CHAPTER 11

A Novel Genetically Engineered Human Osteoblasts for the *in Vitro* Study of Biomaterials

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Summary

Cell and tissue engineering, together with the development of new biomaterials, are fast growing fields of biomedical sciences and technologies. The final goals of the tissue engineering and biomaterials are the *in vitro* and *in vivo* regeneration and development of functionally active tissues. Recent advances in cell biology, molecular genetics and biocompatible materials represent key events in the improvement of clinical applications of the tissue engineering. From an orthopeadic perspective, tissue engineering is focused on the development of innovative materials, which recruit bone progenitor cells and stimulate their *"in vivo"* proliferation. It is worth remembering that biomaterials should allow cell adhesion and proliferation, without inducing alterations in the cell characteristics of the original tissue. In this chapter, we present a novel human cellular model, which was generated for the in vitro study of biomaterials. The study model, derived from a human osteosarcoma cell line, allows the rapid characterization of the cellular parameters. The human osteosarcoma cell line, Saos-2, which maintains the cytological features of the osteoblast cells, was genetically modified to express constitutively the enhanced green fluorescent protein (eGFP) from the jellyfish *Aequorea victoria*. The engineered cell line, named Saos-eGFP, represents a suitable *in vitro* model for studying the biocompatibility, the cell adhesion, spread and proliferation on biomaterials developed for clinical applications.

KEYWORDS: Engineered cell, Enhanced green fluorescent protein, Osteoblast molecular marker, Pre-clinical characterization.

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INTRODUCTION

Tissue engineering is a fast growing discipline that may allow the *in vitro* regeneration of functionally active human tissues, or induce their replacement *in vivo*. New knowledge in many biomedical disciplines, such as cellular and molecular biology, histology, biotechnology and the production of new biomaterials represents the starting point for the development of tissue engineering (1). It is well known that to repair some kinds of bone fractures/defects or to achieve a solid spine fusion after a surgical arthrodesis, often implies a long period of inactivity or hospitalization for patients, with a subsequent low quality level of their life and high social costs for the community (2). For these reasons bone tissue engineering holds a prominent position in clinical applications. At their earliest stages, bone tissue engineering studies were performed for the identification of materials suitable for *in vivo* implantation, with the aim of physical substitution of the damaged bone. The major prerequisite of these materials was that they were to be well tolerated, thus not inducing any "sensitization effects" in the host. More recently, researchers have focused their attention on the development of new biomaterials that are able to induce cellular proliferation and, subsequently, to facilitate bone tissue regeneration (3). It is worth mentioning that biomaterials should be able to enhance cell adhesion and proliferation without interfering with the physiological properties of the cells. Indeed, to obtain biological data on materials it is necessary to perform long term *in vivo* studies, which imply high costs and often ethical problems. In addition, especially for the spine, there is a lack of animal models which represent the human parameters. The considerations mentioned above indicate that orthopaedic surgeons have a good knowledge of the mechanical characteristics of the biomaterials, while they have a poor knowledge of their biological properties.

Herein, we present a cellular model suitable for the *in vitro* assessment of biological parameters. Our model is based on the Saos-2 cell line, a human osteosarcoma cell line which maintains the cellular features of the osteoblasts (4). Saos-2 cells were genetically engineered to express constitutively, as reporter gene, the enhanced green fluorescent protein (eGFP) derived from the jellyfish Aequorea victoria. The engineered cell line was named Saos-eGFP (5).

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MATERIALS/METHODS

Cells and DNA transfection

Saos-2 cells were cultured in DMEM-F12 (BioWhittaker, Milan, Italy) supplemented with 10% foetal bovine serum (FBS) (BioWhittaker, Milan, Italy) and 500 U/ml penicillin/streptomycin (Sigma, Milan, Italy), and maintained at 37°C. in a humidified atmosphere containing 5% CO_2.

To determine in the parental Saos-2 cells the non-toxic concentrations of the antibiotic Genetycin (Invitrogen, Milan, Italy), non-transfected cells were plated in 6-well plates at a density of $5x10^4$ cells/well. The cells were allowed to adhere and then subjected to different concentrations of Genetycin, ranging from 0÷700 µg/ml. The medium was changed twice a week for 15 days. The proportion of viable cells was assessed by microscopy analysis after trypan-blue staining.

DNA transfection of pCMV-EGFP (6) vector was carried out on 50% confluent cell monolayer using the Superfect Reagent (QIAGEN Spa, Milan, Italy) following the manufacturer's instructions. The pCMV-eGFP vector contains the coding sequence for the enhanced green fluorescent protein (eGFP) (7) downstream the Cytomegalovirus (CMV) immediate-early promoter, and the neo gene for Genetycin resistance. Briefly, 5 µg DNA diluted in 150 µl of serum-free medium was incubated at room temperature for 10 minutes with 30 µl of liposome suspension. DNA/liposome complexes, diluted in 1 ml of complete medium, were incubated with cells for 4 hours at 37°C. in an atmosphere of 5% CO_2. Twenty four hours after transfection, the cells were plated at low density in 75 cm^2 flasks. The selection of cells was started 48 hours after transfection, using 500 µg/ml of Genetycin. After 15 days of antibiotic treatment, resistant clones were isolated with sterile glass cylinders (Sigma, Milan, Italy) and expanded as independent cell lines.

Immunofluorescence

For immunofluorescence assays, cells were cultured on 12 mm diameter round glass coverslips for 18 hours and fixed with ice-cold methanol/acetone (1:1) mixture at 4°C. for 20 minutes. The fixed cells were air-dried before incubation with the primary antibody, diluted 1:10 in PBS 1X, for 30 minutes at 37°C. After 3 washes with PBS 1X for 5 minutes at room temperature,

appropriate secondary antibody, diluted 1:10 in PBS 1X, was applied to the cells for 30 minutes at 37°C. in the dark. Following 3 washes in PBS 1X for 5 minutes, at room temperature in the dark, cells were allowed to air dry. For fluorescence microscopy observation, the glass coverslips were mounted with glycerol/PBS 7:3. Observation was performed with a Nikon TE2000E microscope (Nikon S.p.A., Florence, Italy) and digital images were captured using the ACT-1 software for the DXM1200F digital camera (Nikon S.p.A., Florence, Italy).

Cell quantification

Saos-eGFP cells were enzymatically dissociated and resuspended in 2 ml of culture medium. The number of cells was determined by microscopy analysis after trypan-blue staining. Samples containing an increasing number of cells, ranging from $5x10^3$ to $5x10^5$, were centrifuged (2,500 r.p.m., 10 min), washed with 4 ml of PBS 1X, and resuspended in 1 ml of reading buffer (NaH_2PO_4 50 mM, Tris-HCl 10 mM, NaCl 200 mM, pH 8.0). The fluorescence of each sample was measured spectrophotometrically, immediately before data acquisition, at λ=490 nm. The calibration curve was obtained reporting on a graph the number of cells present in each sample with the relative reading at the spectrophotometer.

Cell culture with biomaterials

To test biomaterials with our cellular model, Saos-eGFP cells were cultured in presence of two different cages, which are routinely used to achieve interbody fusion in spine surgery. The two distinct cages were made of carbon fibre reinforced polymer (CFRP) and polyetheretherketone (PEEK), respectively (Fig. 1).

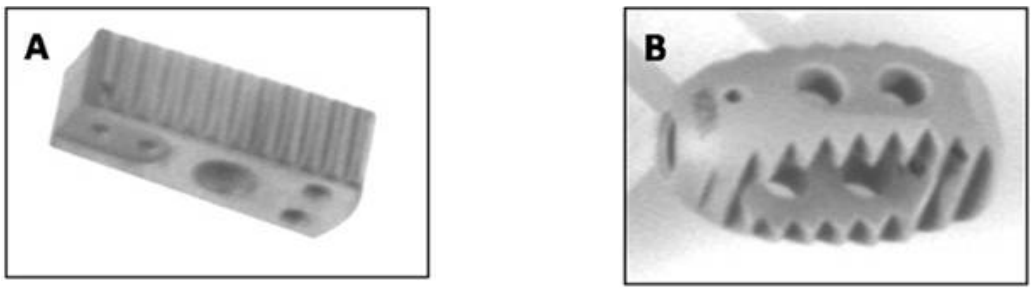

Fig. 1. Images of the two distinct cages, which biomaterials are composed of carbon fibre reinforced polymer (CFRP) (**A**) and polyetheretherketone (PEEK) (**B**), respectively.

Direct observation of the living cells on the two cages was carried out by fluorescence microscopy in order to determine the distribution, colonization and morphology of the human cells on the biomaterials. The number of attached cells, grown on the two different cages, was determined at 36 h. and 84 h. after starting the culture. Cells that detached from the cages by trypsin, were evaluated by spectrophotometry, reading the emitted fluorescence and calculating the number of cells using the calibration curve obtained with the samples containing a known number of cells.

Statistical analysis

All data were obtained from five independent experiments and expressed as a mean value ± standard deviation (SD). Student's t-test was employed to evaluate the statistical significance of the differences in the mean value ± SD between groups. Results were considered statistically significant when p values ≤ 0.05 (5).

RESULTS

Engineered Saos-eGFP cell line

Saos-2 cells were transfected with the pCMV-eGFP construct (5-7) and cultured in the presence of 500 μg/ml of Genetycin. Six resistant clones were isolated and then two of them were propagated as independent cell lines. Expression of the eGFP in the engineered cells was assessed by fluorescence microscopy observation (Fig. 2) (5-7). Stability of eGFP expression was confirmed over a period of 12 weeks as fluorescence intensity. This was determined by monitoring twice a week $1x10^4$ cells for each clone by spectrophotometric analysis at λ= 490 nm. The clone exhibiting the most constant level of fluorescence over this period of time was selected for further studies.

Cell markers

A comparative molecular characterization was carried out with the parental Saos-2 cell line and Saos-eGFP engineered cells. Specifically, we verified whether the expression of the exogenous protein eGFP interferes with the cytological features of the cells. Expression and cellular localization of bone specific markers, such as osteopontin and osteocalcin, were determined by

immunofluorescence assays using the monoclonal antibodies AKm2A1 and V-19, respectively (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Expression of specific markers was confirmed in the parental Saos-2 as well as in Saos-eGFP cells (5). Cellular localization of the osteopontin under investigation was identical for the two cell lines, while osteocalcin labeling did not differ substantially from that of the osteopontin (5) (Fig. 3).

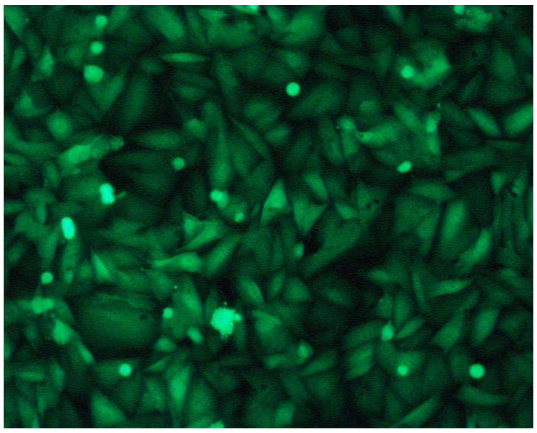

Fig. 2. Microscope fluorescence micrograph showing Saos-eGFP cells distribution on plastic Petri dish.

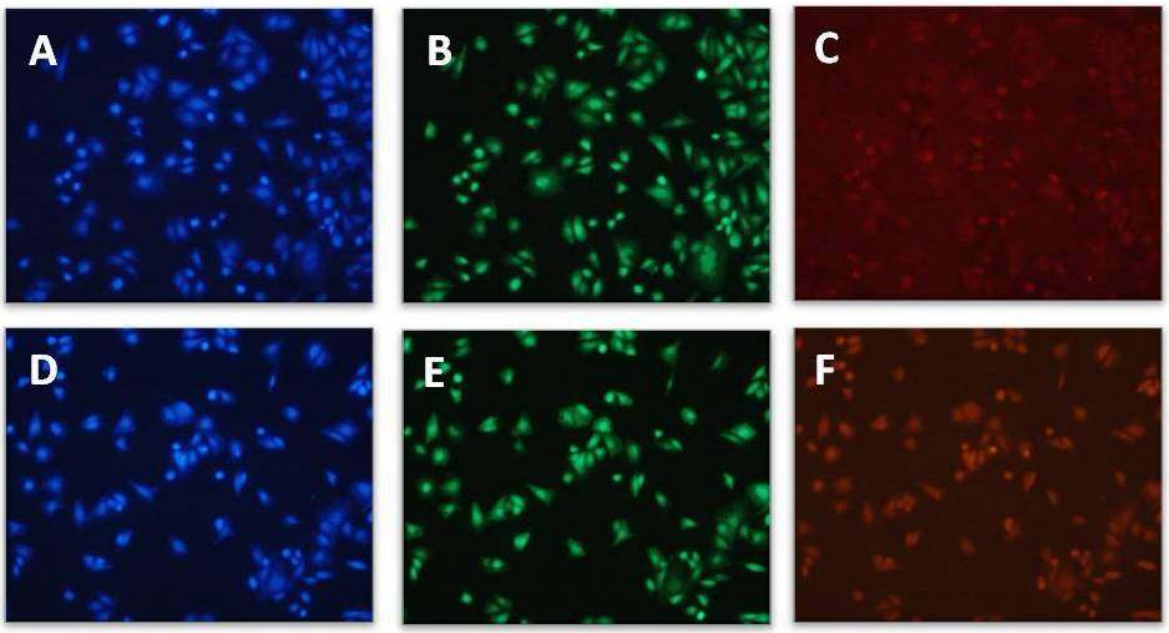

Fig. 3. Microscope fluorescence micrographs showing the cell markers osteopontin OPN (C) and osteocalcin OCN (F) in Saos-EGFP cells. Cell nuclei were counterstained with DAPI (A and D), while unstained Saos-EGFP cells show their constitutive green fluorescence (B and E).

We also assessed if the presence of the exogenous protein could alter the cytoskeletal organization of the Saos-eGFP cells. Actin fibres were therefore visualized using TRITC (tetramethylrhodamine isothiocyanate)-conjugated Phalloidin (Sigma, Milan, Italy). The cytoskeletal architecture of Saos-eGFP cells was indistinguishable from that of parental Saos-2 cells (5). This result indicates that the presence of the exogenous protein eGFP does not affect the cytoskeletal organization of the engineered cells.

Determination of cell number

It has been demonstrated that the fluorescence intensity, due to eGFP expression, is proportional to the amount of eGFP present within a sample (5). Since Saos-eGFP cells express constitutively the green fluorescent protein, the intensity of the fluorescence should depend exclusively on the number of cells present within a sample. In order to confirm the linearity of fluorescence intensity with the number of cells, sets of samples containing $5x10^3$, $1x10^4$, $2x10^4$, $5x10^4$, $1x10^5$, $1.5x10^5$ and $2x10^5$ cells were analyzed by spectrophotometric reading at λ=490 nm. Fluorescence values increased constantly for samples, when the number of cells was in the range of $1x10^4$ - $2x10^5$ cells. Graphical expression of these experiments confirmed that fluorescence intensity values (O.D.) are proportional to the number of cells present within a sample, with a lower threshold of $5x10^3$ cells (5). On this basis, once known the fluorescence value (O.D.), it is possible to extrapolate the number of cells contained within a sample by interpolation with the standard curve (5).

Counting of cells grown on the biomaterials

In our assay, we considered that parental Saos-2 cells need approximately 36 h. to complete a single cell cycle, while the glass was the material used as a comparative internal control (100%) of the CFRP and PEEK cages (5). Cultures analyzed at 36 h. indicated that the percentage of cells grown on PEEK (121%) was slightly higher than those grown on CFRP (116%) (5). Then, cell proliferation on the two different materials was evaluated at 84 h. The percentage of cells grown on PEEK increased in a fashion similar to that observed on the glass (13%), while cells on CFRP increased more than 50% (53%). Our assay clearly indicated that the number of cells, attached on the two distinct biomaterials, CFRP and PEEK, differed substantially at 84 h. of culture (5).

Direct observation of the cells on the biomaterials

The observation of the living cells directly on the cages showed a different distribution on the two different biomaterials (5-7). On the CFRP cage the cells were homogeneously distributed, as a continuous monolayer, like those of the control, while on the PEEK cage the cells were arranged in an irregular non-homogeneous distribution, and associated in clusters (5). To verify whether the cytoskeletal organization of the cells, cultured on the cages, was altered, actin fibres were investigated with the TRITC (tetramethylrhodamine isothiocyanate) conjugated-Phalloidin (Sigma, Milan, Italy). Cytoskeletal architecture on CFRP and PEEK was indistinguishable from that of Saos-eGFP cells grown on plastic petri dish, used as control (Fig. 4).

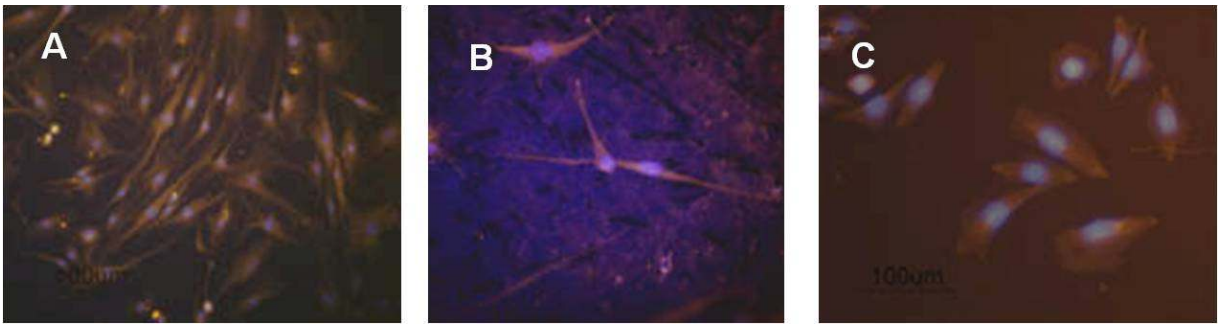

Figure 4. Analysis of the cytoskeletal organization of the engineered Saos-eGFP cells seeded on plastic Petri dish (A), and cages, CFRP (B) and PEEK (C). Actin fibres of the cells were visualized by TRITC (tetramethylrhodamine isothiocyanate)-conjugated Phalloidin.

DISCUSSION

In our investigation, a cellular model suitable for the *in vitro* characterization of cell morphology, spread and proliferation on biomaterials was developed. A human osteosarcoma derived cell line, named Saos-2, which maintained the osteoblastic phenotype (4, 5, 7), and kept an unlimited growing potential *in vitro*, was genetically modified. The parental Saos-2 cells were transfected with the pCMV-eGFP expression vector (6), containing as reporter gene the enhanced green fluorescent protein (eGFP). After Geneticyn selection, an immortal genetically uniform cell clone, the Saos-eGFP, was derived and expanded in culture. The engineered cell line Saos-eGFP maintains the cytological features of osteoblast cells and constitutively expresses the eGFP. This enhanced form of the Green Fluorescent Protein (GFP) was obtained by mutation (9) of the parental gene isolated from the jellyfish *Aequorea victoria* (10). The GFP gene has been demonstrated to ensure the production of the fluorescent protein in heterologous cell systems,

without inducing any cellular toxicity (11). Here, it has been demonstrated that the presence of the eGFP does not alter the physiological expression of molecular markers specific to the bone tissue. Comparative analyses carried out on parental and engineered cells demonstrated that both cell populations express molecular markers specific to bone tissue, such as osteocalcin and osteopontin. These cellular markers are produced by osteoblasts (11), and belong to the extracellular matrix proteins, which are crucial for bone tissue formation. The distribution and cellular localization of the marker proteins were indistinguishable in the two osteoblast cell lines, the parental Saos-2 and engineered Saos-eGFP cells, indicating that functional properties were not modified.

In this study, the cytoskeletal organization of cells was taken into consideration. Indeed, this cellular organization is a key parameter in the bone tissue histology. Previous studies have shown that the actin cytoskeletal element plays an important role in cell attachment and stabilization. Actin bundles coupled with adhesion plaques can transmit forces to the substrate and help to maintain cell shape and facilitate cell adhesion (12, 13). Parental and engineered cells were investigated by immunofluorescence assay for the cytoskeletal architecture. Cytoplasmic actin fibre organization was not affected by the presence of the exogenous protein, since we observed a perfect match in the cytoskeletal architecture between the two cell lines, parental Saos-2 and engineered Saos-eGFP.

It was shown that the measure of eGFP fluorescence is proportional to the eGFP molecule number within each sample (7). This means that, having a genetically uniform clonal cell line, the fluorescence intensity is proportional to the number of cells within each sample. The high expression level of the trans-gene in the genetically modified cell line Saos-eGFP ensures a direct proportion between fluorescence intensity and cell number down to $5x10^3$ cells/sample. This relationship is very useful to easily and rapidly assess the number of cells within any sample, by simply determining the fluorescence intensity by a spectrophotometric reading.

Recently, other cellular models useful to evaluate the biological characteristics of biomaterials have been reported by Ge et al. (14) and Xia et al. (15). Ge et al. (14) used GFP-transfected mesenchymal stem cell-induced osteoblasts to demonstrate the potential of HA–chitin matrixes as a good substrate candidate for tissue engineered bone substitute, while Xia *et al*. assayed eGFP-labelled human mesenchymal stem cells (hMSCs) as an effective tool to trace

the hMSCs fate and evaluate the interactions between cells and ceramics. In particular, these two studies evaluated the osteoinduction, osteoconduction, and osteogenesis which are specific characteristics of mesenchymal stem cells, when they are combined with specific bone substitutes and induced in the osteogenic lineage.

The Saos-eGFP cells described in our study represent a suitable *in vitro* model for the determination of the mature osteoblastic cell morphology, spread and proliferation on biomaterials presenting particular surface, topographical or chemical features. Indeed, different materials with specific morphology, shape and composition may influence the proliferation and the distribution pattern of osteoblasts on their surface. This is the case for the biomaterials evaluated in our study. Mature osteoblasts on the CFRP cage surface showed a more homogeneous spread and a higher rate of proliferation than those on the PEEK cage surface in the same culture conditions. This result demonstrates that the chemical features, the shape of the surface and the morphology of the CFRP cages have an intrinsically different effect on the mature osteobalsts than PEEK cages.

Orthopaedic surgeons are very well informed about the biomechanical characteristics of the biomaterials they use in their procedures, but they require more knowledge of the biological proprieties of these biomaterials which, until now, have rarely been described. In particular, spine surgeons consider fusion of the functional spinal unit as an important goal during most procedures; the possibility to knowing which cages do not alter the cell morphology, specific markers, spread and proliferation could be a fundamental key point to achieve a solid spine fusion. This knowledge is important in all orthopaedic procedures, using different type of biomaterials. In particular, it will become more and more essential in treating osteoporotic traumatic pathology which will be the most frequent pathology of the coming decades. To date, pre-clinical characterization of biomaterials relied mainly on *in vivo* animal model testing. This is certainly the most accurate and reliable approach to assess the biological properties of orthopaedic materials, but it implies high costs, ethical issues and long experimental protocols.

CONCLUSION

In the current study, we described the Saos-eGFP cell line, which can be employed for the *in vitro* preliminary characterization of the cell morphology, spread and proliferation on biomaterials. This cellular model will certainly allow *in vivo* testing of only those materials showing *in vitro* the required properties for successful bone tissue augmentation.

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References

1. Langer R, Tirrel DA. Designing materials for biology and medicine. Nature 2004; 428(6982):487-92.
2. Wade R, Richardson J. Outcome in fracture healing: a review. Injury 2001; 32(12):109-14.
3. Kokubo T, Kim HM, Kawashita M, Nakamura T. Bioactive metals: preparation and properties. J Mater Sci Mater Med 2004; 15(2):99-107.
4. Rodan SB, Imai Y, Thiede MA, Wesolowski G, Thompson D, Bar-Shavit Z, Shull S, Mann K, Rodan GA. Characterization of a human osteosarcoma cell line (Saos-2) with osteoblastic properties. Cancer Res 1987; 47(18):4961-6.
5. Morelli C, Barbanti-Brodano G, Ciannilli A, Campioni K, Tognon M. Cell morphology, markers, spreading, and proliferation on orthopaedic biomaterials. An innovative cellular model for the "in vitro" study. J Biomed Mater Res A 2007; 83(1):178-83.
6. Morelli C, Campioni K, Parolin C, Palù G, Tognon M. Activity of the matrix metalloproteinase 9 promoter in human normal and tumor cells. J Cell Physiol 2004; 199(1):126-33.
7. Cinelli RA, Ferrari A, Pellegrini V, Tyagi M, Giacca M, Beltram F. The enhanced green fluorescent protein as a tool for the analysis of protein dynamics and localization: local fluorescence study at the single-molecule level. Photochem Photobiol 2000; 71(6):771-6.
8. Shupnik MA, Tashijan AH Jr. Epidemal growth factor and phorbol ester actions on human osteosarcoma cells. J Bio Chem 1982; 257(20):12161-4.
9. Cormack BP, Valdivia R, Falkow S. FACS-optimized mutants of the green fluorescent protein (GFP). Gene 1996; 173(1 Spec No):33–8.
10. Prasher DC, Eckenrode VK, Ward WW, Prendergast FG, Cormier MJ. Primary structure of the Aequorea victoria green fluorescent protein. Gene 1992; 111(2):229–33.
11. Chalfie M, Tu Y, Euskirchen G, Ward WW, Prasher DC. Green fluorescent protein as a marker for gene expression. Science 1994; 263(5148):802-5.
12. Anselme K. Osteoblast adhesion on biomaterials. Biomaterials 2000; 21(7):667–81.
13. Sinha RK, Tuan RS. Regulation of human osteoblast integrin expression by orthopaedic implant materials. Bone 1996; 18(5):451–7.
14. Baguenard S. Lim LY, Khor E. Hydroxyapatite-chitin materials as potential tissue engineered bone substitutes. Biomaterials 2004; 25(6):1049-58.
15. Xia Z, Ye H, Locklin RM, Ferguson DJ, Cui Z, Triffitt JT. Efficient characterisation of human cell-bioceramic interactions *in vitro* and *in vivo* by using enhanced GFP-labelled mesenchymal stem cells. Biomaterials 2005; 26(29):5790-800.