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ORIGINAL ARTICLE

Transplantation of human neonatal foreskin stromal cells in ex vivo organotypic cultures of embryonic chick femurs

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Micro mass;
Chick femur;
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Abstract We have previously reported that human neonatal foreskin stromal cells (hNSSCs) promote angiogenesis in vitro and in chick embryo chorioallantoic membrane (CAM) assay in vivo. To examine the in vivo relevance of this observation, we examined in the present study the differentiation potential of hNSSCs in ex vivo organotypic cultures of embryonic chick femurs. Isolated embryonic chick femurs (E10 and E11) were cultured for 10 days together with micro-mass cell pellets of hNSSCs, human umbilical vein endothelial cells (HUVEC) or a combination of the two cell types. Changes in femurs gross morphology and integration of the cells within the femurs were investigated using standard histology and immunohistochemistry. After 10 days, the femurs that were cultured in the presence of hNSSCs alone or hNSSC + HUVEC cells grew longer, exhibited thicker diaphysis and an enlarged epiphyseal region compared to control femurs cultured in the absence of cells. Analysis of cell–femur interactions, revealed intense staining for CD31 and enhanced deposition of collagen rich matrix along the periosteum in femurs cultured with hNSSCs alone or hNSSCs + HUVEC and the most pronounced effects were observed in hNSSC + HUVEC cultures. Our data suggest that organotypic cultures can be employed to test the differentiation potential of stem cells and demonstrate the importance of stem cell interaction with 3D-intact tissue microenvironment for their differentiation.

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1. Introduction

Stem cell therapies hold the promise for curing a large number of age-related degenerative diseases including non-union fractures, osteoporosis, and osteoarthritis. However, appropriate use of cells in the clinic requires the ability of testing the cells in an appropriate model predictive for in vivo phenotype (Wei et al., 2013).

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Traditionally, ex vivo cell cultures have been utilized to test the differentiation potential of stem cells. While useful, culturing the cells in 2-D environment does not reflect the in vivo situation. On the other hand, ectopic transplantation of stem cells in vivo can provide biologically relevant information but these assays are expensive, require the utilization of large numbers of animals, and results may vary depending on animal species and strain due to systemic influences (Pearce et al., 2007).

Ex vivo organotypic cultures provide an alternative model system that bridges the gap between in vitro culture and in vivo testing and have the advantage of maintaining the normal extracellular matrix and 3D-tissue architectures. The ex vivo embryonic chick femur model has been utilized in various experimental setups that include studies of the skeletal development and the effects of various growth factors and morphogenesis on bone formation and bone resorption (Smith et al., 2013).

A number of studies have demonstrated multipotency of human neonatal skin stromal cells (hNSSCs) that include differentiation into adipocytes, osteoblasts, chondrocytes, hepatocytes and insulin-producing cells (Vishnubalaji et al., 2012a). Recently, our group has reported that hNSSCs can differentiate into endothelial like cells that are positive for CD31, CD34, vWF (von Willebrand factor) and SMA (smooth muscle actin), and formed characteristic tubular formation when cultured on Matrigel® in vitro (Vishnubalaji et al., 2012b). Moreover, in vivo CAM assay, transplantation of endothelial-differentiated hNSSCs resulted in the formation of a higher number of blood vessels containing hNSSCs that expressed vWF, CD31, SMA and factor XIIIa (Vishnubalaji et al., 2015).

In order to test the differentiation potential of hNSSC in mature tissue context which is relevant for clinical use of the cells, the aim of the present study was to examine the differentiation potential of hNSSCs role in ex vivo embryonic chick femurs.

2. Materials and methods

2.1. Cell isolation and culture

hNSSCs were isolated and cultured according to the method previously reported (Vishnubalaji et al., 2012b). Neonatal foreskins were received from voluntary circumcisions of 2–3 days male babies. Cells were isolated in an explant cultures. Tissues were washed and the epidermis was removed. Tissues were placed in culture dishes with the epidermis layer facing upward and the dermis area in contact with the plastic surface with a droplet of culture medium. The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with D-glucose 4500 mg/L, 4 mM L-Glutamine and 10% Fetal Bovine Serum (FBS) (all purchased from Gibco-Invitrogen, USA).

Human umbilical vein endothelial cells (HUVEC) were isolated from umbilical cord tissue. The tissues were cleaned with 70% ethanol and PBS and digested using collagenase B (0.005 g/ml) for one hour at room temperature. Cell pellets were obtained following centrifugation at 1100 rpm for four min. The cell pellets were cultured in endothelial cell culture medium (ECM) (Medium 199 with Earle's salts, L-glutamine and sodium bicarbonate (PAA; E15-834) + 10% FCS, 1% Pen-strep, ECGS/H (3 mg/ml Protein + Heparin) (Promocell; C-30120), 2 ng/mL VEGF (R&D systems, USA).

2.2. Immunophenotyping by flow cytometry

Phenotypic analysis of hNSSCs was performed as described in our previous study (Vishnubalaji et al., 2012b). Briefly, cells were washed twice in ice-cold PBS supplemented with 0.5% BSA and re-suspended at 10^6 cells per ml. Ten microliters of PE-conjugated mouse anti-human CD146, CD73, CD29 and HLA-DR, FITC-conjugated mouse anti-human CD34, CD90, CD45, CD13, CD184 and CD31, or APC-conjugated mouse anti-human CD105, CD14 and CD44 antibodies (all from BD Biosciences, except the anti-human CD105, which was purchased from R&D systems) was added to 100 µl of cell suspension (105 cells). Negative control staining was performed using a FITC, PE, or APC-conjugated mouse IgG1 isotype control antibodies, respectively. Cells were incubated for 30 min at 4 °C in the dark, then were washed with PBS to remove excess antibodies, and then were resuspended in 500 µL of PBS and were analyzed using BD FACS Calibour flow cytometer (BD Biosciences. Data were analyzed using Cell Quest Pro Software Version 3.3 software (BD Biosciences).

2.3. Cell differentiation

Cell differentiation into osteoblasts and adipocytes was performed as described in our previous study (Vishnubalaji et al., 2012b; Al-Nbaheen et al., 2013). Briefly, for osteoblast differentiation, the cells were seeded at a density of 0.05×10^6 -cell/ml in 6-well plates and were grown for 24 h in standard DMEM growth medium. At 70–80% confluence, cells were induced for 15 days with osteogenic medium that was composed of basal medium supplemented with 50 µg/mL L-ascorbic acid (Wako Chemicals GmbH, Neuss, Germany), 10 mM b-glycerophosphate (Sigma), and 10 nM calcitriol [(1a,25-dihydroxy vitamin D3) (Sigma)], 10 nM Dexamethasone (Sigma).

For adipocyte differentiation, the basal medium was supplemented with 10% Horse Serum (Sigma), 100 nM dexamethasone, 0.45 mM isobutyl methyl xanthine [(IBMX) (Sigma)], 3 µg/mL insulin (Sigma), and 1 µM Rosiglitazone [(BRL49653). Controls cells were cultured in the basal medium supplemented with vehicle.

2.4. Micromass pellet cultures

hNSSCs were cultured as previously described (Vishnubalaji et al., 2015). Briefly, following trypsination cell pellets were re-suspended in 10 mL ECM medium. The cell density was adjusted to 3×10^5–5×10^5/mL to make pellets by centrifugation at 400 g for 10 min. Cell pellets were incubated at 37 °C for 2 days. Cell pellets were then transferred to the confetti (hydrophilic PTFE (polytetrafluoroethylene) and then placed on the membrane inserts (0.4 µm pore size, 30 mm diameter); Millipore; UK).

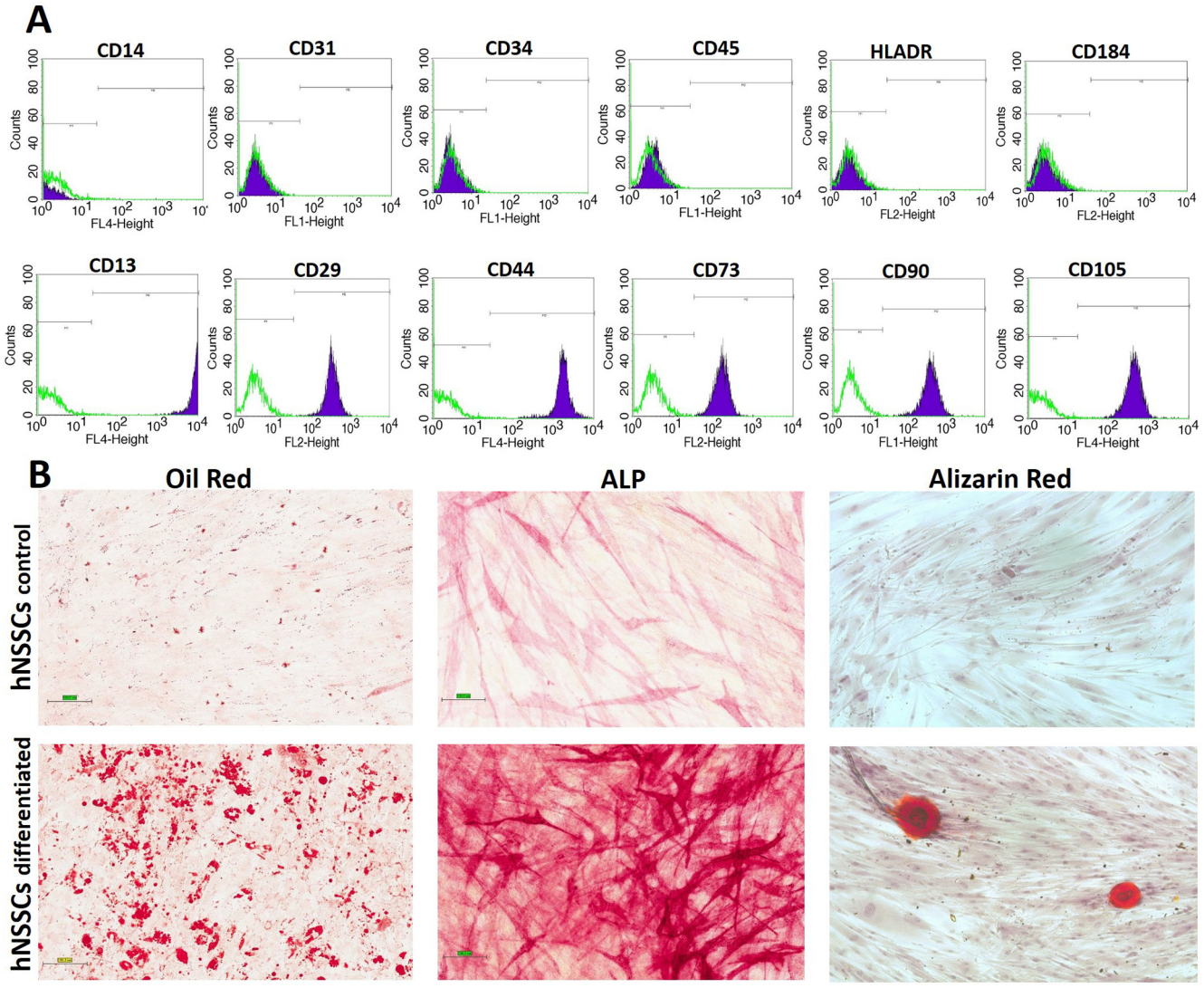

Figure 1 Phenotypic analysis and multilineage differentiation potential of hNFSSCs. Flow cytometry analysis of cell surface protein expression of stromal cells, endothelial and hematopoietic lineage associated markers. Filled histograms represent cells stained by the corresponding isotype control antibody. Five thousand events were acquired for analysis. Cells that were induced for 15 days under adipogenic conditions were assessed by the development of neutral lipid vacuoles stainable with Oil Red O, while cells induced for 15 days under osteogenic conditions then were assessed for alkaline phosphatase activity (ALP) and Alizarin Red to measure osteogenesis (shown at a magnification of 10X = 100 μ).

2.5. Preparation of chick femurs for ex vivo culture

Whole femurs were isolated from embryonic day 11 chicks, the soft tissue and adherent muscles and ligaments were carefully removed. Femurs were washed in PBS and placed onto the membrane inserts next to the micromass cell pellets on the membrane inserts at the interface between the air and the basal culture media (1 mL of basal tissue culture media consisting of a-minimum essential medium (a-MEM), penicillin (100U/mL), streptomycin (100 mg/mL), and ascorbic acid 2-phosphate (100 mM)) in 6-well tissue culture plates. Femurs along with cell pellets were cultured for 10 days. The medium was replaced every 24 h. Femurs were then fixed in 4% paraformaldehyde (PFA) for 24 h.

2.6. Histological and immunohistochemical analysis of the femurs

Chick femurs from each group were dehydrated through a series of graded alcohols and embedded in low-melting point paraffin using an automated Shandon Citadel 2000. Six micrometers tissue sections were cut and stained with Weigert's hematoxylin, followed by staining with 0.5% Alcian blue for proteoglycan-rich cartilage matrix and 1% Sirius red for collagenous matrix.

To identify human endothelial cells, tissue sections were stained for human CD31 using human specific antibody (GeneTex). In brief, after quenching endogenous peroxidase activity with 3% H_2O_2 and blocking with 1% bovine serum albumin (BSA) in 1X PBS, sections were incubated overnight at 4 °C

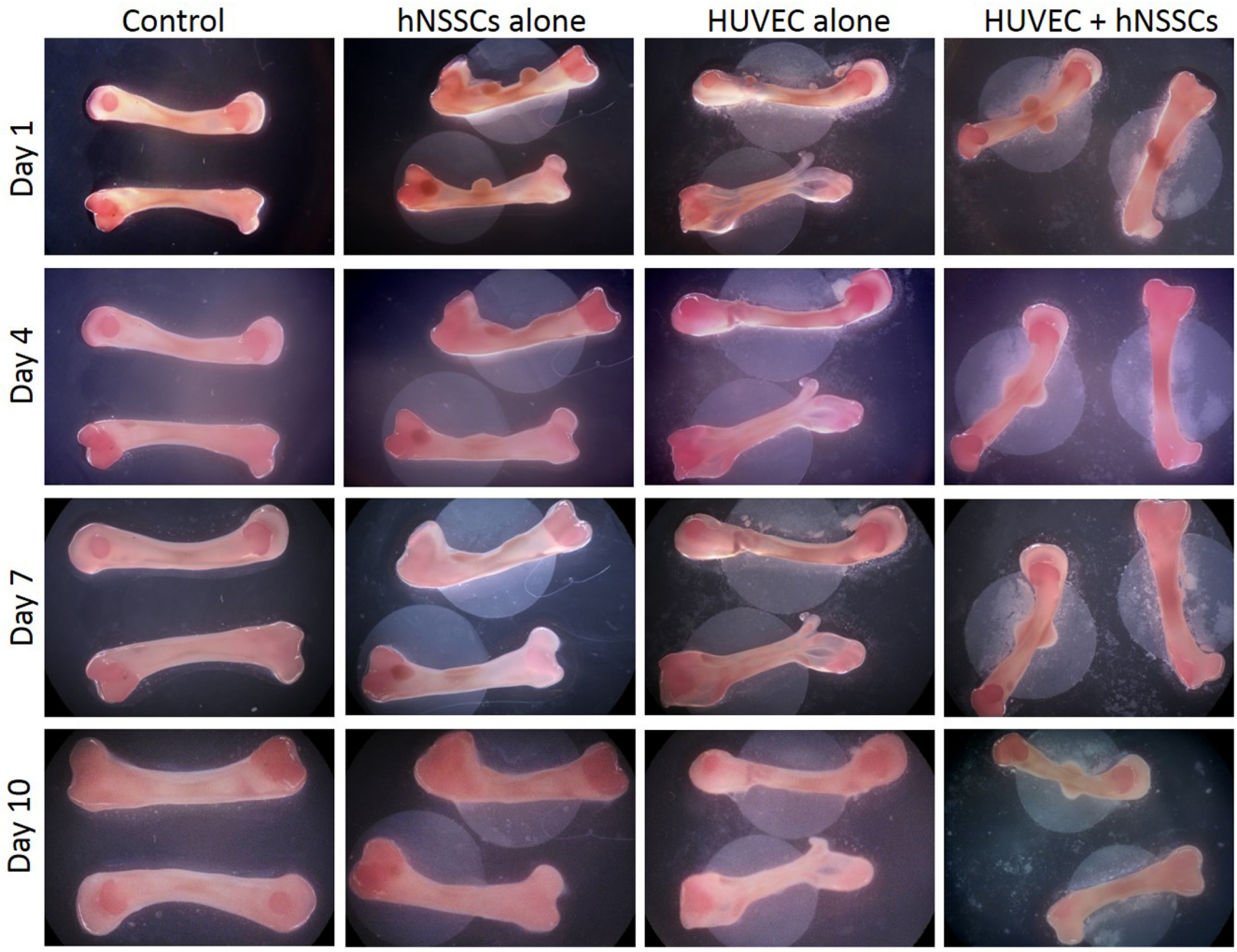

Figure 2 Comparative analysis of micromass on ex vivo chick femur model. hNSSC and HUVEC alone, and hNSSC + HUVEC co-cultured were compared to control chick femur. Day1 hNSSC pellets were attached with femur and fully migrated on Day10. HUVEC pellets were not attached and the pellets were dissociated and lost the micromass nature. The co-cultured (hNSSCs + HUVEC) micro mass attached and migrated over the chick femur.

with anti-CD31 primary antibody diluted appropriately in 1% BSA in PBS. Following primary antibody incubations, sections were incubated with biotin-conjugated secondary antibody anti-rabbit IgG (DAKO A/S; 1:100) diluted appropriately in 1% BSA in PBS. Visualization of the immune complex involved the avidin–biotin method linked to peroxidase and 3-amino-9-ethylcarbazole, resulting in a reddish brown reaction product. Sections were counterstained for light green and Alcian blue. Control staining was performed by omitting the primary antibody. Slides were digitized using high-resolution whole slides digital ScanScope scanner (Aperio Technologies, Inc.).

3. Results

3.1. hNSSC phenotype, adipocytic and osteoblastic differentiation

The hNSSCs were positive for known stromal cell-associated CD markers: CD105, CD90, CD73, CD29, CD13, and CD44, and were negative for endothelial and hematopoietic lineage markers (Fig. 1A).

To confirm the multipotent differentiation capacity of primary hNSSCs, the cells were induced to differentiation into adipocytes and osteoblasts. In the presence of adipogenic medium, hNSSCs formed adipocyte-like cells filled with intracellular lipid droplets that stained positive for Oil Red O (Fig. 1B). Similarly, cells cultured under osteogenic differentiation conditions formed osteoblast-like cells that were positive for alkaline phosphatase (ALP) which is an osteoblastic cell commitment marker (Fig. 1B) and the cultures contained areas of mineralized matrix that stained positive for alizarin red (Fig. 1B), Non-induced control cultures did not reveal any evidence for matrix mineralization.

3.2. Comparison of hNSSC, HUVEC, and hNSSC + HUVEC co-culture pellets using ex vivo chick femur

Fig. 2 shows gross morphological changes in femurs during the 10 day incubation period. Significant differences in the gross

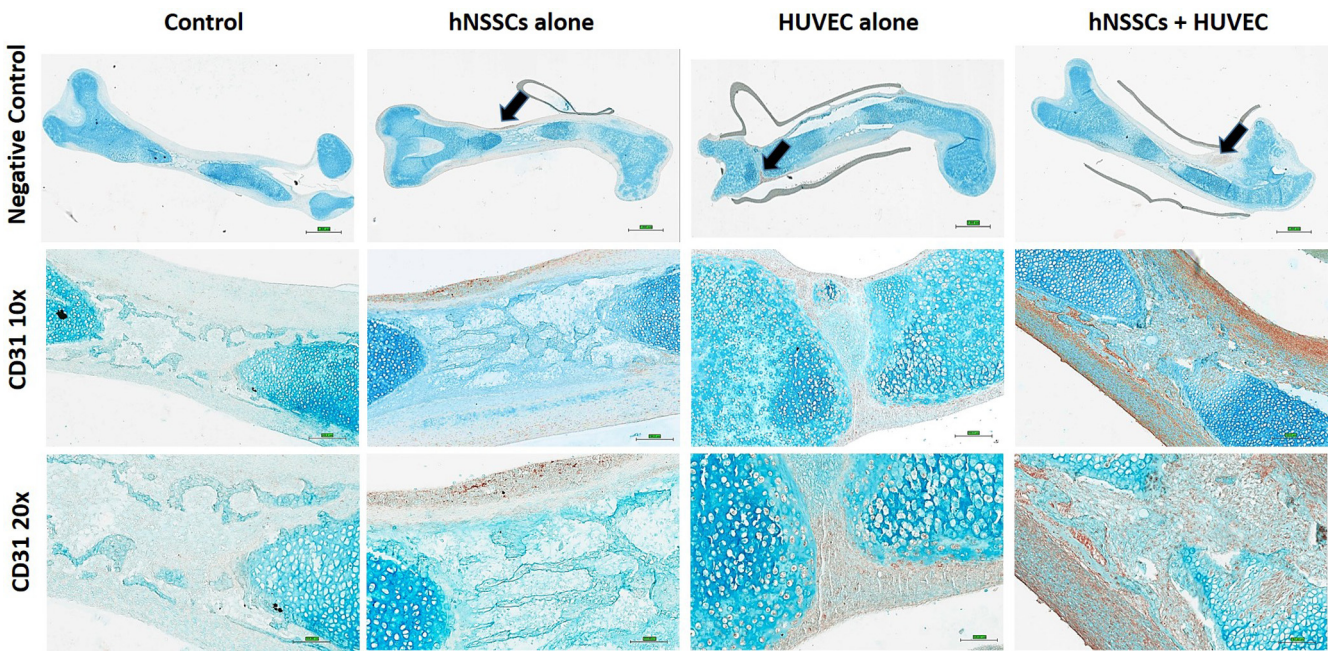

Figure 3 Comparative analysis of CD31 expression on hNSSCs and HUVEC alone, and hNSSC + HUVEC cultured chick femur. Immunohistochemical analysis, brown color indicates the areas with positive staining for CD31 marker. To illustrate more clarity magnified images were shown in different scales. Scale bar: Experiment negative control = 1 mm (1st row), Test = 200 μM and 1000 μM. Arrow marks indicate the attached cell pellet.

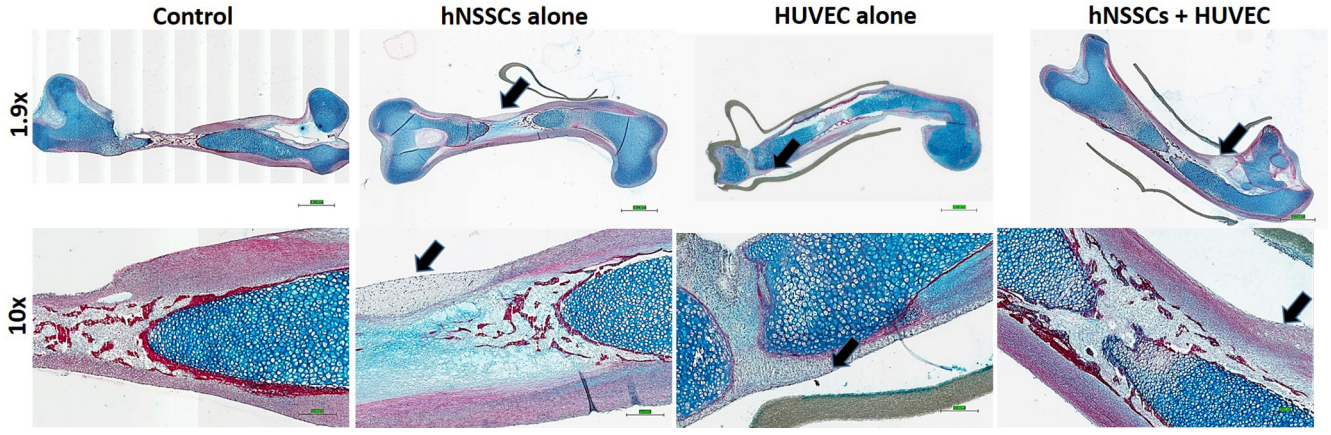

Figure 4 Analysis of collagen (Sirius red) and proteoglycan-matrix components (Alcian blue) of chick femur cultured with micromass. Histological analysis of the embryonic femurs cultured in the three conditions with control for Alcian blue/Sirius red, embryonic chick femurs (E11) after 10 days in the cultures. Scale bar: Experiment negative control = 1 mm (1st row), Test = 200 μM. Arrow marks indicate the attached cell pellet.

morphology were observed between chick femurs cultured in different conditions. Embryonic chick femurs cultured in the presence of hNSSC micromass pellets were longer, exhibited thicker diaphysis and an enlarged epiphyseal region compared with control femurs (Figs. 2 and 3). Femurs cultured in the presence of HUVEC cell pellets did not reveal these changes and were comparable to the controls. Femurs cultured in the presence of hNSSC + HUVEC cell micromass pellets exhibited similar morphology to those cultured in the presence of hNSSC cell micromass pellets. Histological analysis (Fig. 3) revealed migration and adherence of hNSSCs to the femurs compare to HUVEC cell pellets. Notably, on day one hNSSC pellets were attached to the femurs and subsequently started to migrate along the periosteum. At day 10, they covered the outer perimeter of the femurs. By contrast, HUVEC pellets showed limited cell migration and adherence to the femurs. (Figs. 2 and 3). Nonetheless, the co-cultured hNSSC + HUVEC pellets exhibited enhanced adherence and migration which was more pronounced than hNSSCs alone (Fig. 3) suggesting the importance of hNSSCs and HUVEC cell–cell interaction.

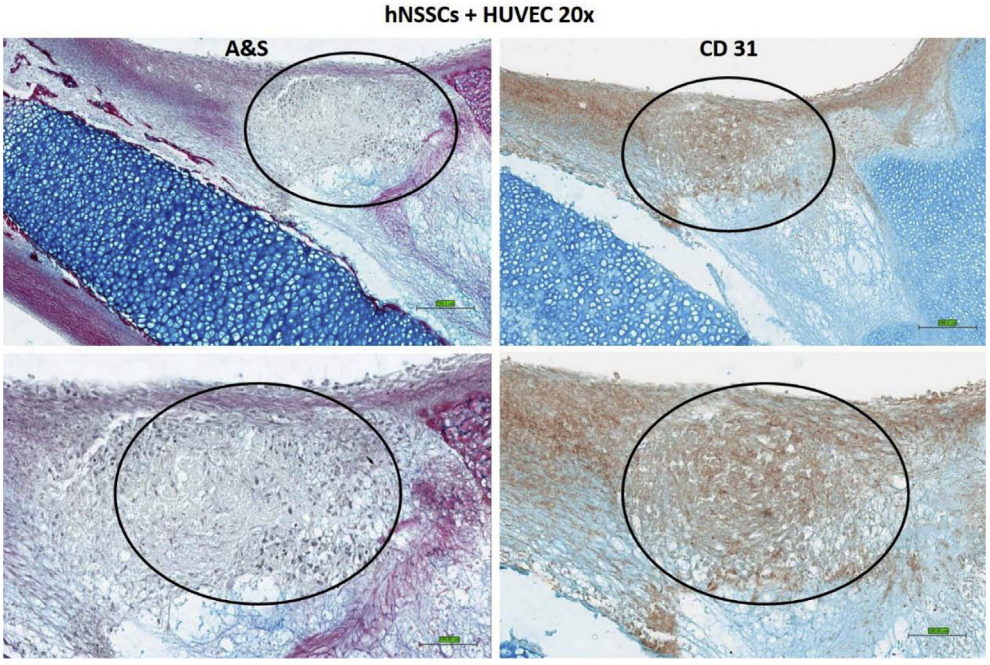

Figure 5 Comparison of Alcian blue/Sirius red (A&S) stain with CD31 expression of chick femur cultured with hNSSC + HUVEC micromass. Images were showing the same location of co-cultured pellets attached. Cells migrating out from the pellets were shown to be collagen positive and the cells which are negative showing positive for CD31. Circle marks indicate the attached cell pellet.

3.3. Analysis of cellular distribution of CD31, Sirius red and Alcian blue staining

Some positive staining for CD31 was observed in hNSSC cultures and HUVEC cultures but was most pronounced in cultures of hNSSC + HUVEC cell pellets (Fig. 3). Collagen staining with Sirius red corroborate the gross morphology of increased collagen deposition in femurs cultured in the presence of hNSSCs or hNSSC + HUVEC cell pellets and the staining was localized at a small area of the femurs periosteum in the case of no cell control, compared to wider distribution along the periosteum in cultures of hNSSCs + HUVEC (Fig. 4, 1.9×). Proteoglycan-rich matrix staining with Alcian blue appeared to be similar in all treatments (Fig. 4). Fig. 5 shows a higher magnification of the area of contact between hNSSC + HUVEC cell micromass pellets and the femur. The cells stained positive for CD31 and cells lying along the periosteum produced a large amount of collagen rich matrix as demonstrated by positive staining with Sirius red.

4. Discussion

In the present study, we demonstrated that ex vivo embryonic chick femurs could be employed as organotypic cultures to test the differentiation potential of stem cells. Using this assay, we demonstrated the presence of dynamic interaction between hNSSCs and the intact femur microenvironment that enhanced hNSSC cell differentiation into CD31 + vascular cells that were able to deposit collagen rich extracellular matrix. On the other hand, hNSSC cells produced factors that enhanced femur growth.

Bone is a complex multicellular 3D-mciroenvironment that allows dynamic interaction between cells and extracellular matrix. However, testing differentiation potential of stem cells with bone forming capacity has traditionally been studied in 2D-culture dishes and thus results from the 2D-cutlures were variable, dependent on culture conditions and not always predictable to the in vivo behavior of the cells. On the other hand, in vivo stem cell implantation is expensive, labor intensive and time consuming. Employing the embryo chick femur model to test cell differentiation potential has the main advantage of maintaining the relevant 3D-mciroenvironment important for cell differentiation. In addition, the model is easy to establish and has a quick turnover time. This model can be used not only to test cell differentiation potential but also the effects of different hormones and growth factors on bone growth, bone development and bone repair. For example, Smith et al have studied the delivery of bioactive growth factors from a novel alginate/bECM hydrogel to augment skeletal tissue formation as an approach for skeletal tissue regeneration (Smith et al., 2014a,b). We have employed histological methods to examine for cellular changes, but previous studies have demonstrated the possibility of combining histological methods with μCT-scanning of the femurs for more precise quantitation of bone mass and bone architecture (Kanczler et al., 2012).

We observed the ability of hNSSCs to differentiate into CD31 + vascular cells and to be integrated within the femur tissues. Our results corroborate findings from our previous studies that showed the ability of hNSSCs to differentiate into endothelial-like cells in vitro and in vivo (Vishnubalaji et al., 2015, 2012b). In addition, the ability of hNSSCs to migrate to biological surfaces was similar to what we have reported previously in real-time migration analysis, and scratch assay (Vishnubalaji et al., 2015). Thus, the chick embryo femur assays confirms that the observed differentiation potential of hNSSCs is not a culture artifact but it may have an in vivo relevance.

The use of chick embryo femur assay has a limitation that it is not a complete in vivo system and it lacks the systemic environment of the living organism. Nonetheless, it can be considered as a good screening tool to be utilized prior to in vivo studies. Also, the available of molecular biology techniques and tools to analyze the femurs will allow more sophisticated molecular studies in the context of 3D-tissue microenvironment that cannot be performed in 2D cultures (Pitsillides and Rawlinson, 2012).

In conclusion, hNSSCs alone and in combination with HUVEC support the bone development of embryonic chick femurs and demonstrate the ability to differentiate into vascular cells and ability to integrate and be functional within the 3D-bone environment. Our findings encourage testing the possible use of hNSSCs in bone tissue regeneration in vivo models.

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