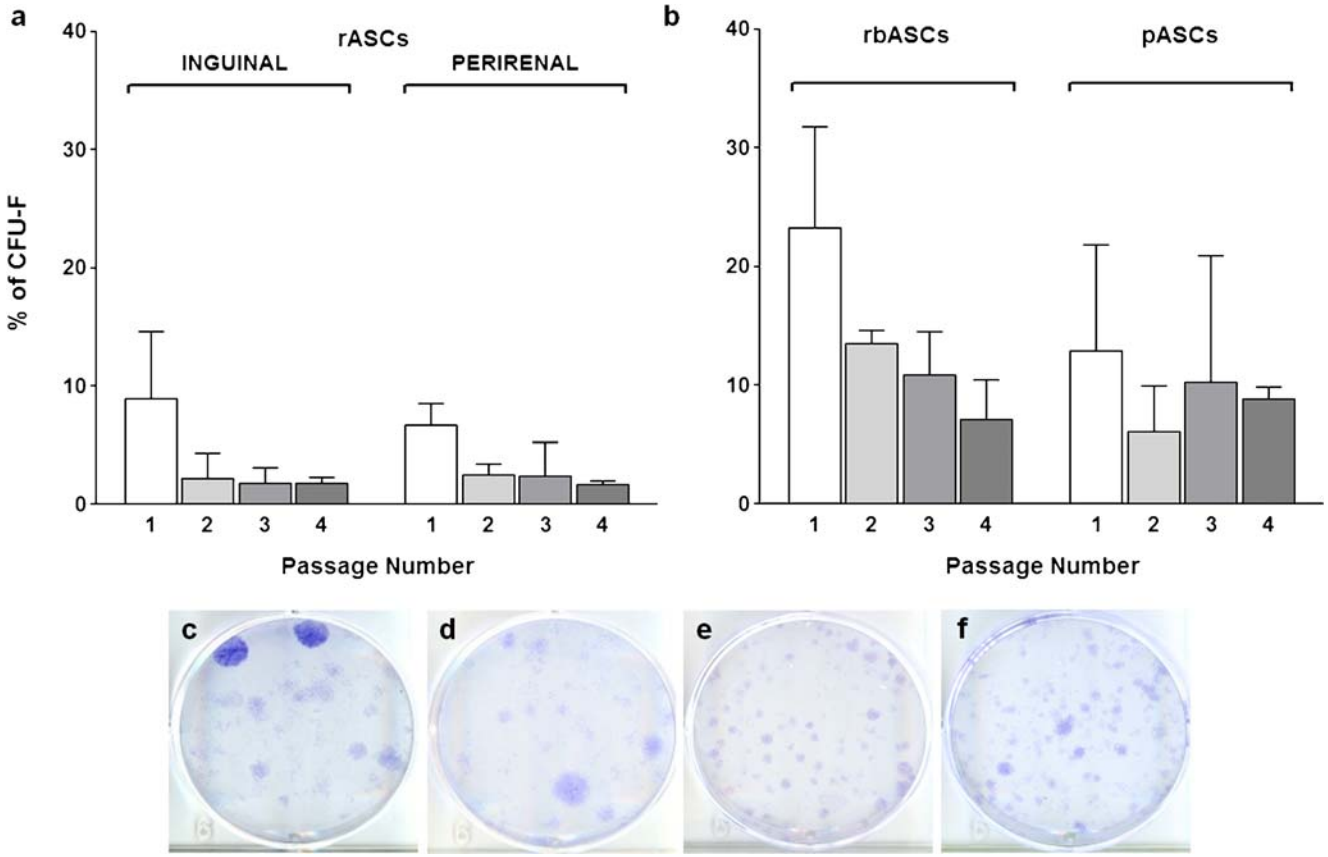

Fig. 3 CFU-F assays performed with the ASCs at various passages in culture. **a**, **b** Percentage of CFU-F cells was calculated as (number of colonies/number of plated cells)×100. **c**–**f** Microphotographs of colonies at passage 1 stained with crystal violet for inguinal (**c**) and perirenal (**d**) rASCs, rbASCs (**e**) and pASCs (**f**)

increase (+22%) after 21 days of osteo-differentiation (Fig. 4j–l).

In order to evaluate further the osteogenic differentiation ability of rbASCs and pASCs, we examined osteocalcin and osteonectin protein expression by Western blot analysis and by immunofluorescence. Both osteo-induced rbASCs and pASCs expressed osteocalcin and osteonectin: these proteins were upregulated in osteogenic differentiated rbASCs by about 152% and 51% and in pASCs by 6% and 121%, with respect to undifferentiated cells, respectively (Fig. 5a). Moreover, both osteocalcin (Fig. 5d) and osteonectin (Fig. 5h, j) were expressed in ASCs osteo-differentiated for 28 days but not in undifferentiated cells (Fig. 5b, f). The expression of osteocalcin and osteonectin was thus induced by the differentiation process, although we observed that not all the cells were homogeneously stained, in particular for the expression of osteonectin, suggesting that differentiated rbASCs and pASCs were not synchronized.

Furthermore, cryopreservation did not affect the stem cell features or the osteogenic differentiation ability of rASCs, rbASCs and pASC, as previously shown for hASCs (de Girolamo et al. 2008).

Interaction of ASCs and HA scaffold

Since the final aim of our in vitro study was to test cells suitable for future in vivo models, we needed to check whether ASCs were able to adhere to scaffolds routinely used in the orthopaedic field, such as HA granules. rbASCs and pASCs tightly adhered to the biomaterial and actively proliferated. Moreover, both cell populations properly colonized the surface and the pores of HA granules and no cellular toxicity was produced by the scaffold (Fig. 6a; data not shown). Both rbASCs and pASCs, seeded on HA and differentiated for 14 days, produced a 31% and 34% increase in ALP activity compared with undifferentiated cells, respectively (Fig. 6b, d). Then, when cultured in a 3D environment, both undifferentiated rbASCs and pASCs showed greater ALP activity in comparison with that of undifferentiated cells cultured in monolayer (31% and 303%, respectively). In addition, calcium depots, produced by rbASCs differentiated for 21 days in the presence of HA, were upregulated by 98% compared with cells maintained in control medium (Fig. 6c). In contrast, pASCs induced to differentiate in the presence of HA showed an increase of

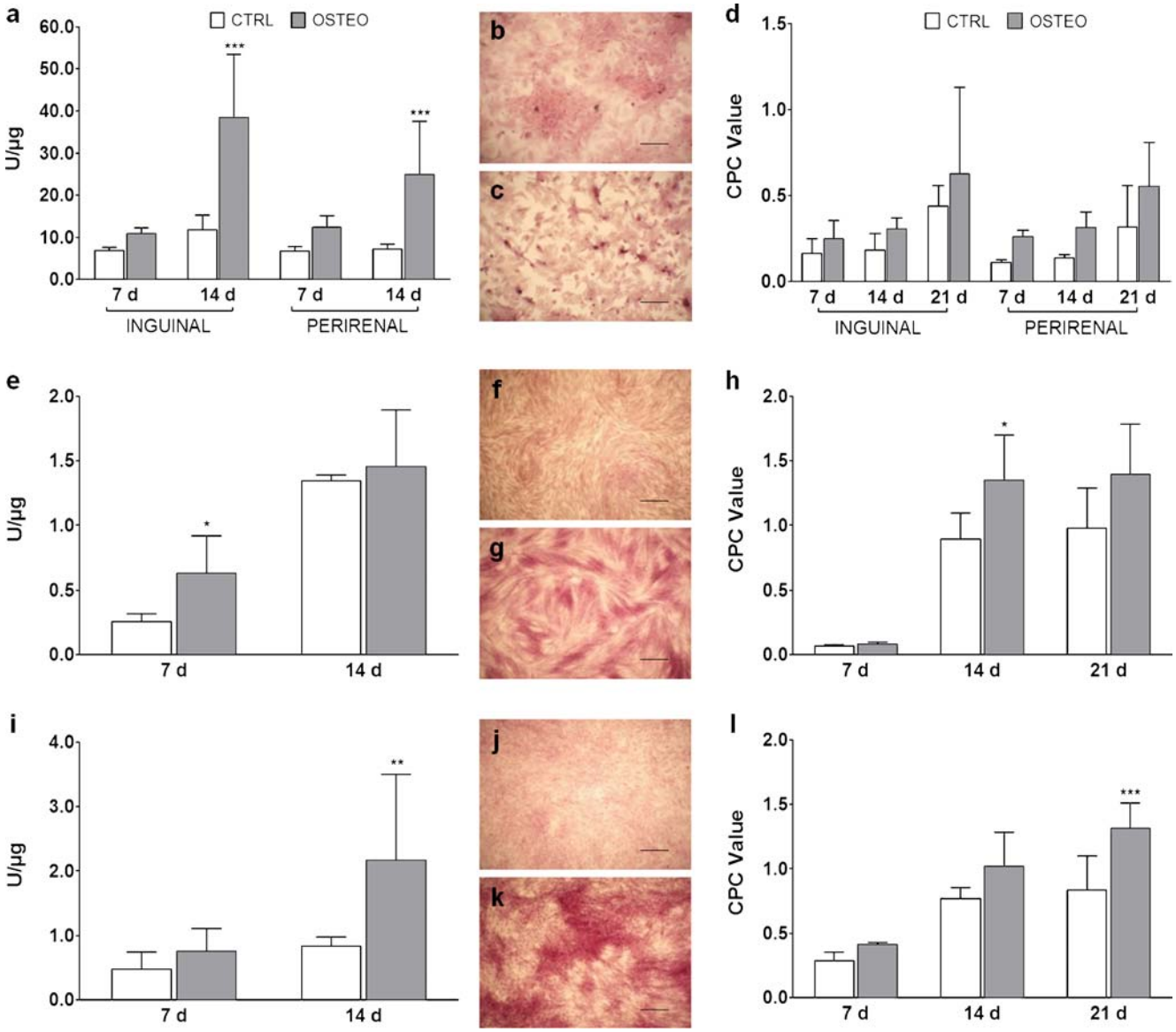

Fig. 4 Osteogenic differentiation assessment of rASCs (**a**-**d**), rbASCs (**e**-**h**) and pASCs (**i**-**l**) (*CTRL* undifferentiated cells in control medium, *OSTEO* differentiated cells in osteogenic medium, *d* days). **a**, **e**, **i** ALP activity (means±SD of ALP U/µg proteins, *n*=3) for each species and for various time points. **b**, **c**, **f**, **g**, **j**, **k** Microphotographs of alizarin red-S staining at 21 days of culture for rASCs and at 21 days for rbASCs and pASCs in control (**b**, **f**, **j**) and osteogenic (**c**, **g**, **k**) medium (light microscopy). *Bars* 200 µm. **d**, **h**, **l** Quantification of calcium deposition by cetylpyridinium chloride (*CPC*) extraction at various time points (means±SD, *n*=3). OSTEO vs CTRL: *P<.05, **P<.01, ***P<.001

just about 20% in extracellular calcified matrix deposition without a significant difference with respect to cells cultured on plastic (Fig. 6e).

Discussion

In this study, we have compared the features of ASCs isolated from rat, rabbit and pig, with particular reference to their osteogenic differentiation potential.

In rat, the site of tissue harvesting seems to have little effect on the characteristics of ASCs. Indeed, the number of cells per millilitre of raw adipose tissue is higher from the perirenal site than from the inguinal area. However, as early as 1 week after isolation, we have observed a faster proliferation rate of the cells derived from the inguinal region with respect to those from the perirenal area, thereby balancing the final cellular yield. In addition, the clonogenic ability appears similar for both cell populations showing a similar decrease during the passages in culture.

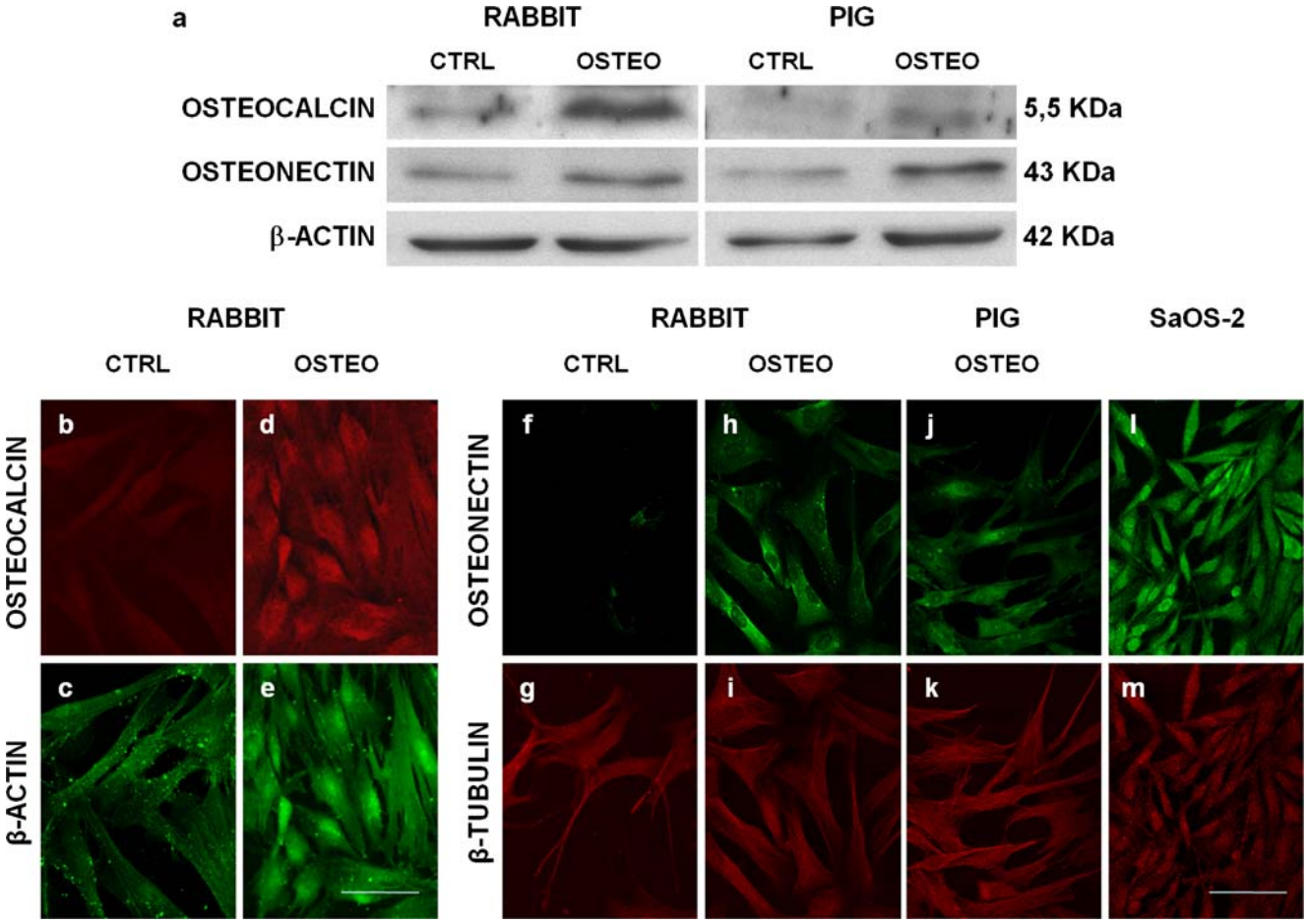

Fig. 5 Osteocalcin and osteonectin expression in undifferentiated and osteogenically differentiated rbASCs and pASCs (*CTRL* undifferentiated cells in control medium, *OSTEO* differentiated cells in osteogenic medium). **a** Western blots. **b–m** Immunofluorescence analysis (light microscopy). Osteocalcin expression (**b**, **d**). Osteonectin expression (**f**, **h**, **j**, **l**). β-Actin (**c**, **e**) and β-tubulin (**g**, **i**, **k**, **m**) as housekeeping proteins. Human osteosarcoma cells (*SaOS-2*) as positive controls (**l**,**m**). *Bars* 100 μm

The number of ASCs from the interscapular rabbit adipose tissue is significantly lower in comparison with those from the rat and pig. However, as also demonstrated by Peptan et al. (2006), rbASCs proliferate more rapidly and show a marked clonogenic ability that mildly decreases during passage, as also previously described for hASCs (de Girolamo et al. 2008). Porcine adipose tissue contains an abundant population of pASCs, showing a similar proliferative ability as the other animal ASCs but, interestingly, these cells are better able to maintain their high clonogenic potential throughout the period of culture that we have analysed. Furthermore, all these ASCs have been tested after cryopreservation and no differences have been observed, suggesting their use for cell-based therapies, even after a long period of time after their isolation (data not shown).

All these cell types possess suitable osteogenic potential as assessed by the upregulation of markers of osteogenic differentiation such as ALP activity, osteocalcin and osteonectin and as confirmed by the ability of these cells to produce a large amount of calcified extracellular matrix. Both rASC populations, if opportunely stimulated, acquire some of the features of osteoblastic-like cells and, to date, we have not determined any relevant difference that would lead us to prefer one or the other cell population.

Our data demonstrate that osteo-differentiated rabbit ASCs exhibit high levels of ALP activity just after 7 days of differentiation, in contrast to results previously obtained for hASCs and also reported by Peptan et al. (2006). These observations might be useful in the perspective of future in vivo applications because they suggest that the time of *ex vivo* manipulation can be reduced before ASC reimplantation.

In addition, the ability of these cells to adhere and grow on an HA scaffold suggests that this biomaterial may be an efficient support for future in vivo applications, even if HA does not exert any significant osteoinductive effect on rbASCs and pASCs differentiation,.

Before performing pilot clinical studies, the regenerative capacity of human mesenchymal stem cells in both athymic

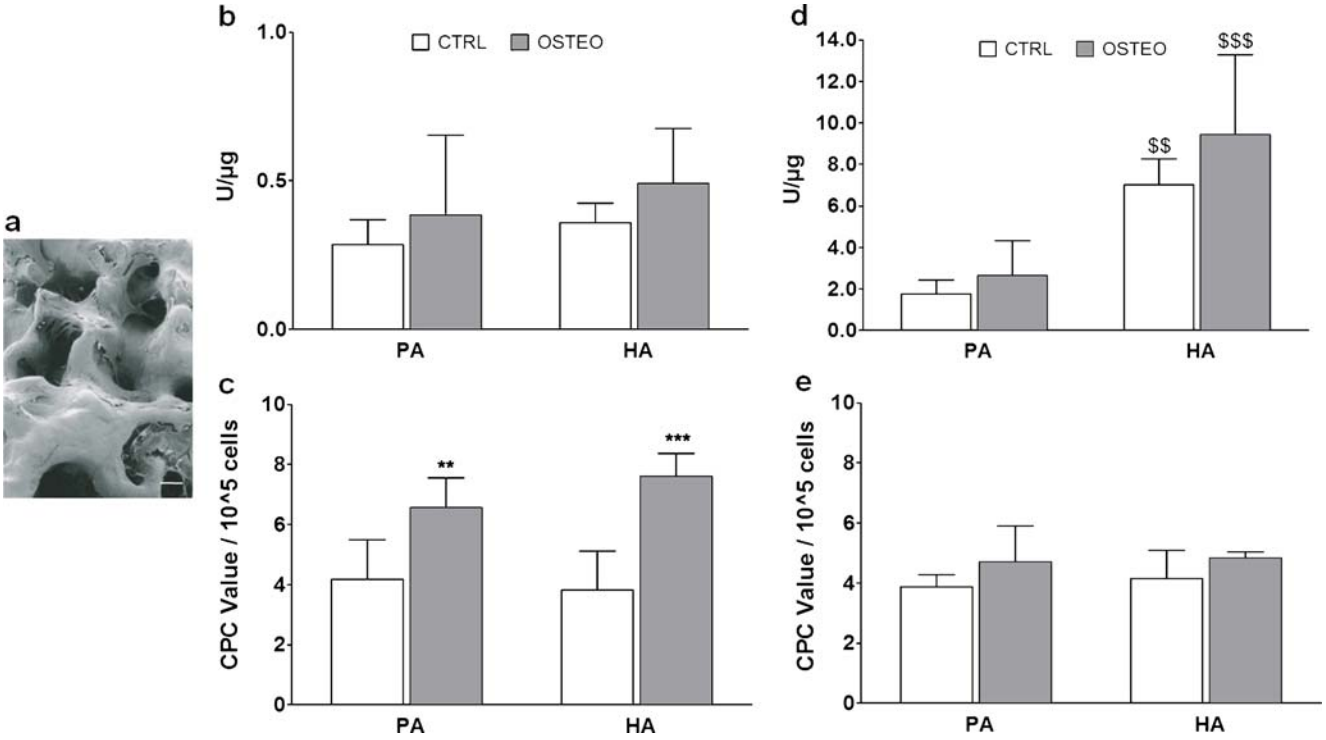

Fig. 6 rbASCs (**a**) seeded on a hydroxyapatite scaffold cultured in osteogenic medium (scanning electron microscopy). *Bar* 100 µm. ALP activity of undifferentiated (*CTRL*) and osteo-differentiated (*OSTEO*) rbASCs (**b**) and pASCs (**d**) cultured for 14 days both on plastic (*PA*) and on hydroxyapatite (*HA*); means±SD of ALP U/µg proteins, n=8. Calcified extracellular matrix deposition of undifferentiated (*CTRL*) and osteo-differentiated (*OSTEO*) rbASCs (**c**) and pASCs (**e**) cultured for 21 days on PA and in the presence of HA were quantified by CPC extraction; means±SD of CPC value/10^5 cells, n=8. OSTEO vs CTRL: $^{**}P<0.01$, $^{***}P<0.001$; HA vs PA: $^{\$\$}P<0.01$, $^{\$\$\$}P<0.001$

and immune-competent pre-clinical animal models has to be tested. However, although the nude model allows us to test the in vivo behaviour of human-derived cells, it presents limitations particularly because of the lack of the immunological system, which is usually involved in every regeneration process; this could delay the healing of any experimental defects. Moreover, nude animals are more prone to infections and systemic illness. Thus, these disadvantages could mask the potential role of cells in the healing process (Tholpady et al. 2003). Thus, immune-competent animal models lacking these drawbacks might better resemble the relevant biology in a clinical autologous transplantation procedure.

Rabbits and pigs are promising animal models in the field of regenerative medicine. In particular, rabbits are useful for screening several experimental conditions because of their low cost of maintenance, whereas pigs represent a more predictive model because of their greater similarity to human beings with regard to diet, weight and working load. The femoral defect in the rat also represents a valid model in the orthopaedic field (Cuomo et al. 2009; Smietana et al. 2009). However, we are interested in an autologous cell-implant model, which we believe will not be easy to obtain in rat. Moreover, the small dimension of rat bones requires an extremely precise surgical approach, which could be subjected to possible errors with resultant effects on the final regeneration process.

Another important problem that researchers have to face in isolating ASCs from these animals remains the lack of reagents to evaluate specific mesenchymal stem cell markers that might be partially overshadowed by the similar morphological and biological features of both rASCs, rbASCs and pASCs compared with human mesenchymal stem cells (Pittenger et al. 1999; Zuk et al. 2001).

In conclusion, our results could have relevant implications in the setting up of adequate bone regeneration strategies based on the reimplantation of autologous ASCs either in the undifferentiated or the pre-differentiated state. We have just established an autologous rabbit model of bone critical-size lesions by using ASCs in association with disks of HA in order to assess the in vivo integration of the cells/scaffold constructs with the surrounding bone tissue and the effect of the physiological microenvironment (experiments in progress). Adipose tissue represents a precious source of multipotent cells with a great potential for several clinical applications, above all in the field of the

regenerative medicine. We strongly believe that a large animal model, such as the pig, has to be tested, before moving on to a pilot clinical study.

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