Genotype and expression of the enhanced green fluorescent protein in LEW-Tg (EGFP) F455.5/Rrrc rats

Genótipo e expressão da proteína fluorescente verde melhorada em ratos da linhagem LEW-Tg (EGFP) F455.5/Rrrc

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Abstract

The Green fluorescent protein (GFP) was first described after being extracted from *Aequorea victoria* in 1987; Since then, GFP and its derivatives have been widely used in several experiments as cell and protein marker. In the present study it was verified the genotype of the offspring from crosses between heterozygote Lewis LEW-Tg (EGFP) F455.5/Rrrc rats and analyzed the expression of the enhanced green fluorescent protein (EGFP) in different cell types and genotypes. The genotype of the offspring was assessed by PCR and analysis of EGFP expression in different cells and genotypes, including mesenchymal stem cells (MSC) derived from adipose tissue and calvarial osteoblast cells. Expression of EGFP was verified by flow cytometry, fluorescence microscopy, and immunostaining. Through these methods, it was identified the genotypes of the offspring and determined the levels of expression of EGFP in two cell types. A difference in expression between the (*EGFP* +/+) and (*EGFP* +/-) genotypes was also observed in addition to the presence of autofluorescence. Further studies on the natural fluorescence of cells with the (*EGFP* +/-) genotype and that induced by presence of the EGFP are necessary.

Keywords: Enhanced green fluorescent protein. EGFP. Autofluorescence. Difference in expression.

Resumo

A proteína fluorescente verde (GFP) foi descrita pela primeira vez após ter sido extraída de *Aequorea victoria* em 1987. Desde então, a GFP e seus derivados têm sido amplamente utilizados em várias experiências como marcador celular e de proteínas. O objetivo do presente estudo foi o de verificar o genótipo dos descendentes de cruzamentos entre ratos Lewis LEW-Tg (EGFP) F455.5/Rrrc heterozigotos e de analisar a expressão da proteína fluorescente verde melhorada (EGFP) em diferentes tipos celulares e genótipos. O genótipo da descendência foi avaliado por PCR e pela análise da expressão da EGFP em diferentes células e genótipos, incluindo-se as células-tronco mesenquimais (MSC) derivadas de tecido adiposo e de osteoblastos de calvária. A expressão da EGFP foi verificada por citometria de fluxo, microscopia de fluorescência e imunocoloração. Foram, identificados os genótipos da descendência e determinados os níveis de expressão de EGFP em dois tipos de células. Foi também constatada uma diferença de expressão entre os genótipos (*EGFP* +/+) e (*EGFP* +/-) além da presença de autofluorescência. Mais estudos são necessários para esclarecer a fluorescência natural de células com o genótipo (*EGFP* +/-) e aquela induzida pela presença da EGFP.

Palavras-chave: Proteína verde fluorescente melhorada. (EGFP). Autofluorescência. Diferença de expressão.

Introduction

In 1955, Devanport and Nicol[1] discovered that the jellyfish *Aequorea Victoria* was bioluminescent and emitted a green light when exposed to ultraviolet (UV) radiation. This species looked like a hemispherical umbrella, with light-emitting organs along its edge[2,3,4,5,6,7]. Two proteins were discovered after extraction and purification of the components

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isolated from its bioluminescent organs: *Aequorin*, a protein capable of emitting blue light in presence of Ca^{2+} even in the absence of oxygen, and the Green Fluorescent Protein (GFP), which could emit yellow light in the presence of tungsten and green light under UV radiation[3,4,8,9,10,11,12,13]. A chromophore inside the jellyfish can absorb and emit light after calcium ion release by the *Aequorea*. After binding to *Aequorin* protein, these ions emit a blue light, which is absorbed by the GFP and converted to green light to be emitted. No additive (calcium, a prosthetic group) is necessary for Aequorin to emit light[4,8,9,12,14,15,16].

GFP has two chemically different groups: a neutral chromophore (major peak of absorption; excitable at 395 nm) and an anionic chromophore (minor peak; excitable at 475 nm). Both groups emit a visible green light in the maximum emission peak (505-509 nm)[14,15,17,18,19,20,21]. The composition of GFP, an 11-stranded β-barrel wrapped around an α-helix, is its most distinctive feature. Its chromophore is located in the central part of the barrel. Almost the whole primary sequence of this protein is utilized to build the β-barrel and central α-helix. Each string consists of approximately 9 to 13 residues of amino acids (Figure 1)[4,6,12,19,20,22,23].

There are seven classes of fluorophores derived from the wild-type GFP. In the present study, we investigated the enhanced green fluorescent protein (EGFP) containing a phenolate anion chromophore (class 02 of chromophores), the class most widely used in different biological techniques. The reason for this choice is its capacity to emit intense light with the excitation and emission peaks at a wavelength very similar to that of fluorescein. The Ser65Thr substitution is the most used mutation that causes ionization of the phenol group in the chromophore[12,14,16,17,20,21,24,25,26]. There are currently several biological techniques that use EGFP, including its introduction in live animals. Lewis rats of the LEW-Tg (EGFP) F455.5/Rrrc line were modified to express EGFP in every cell of the body. Due to the high reproductive rate of the heterozygote animal genotype, it is necessary not only to confirm the presence of EGFP in the offspring, but also to evaluate the fluorescence intensity in these animals. The present study, aimed to identify the genotypes in the offspring of heterozygous animals and to analyze protein expression in different tissues and genotypes. For this purpose, it was cultured mesenchymal stem cells derived from adipose tissue and calvarial osteoblast cells (COB).

Material and Methods

Animals

Lewis rats of the line LEW-Tg (EGFP) F455.5/Rrrc (Rat Resource and Research Center, University of Missouri, MI, USA) are modified animals that express EGFP. The highest peak in the reproductive rate occurs in their first two months of life, and heterozygous rats are more fertile than the homozygous ones. Decline in the reproductive rate occurs between the sixth and eighth months of life. The wild-type GFP has 238 amino acid residues and 26.9-kDa molecular weight[4,8,12,14,18,19,21,27]. The sequence of amino acids of GFP is given below, and the residues in bold type refer to the fluorophore[4]:

MSKGEELFTGVVPVLVELDGDVNGQKFSVSGE
GEGDATYGKLTLNFICT

TGKLPVPWPTLVTT**FSYG**VQCFSRYPDHMKQ
HDFFKSAMPEGYVQERTI

FYKDDGNYKTRAEVKFEGDTLVNRIELKGIDF
KEDGNILGHKMEYNYNS

HNVYIMGDKPKNGIKVNFKIRHNIKDGSVQL
ADHYQQNTPIGDGPVLLP

DNHYLSTQSALSKDPNEKRDHMILLEFVTAARI
THGMDELYK

DNA extraction

Blood samples were collected from the upper part of the tail of adult (2 months) Lewis rats. The animals

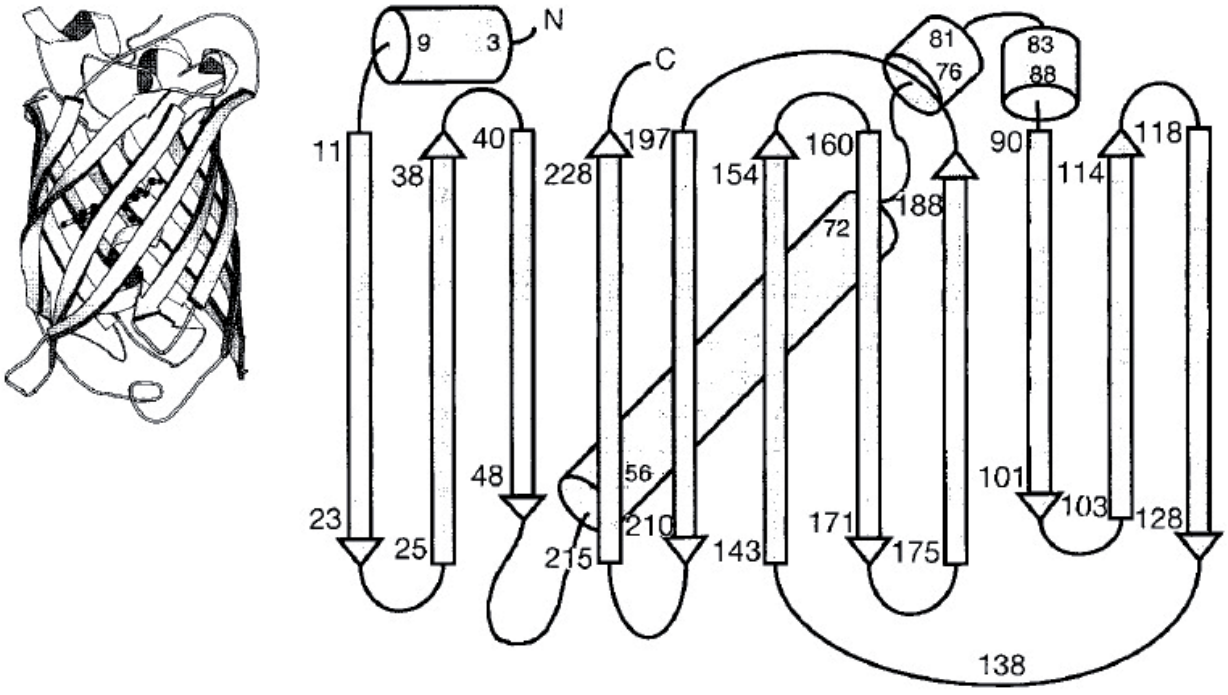

Figure 1 - Representation of 3D structure of GFP
Legend: Its structure is composed of an 11-stranded β-barrel wrapped around an α-helix [backbone]. The chromophore is located in the central part of the barrel. Source: Örmo et al., 1996

were anesthetized (10% ketamine and 2% xylazine) and fur-marked with picric acid solution. Isolation of genomic DNA (50 μL of blood) was performed using DNAzol reagent (Invitrogen, City, ST, Country), following the supplier's instructions[25].

Table 1 - Primer sequences used in PCR reactions

Primers	Sequences	pb
LWS 455 5F	AACCTCCCAGTGCTTTGAACGCTA	24
LWS 455 5R	GGTGCCAAGCCTCAACTTCTTTGT	24
U3r-4	ATCAGGGAAGTAGCCTTGTGTGTG	24

Polymerase Chain Reaction (PCR)

The extracted DNA was used in PCR reactions with the following primers: LWS 455 5F, LWS 455 5R, and U3r-4 (Invitrogen, City, ST, USA). Their sequences are shown in table 1. The other components (and their final concentrations) were: dNTPs (0.2 mM), Taq DNA Polymerase (Fermentas; 0.026 u/μl), Taq Buffer (1 of 5x), $MgCl_2$ (1.5 mM), primers 1, 2 and 3 (0.4 mM), and DNA (≥15 ng). The thermocycler conditions were as follows: initial denaturation step (94 °C; 3 min) followed by 94 °C (34 cycles; 30 s), 64 °C (30 s), 72 °C (30 s/cycle), and 72 °C (final extend; 10 min). PCR products were analyzed on agarose gel (3%) with SyberSafe stain solution (Supplier, City, ST, Country). Analysis of the amount of amplicon base pairs was performed by comparison with a molecular-weight standard of 1-kb DNA ladder (Life technologies, City, ST, Country). This protocol followed the protocol provided by the institution that supplied the animals described above[28].

Cell culture

Standard culture medium was used for mesenchymal stem cells (MSC) and a Roswell Park Memorial Intitute medium (RPMI) with Fetal Bovine Serum (FBS) was used for the calvarial osteoblast culture.

Standard culture medium or Dulbecco's Modified Eagle Medium (DMEM; Gibco, City, ST, USA) + fetal bovine serum (FBS)(10%) (Gibco, City, ST, Country) + penicillin/streptomycin/amphotericin B (Gibco, City, ST, USA; 10%)], and the other medium , Roswell Park Memorial Institute (RPMI; Gibco, City, ST, USA) + FBS (10%) (Gibco, City, ST, USA) were used for osteoblast culture. The culture media were prepared following supplier's instructions.

Isolation of MSC derived from adipose tissue (AT)

The MSC-AT were isolated from adult (4 months) Lewis rats. Rats were sacrificed after an anesthesia combination of Ketamine (10%) and Xylazine (2%). The inguinal adipose tissue was collected and stored in falcon tubes (50 mL) with DMEM (Gibco, City, ST, USA). The inguinal adipose tissue was then rinsed in sterile PBS and then transferred to a new tube to be sliced, macerated and treated with collagenase (type II; Sigma, City, ST, Country; 15%, p/v) diluted in PBS (0.15 M). This process lasted 1 h in the incubator (Revco-Elite II, City, ST, Country; 37 ºC, 5% CO_2), with gentle agitation (every 15 min). Collagenase was inactivated with DMEM (10 mL) + FBS (10%; Gibco, City, ST, USA). The cellular pellet was then resuspended in DMEM (10 mL) + FBS (10%; Gibco, City, ST, Country) and plated into T75 tissue culture flasks (Techno Plastic Products, City, ST, Switzerland), where they were allowed to grow in a controlled incubator (5% CO_2; 95% humidity; 37 ºC). All animal experimental procedures were approved by the local Committee for Ethical Use of Experimental Animals (CETEA for *Comitê de Ética em Experimentação Animal*; protocol Nº. 003/06), UFMG, MG, Brazil[29].

Isolation of osteoblastic cells

Osteoblastic cells were isolated from the calvaria of fetal (5 days) Lewis rat's. Rats were sacrificed after an anesthesia combination of Ketamine (10%)(60 mg/Kg of the animal) and Xylazine (2%)(7.5 mg/Kg of the animal) intraperitonally according to the CETEA regulation (protocol 079/2007). The calvarias were isolated, cut into small pieces and rinsed in sterile PBS into a falcon tube (50 mL). The calvaria pieces were treated with trypsin [type I; 1%; diluted (dil.) in PBS; 0.15 M; 10 min], followed by 4 sequential digestions with collagenase (2%; dil. in 0.15 M PBS; 20 min each), and incubated (37 ºC). The supernatant of the first digestion was discarded in order to eliminate fibroblasts, and then collagenase (2%; dil. in PBS; 0.15 M; 3 mL) was added, repeating the incubation process (30 min). The cell suspension was collected and centrifuged (Jouan CR4.12; City, ST, Country; 1400 rpm; 10 min). Cell pellet was resuspended in RPMI (Gibco City, ST, Country) + FBS (Gibco City, ST, Country; 10%), plated into tissue culture flasks (TPP City, ST, Country; T25) and allowed to grow in an incubator (5% CO_2; 95% humidity; 37 ºC). This protocol was described by Valerio et al.[21] with some modifications.

Flow Cytometry analysis

The MSC were detached from the cell culture surface by treatment with trypsin solution. They were centrifuged and approximately $5x10^5$ cells/well were ressuspended (PBS, 0.15 M, 100 µL) and analyzed by flow cytometry (FACScan; BD Immunocytometry System, City, ST, Country). Approximately 15,000 events were acquired (CellQuest software; BD Biosciences, City, ST, USA). These data were analyzed (WinMDI v2.9 software, supplier, City, ST, Country) and the population tested was determined on the basis of its size and granularity. Unstained cells also served as a negative control and are represented in the histogram graphs[30].

Fluorescence Microscopy

Cultured COB of the *EGFP* $^{-/-}$ genotype and MSC of the *EGFP* $^{-/-}$, *EGFP* $^{+/-}$, and *EGFP* $^{+/+}$ genotypes were incubated (5 min; 37 ºC) after their nuclei stained (Hoechst; 0.2 µg/mL; in PBS). Their natural

fluorescence was observed by confocal microscopy (Olympus Evolt E-300, City, ST, Country) without use of antibody.

Immunofluorescence assay for intracellular structures

Cells were rinsed with PBS (0.15 M) and fixed with paraformaldehyde (4%) or cold methanol (15 and 3 min, respectively; -20 ºC). They were rinsed again and permeabilized with Triton X-100 (Sigma, City, ST, Country; 0.1%; 3 min). Cells were rinsed once again and added block solution [Bovine Serum Albumin (BSA, City, ST, Country; 1%, w/v) and goat serum (5%, v/v) in PBS], and incubated (1 h; room temp). Cells were labeled with rabbit anti-GFP antibody (ABCA, City, ST, Country; 2 h; room temp) and then with goat anti-rabbit antibody (*Alexa fluor 555*, Molecular Probes-Invitrogen, City, ST, Country; 1 h). *Hoechst* staining was then utilized to identify the cell nuclei. This protocol was described by Leite et al.[30], Echevarria et al. apud Gomes[26], and used by Breyner et al.[31] with some modifications.

Results

PCR

Agarose gels revealed the three types of genotype (Figure 2). Comparison with the standard (2A) and blank (2B; control) columns allowed identifying the first sample (2C) of a low molecular-weight (129 pb) band with homozygous EGFP-positive cells, the second one (2D) of a higher molecular-weight (438 pb) band with homozygous EGFP-negative cells, and the last one (2E), of two (129 and 438 pb) bands, indicating a heterozygous genotype.

Flow Cytometry

As shown in figure 3, the circled area delimits fluorescence of the analyzed population into a graph of size versus granularity (A). According to this figure, the population of the (*EGFP* $^{+/+}$) genotype showed a high

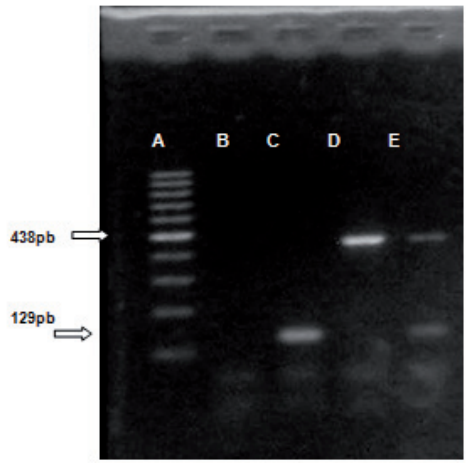

Figure 2 - Agarose gel electrophoresis of EGFP
Legend: The polymerase chain reaction (PCR) products were analyzed on agarose gel. The visible bands correspond to base pairs of amplicons with different molecular weights. Lane A: standard; lane B: blank (no contaminant from the agarose gel); lane C: homozygous positive sample; lane D: homozygous negative sample; lane E: heterozygous sample

number of cells expressing green fluorescence. The (*EGFP* $^{+/+}$) genotype showed 99.4% of cells expressing green fluorescence. On the other hand, the (*EGFP* $^{-/-}$) genotype showed only 0.86% of cells expressing the same fluorescence, and the (*EGFP* $^{+/-}$) genotype showed 3.55% a green color, same color exhibited by EGFP. Analysis of autofluorescence was performed to confirm the above results. According to this analysis, 1.18% of cells with the (*EGFP* $^{-/-}$) genotype, 2.59% of cells with the heterozygous genotype, and 0.55% of cells with the (*EGFP* $^{+/+}$) genotype emitted intense red fluorescence.

Fluorescence Microscopy

Fluorescence of MSC (Figure 4) of the (*EGFP* $^{+/+}$) genotype (A) was higher than that of the (*EGFP* $^{+/-}$) genotype (B) when exposed to UV light. In contrast, MSC of the (*EGFP* $^{+/-}$) (B) and (*EGFP* $^{-/-}$) (C) genotypes showed red autofluorescence when exposed to the radiation above, and expression of EGFP fluorescence was lower than that of MSC with the (*EGFP* $^{+/+}$)

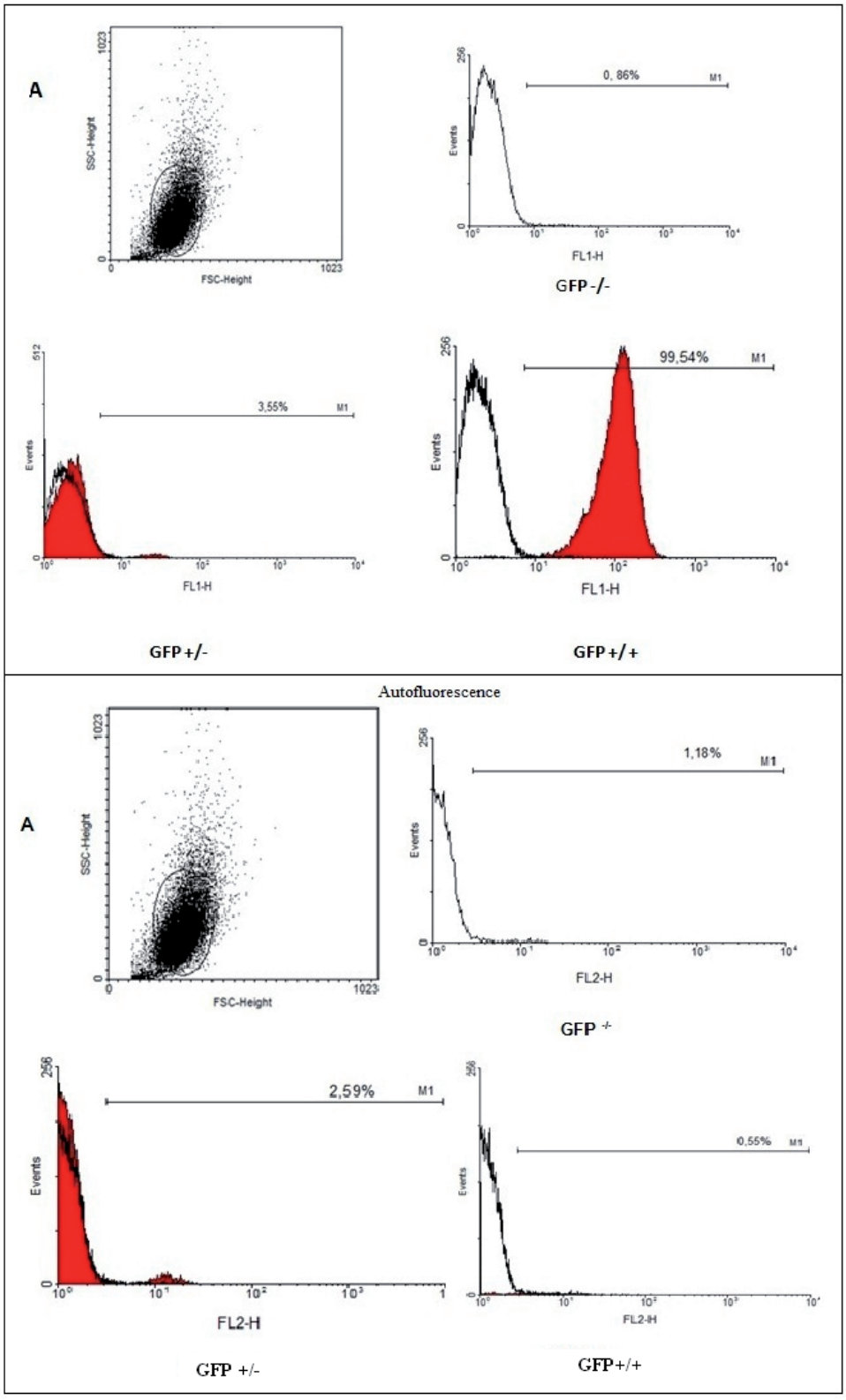

Figure 3 - Characterization of genotypes of MSC-TA and autofluorescence
Legend: Peak heights refer to different fluorescence and autofluorescence responses of samples with MSC-TA of different genotypes [(*EGFP* $^{+/+}$), (*EGFP* $^{+/-}$), and (*EGFP* $^{-/-}$)] to UV exposure

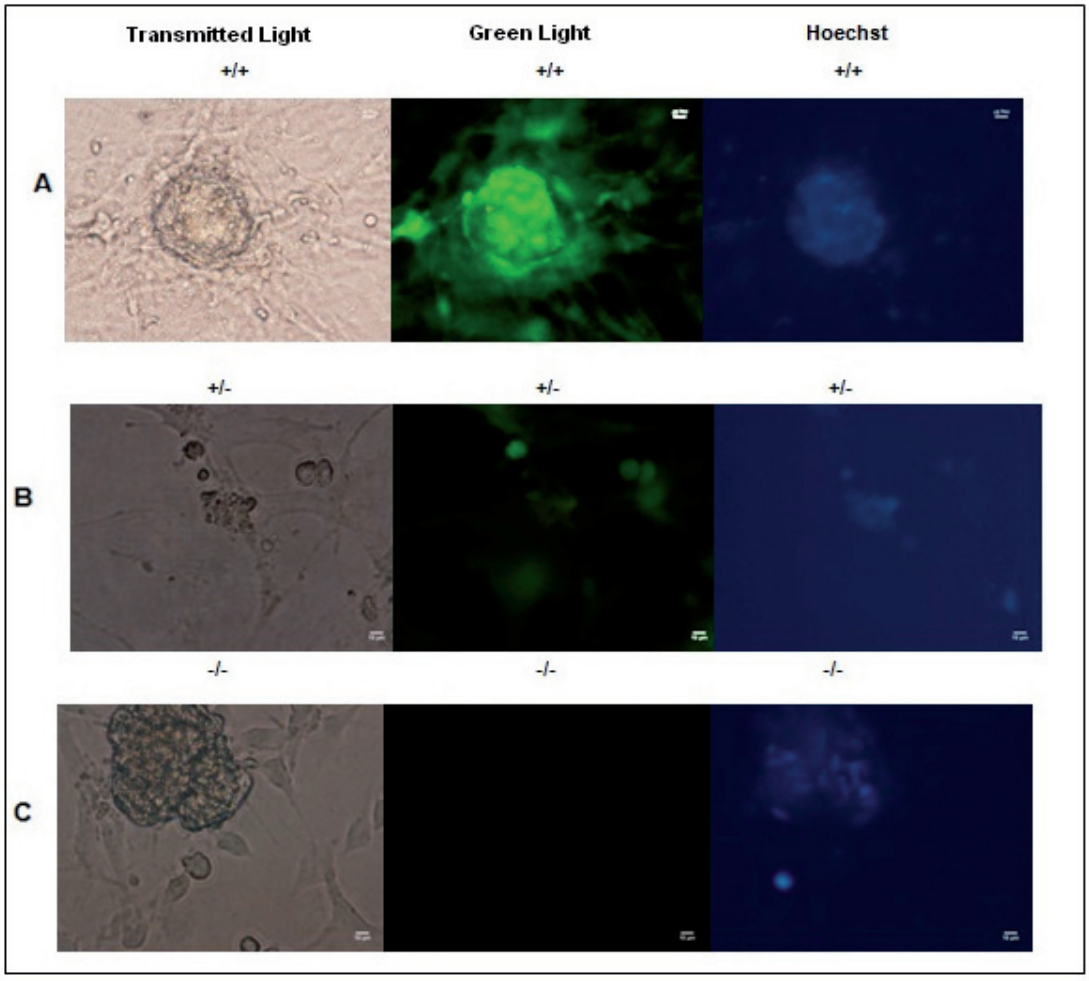

Figure 4 - MSC-TA with genotypes A: $^{+/+}$/(*EGFP* $^{+/+}$), B: $^{+/-}$/(*EGFP* $^{+/-}$), and C: $^{-/-}$/(*EGFP* $^{-/-}$). (Magnification: 30x)
Legend: MSC-TA with genotypes A: $^{+/+}$/(*EGFP* $^{+/+}$), B: $^{+/-}$/(*EGFP* $^{+/-}$), and C: $^{-/-}$/(*EGFP* $^{-/-}$). MSC-TA, which express EFGP, showing fluorescence upon exposure to UV light without antibody labeling (Magnification: 30x)

genotype (A) or inexistent. No cell was labeled with antibody for this analysis. Besides MSC, other cells, such as COB isolated from neonatal Lewis rats, can express EGFP. Since cells of the (*EGFP* $^{-/-}$) genotype do not emit fluorescence (since they do not express EGFP), their nuclei were labelled with *Hoechst* staining in order to detect their presence (Figure 5).

Fluorescence analysis of MSC by confocal microscopy

Confocal analysis was performed to verify fluorescence of cells labeled (Figure 6) and not labeled with antibody (Figures 7 and 8). Moreover, it was utilized to analyze difference between the (*EGFP* $^{+/+}$) and (*EGFP* $^{+/-}$) genotypes (08A and 08B, respectively). These figures show different fluorescence emitted by cells with different genotypes. Fluorescence due to antibody labeling exhibited by the (*EGFP* $^{+/+}$) genotype was more intense than that exhibited by the (*EGFP* $^{+/-}$) genotype.

Discussion

The animals selected for this study had the (*EGFP* $^{+/-}$) genotype and their reproductive rate was higher than that of animals with the (*EGFP* $^{+/+}$) and (*EGFP* $^{-/-}$) genotypes. Because of this difference, identifying the newborns

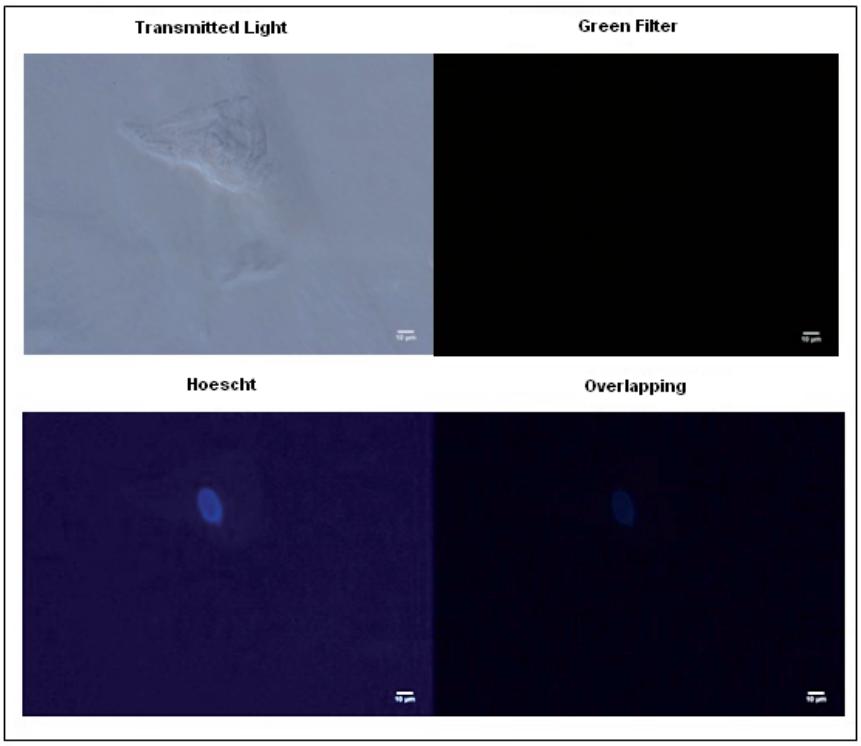

Figure 5 - Calvarial osteoblast cells (COB) of the $^{-/-}$/(*EGFP* $^{-/-}$) genotype; nucleus labeled with Hoechst (Magnification: 30x)
Legend: Calvarial osteoblast cells (COB) of the $^{-/-}$/(*EGFP* $^{-/-}$) genotype; nucleus labeled with Hoechst (Magnification: 30x)

genotype was necessary. In the present study, identification of genotypes was characterized by PCR. As shown in figure 2, the standard lane presented a 100-pb DNA ladder (Fermentas), and absence of contamination is shown in the second lane (blank control). In the third lane (DNA of Lewis rats), we can observe only one band with low molecular weight (129 bp), which features the transgenic (*EGFP* $^{+/+}$) allele and characterizes a positive homozygous allele for expression of EGFP. The fourth lane showed only one band with high molecular weight (438 pb), featuring the wild-type (*EGFP* $^{-/-}$) allele and characterizing a negative homozygous for expression of EGFP. In the last lane, we can observe two distinct bands (129 and 438 pb), which characterizes a heterozygous (*EGFP* $^{+/-}$) genotype. This technique is advantageous because the animal can be utilized in future projects in the lab. Even so, we were not able to detect any difference in the intensity of expression between cells of the (*EGFP* $^{+/+}$) and (*EGFP* $^{+/-}$) genotypes. To verify that difference, we performed the flow cytometry assay.

Cells of the (*EGFP* $^{-/-}$) genotype expressed green fluorescence (0.86%). Cells of the (*EGFP* $^{+/+}$) genotype expressed green fluorescence (99.5%), confirming that it is a good marker. Although cells of the (*EGFP* $^{+/-}$) genotype were expected to have an intermediate number expressing green fluorescence, only 3.55% of them showed this capacity. Until now, no study on the difference in fluorescence intensity could be found. However, from this study we can suggest the convenience of labeling cells of the (*EGFP* $^{+/-}$) genotype with antibody before utilizing them.

It is known that, in most species, cells (including their metabolites and structural components) exhibit natural fluorescence, also called "autofluorescence". A common question in almost

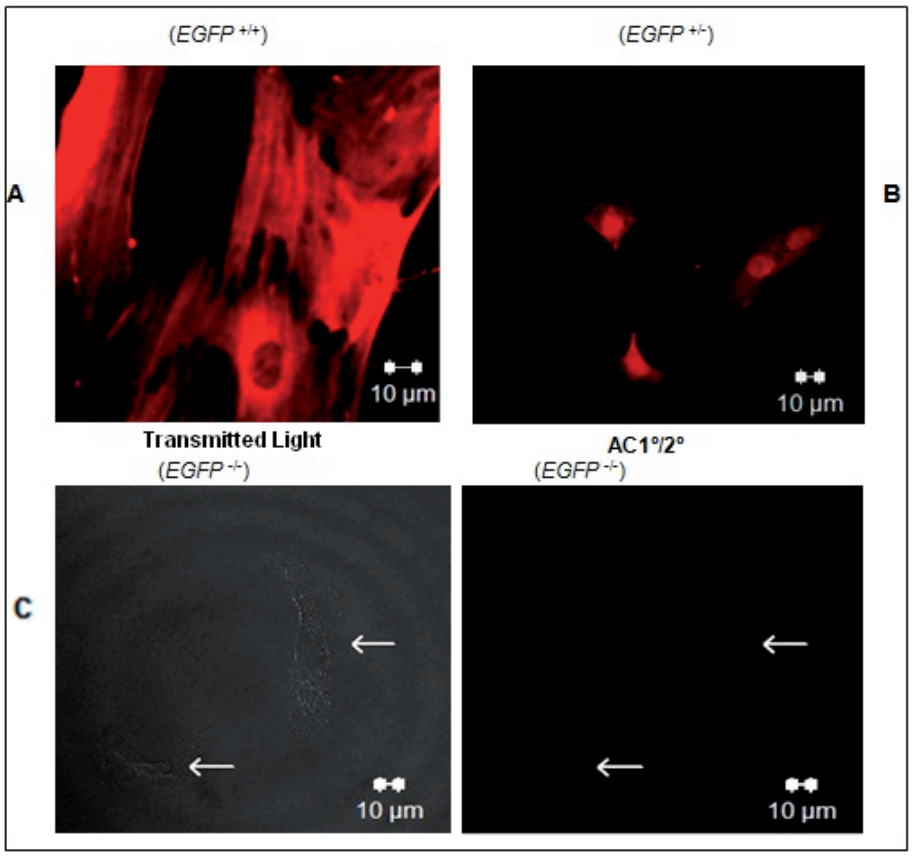

Figure 6 - MSC-TA Cells labeled with first and second antibodies\
Legend: MSC-TA Cells labeled with first and second antibodies. Cells with genotypes A: $^{+/+}$/(*EGFP* $^{+/+}$), B: $^{+/-}$/(*EGFP* $^{+/-}$), and C: $^{-/-}$/(*EGFP* $^{-/-}$) analyzed by confocal microscopy for evaluation of their fluorescence after antibody labeling

all experiments is whether the observed fluorescence is autofluorescence or a real fluorescence process. Unless GFP is very highly expressed or densely localized in the cell, it is possible that the GFP fluorescence signal is contaminated with endogenous cellular autofluorescence[32]. According to the analysis1.18% of cells that showed autofluorescence had the (*EGFP* $^{-/-}$) genotype, a higher number as compared to those that emitted green fluorescence. We can state that the observed green emission was actually autofluorescence, since this type of emission has a usually broad spectrum, which encompasses most of the emission spectra in the visible range of the electromagnetic radiation, overlapping the spectrum of EGFP and many of its derivatives[3]. Only 0.55% of cells that showed autofluorescence had the (*EGFP* $^{+/+}$) genotype, confirming their excellence in expressing EGFP. Interestingly, 2.59% of the cells with the (*EGFP* $^{+/-}$) genotype showed autofluorescence, a figure very close to that [of cells], with the same genotype, which express the EGFP. Therefore, we state again that antibody staining is necessary in the use of cells with this genotype.

A variety of cells and organisms, such as bacteria, fungi, plants, and mammals, showed a high capacity to express GFP and many of its derivatives. This capacity is due to favorable properties such as: high stability, minimum toxic effect, non-invasive detection, and their capacity to express fluorescence *in vivo* without external cofactors[2,14,32,33]. In the present study, we

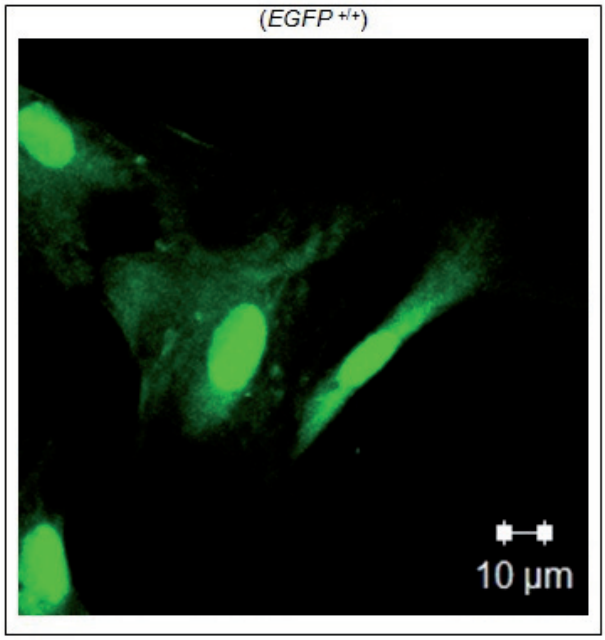

Figure 7 - Fluorescence of MSC-TA cells with the (*EGFP* $^{+/+}$) genotype, unlabeled with antibody
Legend: MSC-TA Cells of the (*EGFP* $^{+/+}$) genotype were analyzed by confocal microscopy for evaluation of their fluorescence (unlabeled with antibody)

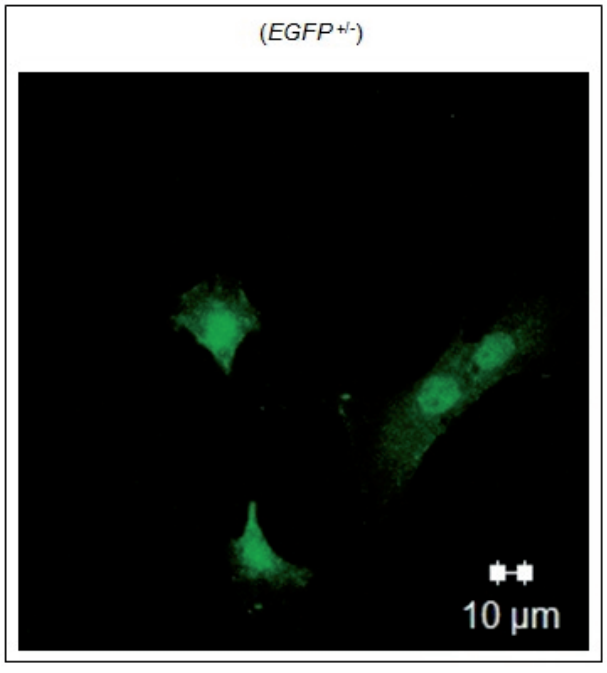

Figure 8 - Fluorescence of MSC-TA cells with the (*EGFP* $^{+/-}$) genotype, unlabeled with antibody
Legend: MSC-TA Cells of the (*EGFP* $^{+/-}$) genotype were analyzed by confocal microscopy for evaluation of their fluorescence (unlabeled with antibody)

showed that EGFP can be expressed in two cell types, MSC and COB.

Autofluorescence as well as the difference in fluorescence emission by the (*EGFP* $^{+/-}$) and (*EGFP* $^{+/+}$) genotypes were observed in MSC. The figures show that fluorescence exhibited by cells with the (*EGFP* $^{+/-}$) genotype is lower than that by cells with the (*EGFP* $^{+/+}$) genotype. Cells of both (*EGFP* $^{+/-}$) and (*EGFP* $^{+/+}$) genotypes were able to emit green light after they were exposed to UV radiation, with and without antibody staining. Although emission by cells with the (*EGFP* $^{+/+}$) genotype is more intense than that by cells with the (*EGFP* $^{+/-}$) genotype, this observation confirms the results of fluorescence microscopy and flow cytometry. Cells of the (*EGFP* $^{-/-}$) genotype were not able to emit green fluorescence[32].

Concluding Remarks

Through the PCR technique, it was identified the genotypes of the offspring of a cross between two heterozygous Lewis rats. However, no variation between the (*EGFP* $^{+/-}$) and (*EGFP* $^{+/+}$) genotypes was detected in the emission of green fluorescence. In the present study, it was possible to confirm the presence of EGFP in two cellular types, COB and MSC, of Lewis LEW-Tg (EGFP) F455.5/Rrrc rats, confirming the information given by the Rat Resource & Research Center. In the confocal microscopy and fluorescence microscopy photographs, a visual difference was observed in the intensity of EGFP fluorescence. In the analysis of flow cytometry data, a high percentage difference was observed between cells of the (*EGFP* $^{+/-}$) and (*EGFP* $^{+/+}$) genotypes that are able to express EGFP. Regarding autofluorescence of cells that are able to express EGFP, the number of those with the (*EGFP* $^{+/-}$) genotype was very close to the ones expressed in the auto-fluorescence analysis. Therefore, we recommend that cells with this genotype be utilized with antibody labeling.

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