Fetal Cells Homing in Maternal Bony Defects: A Preliminary *in vivo* Investigation

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Introduction: Fetal cells are present in maternal tissue during pregnancy as well as post-partum. Although their clinical significance is not clear, these cells can be found in injured, diseased and normal tissue. In this study, the authors sought to assess the possibility of fetal cells' homing in iatrogenic maternal jawbone defects. **Materials and Methods:** Eight wild female mice were bred with eight male mice carrying the green fluorescent protein (GFP). Two mice with the same specification were bred with non GFP male mice. A two-millimeter defect was created in pregnant mice mandibles on day 12.5 of pregnancy. The mice were euthanized 4 days later. **Results:** GFP+cells were investigated in mandibular defects using immunoflurescent, immunohistochemical staining, and quantitative polymerase chain reaction. GFP+cells were present at defect margins of four cases by all evaluations. GFP+cells were absent in normal tissues and in control mice. **Conclusion:** Fetal cells were distinguishable at the margin of iatrogenic jaw bone defect in the mice but their function remain to be elucidate.

Keywords: Bone Defect; Fetal Cell; Microchimerism; Green Fluorescent Protein; Tissue Repair; Bone Regeneration

Introduction

Stem cell recruitment is a crucial step in bone healing process and decreased mesenchymal stem cell (MSC) pool can significantly correlate with the failure of bone regeneration (1). In this regard, homing of MSCs and their mobilization are essential for bone formation (2). MSCs are attracted from bone marrow, around tissue, and circulating blood (3). Various mechanisms have been reported to explain the homing of stem cells in the defect sites (4). In "natural tissue engineering" fetal cells showed a precisely homing mechanism that is not clear yet (5, 6).

Fetal cells represented a remarkable capacity to migrate through the placenta and homing in maternal tissues especially in injured tissues and site of disease (7, 8). Fetal cells can be found in maternal tissue even long after pregnancy (9). However, the clinical significance of fetal cells (microchimeric cells) has not been ascertained yet. It has been shown that fetal cells act like stem cells and they play a significant role in natural tissue regeneration (5, 10, 11). Khosrotehrani and Bianchi hypothesized that fetal microchimeric stem cells may relate to damaged maternal tissues as part of tissue repair response (12). Tissue injury can induce fetal cells in liver, kidneys, heart and brain of maternal mice (13-16). The role of fetal cells in "natural tissue engineering", however, has not been fully understood (5, 6). Cultured fetal cells harvested from maternal bone marrow have been shown the differentiation towards different cell lineages (17, 18). It has been assumed that the diversity of their phenotypes and their presence within the damaged tissue may aid the tissue healing process (19-21).

Knowledge of the migrated fetal cell type and molecular mechanisms that permit for the migration, homing, and multilineage differentiation potential of these cells can improve our whole strategies for homing in bone regeneration and stem cell-based therapies of bone defects. Moreover, this knowledge can enhance the prospects for minimally invasive delivery of stem cells for cytotherapeutic repair of maternal tissues. In the current study, we hypothesized a smart homing of fetal cells in bony defects, and we aimed to assess the presence of fetal cells in iatrogenic maternal mandibular bone defects.

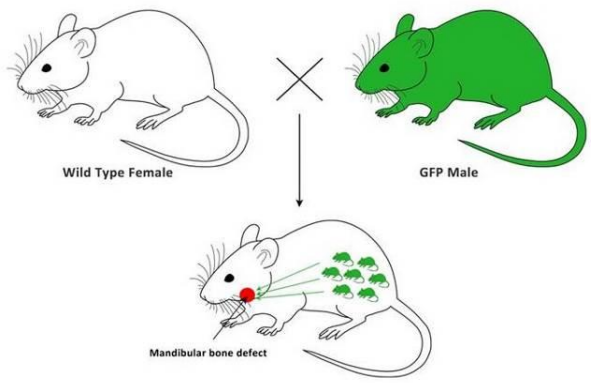

Figure 1. Study design

Materials and Methods

Animals

This study was approved by The Institutional Animal Care and Use Committee of the Shahid Beheshti University of Medical Sciences. To investigate the fetal-maternal microchimerism, 10 wild type virgin C57Bl/6 female mice (6 to 8 weeks-old, Pastor Institute, Tehran, Iran) were bred with 8 congenic male mice (6 to 8 weeks-old, originally generated by Osaka University, Japan) carrying an eGFP gene as test group and 2 wild type as control group. The homozygous transgenic mice (in C57Bl/6 background) carried an eGFP gene, which was under the control of a chicken beta-actin promoter and cytomegalovirus enhancer.

Surgical injury

On the 12.5 day of pregnancy of female C57Bl/6 mice (n=8), they were anaesthetized using ketamine (80 mg/Kg); then access to mandibular body was obtained via a delicate mucosal incision and subperiosteal flap elevation. Surgical round burs (Meisinger, Neuss, Germany) at a speed of 20,000 (rev/min) were used under sufficient irrigation of normal saline to create a round bone defect in the lower jaw. The incision was closed with a running suture (5–0 of Vicryl, Ethicon Inc., Somerville, NJ, USA). The same procedure was performed on two virgin female C57BL/6J mice as negative controls.

Tissue collection

Mice were euthanized by chloroform inhalation 4 days after surgery. One GFP+male mouse was also involved in the study as a positive control. Their mandible bones were separated from the surrounding soft tissues and fixed in 10% formaldehyde.

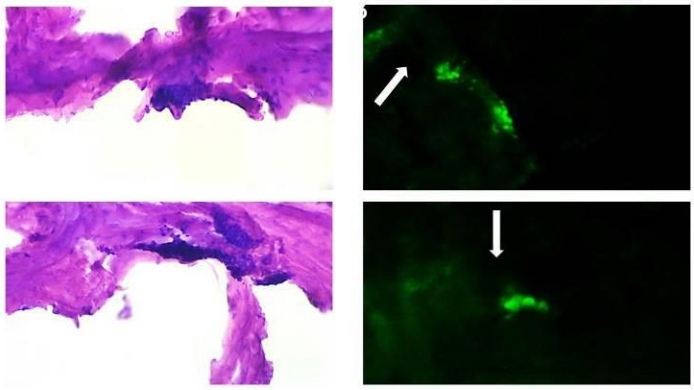

Figure 2. *A*) Histological evaluation of a maternal jaw defect shows no necrosis (H&E staining) (40×); *B*) Immunofluorescence staining of GFP+cell in the jaw bone (White arrows shows the positive cells)

Histology

The specimens were decalcified in 10% nitric acid and embedded in paraffin. Thin 5-micrometer sections of bone specimens were stained with hematoxylin–eosin to detect fibrosis. Sections were evaluated histologically, focusing on general structure, amount of inflammation and presence of steatosis or necrosis.

Immunohistochemistry and immunofluresecent staining

For immunohistochemistry, Anti-GFP rabbit polyclonal antibody (Abcam, Cambridge, MA, USA) was used on decalcified sections to detect fetal GFP+ cells. A HRP-conjugated goat anti-rabbit secondary antibody (Abcam, Cambridge, MA, USA) for DAB labeling according to standard protocol 21 was used as a secondary antibody. The sections were observed using a light microscope (Nikon, Tokyo, Japan). For immunofluresecent staining, anti-GFP mouse monoclonal antibody [LGB-1] (FITC) (Abcam, Cambridge, MA, USA) was used as a primary antibody and conjugated anti-mouse IgG antibody (Abcam, Cambridge, MA, USA) were used as a secondary antibody.

Quantitative real time-PCR (qRT-PCR) analysis

Three fresh frozen of mandibular bone specimens from each group was stored at -70°C until upcoming assessments. The specimens were air died at room temperature and immediately dipped in liquid nitrogen until reached a chilly -195 °C, then transferred into an RNAs free crucible in order to make bone powder. Total RNA were extracted, using RNeasy Micro Kit (QIAGEN, Valencia, CA). Then, extracted RNA were evaluated by NanoDrop 2000c Spectrophotometer (Thermo Scientific NanoDrop Products, Wilmington, Delaware, USA).

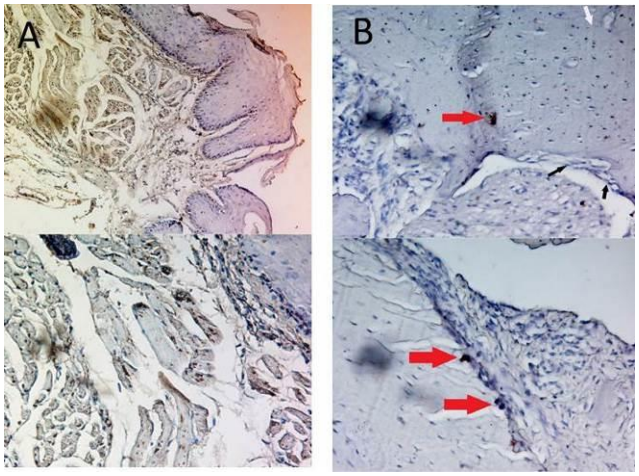

Figure 3. A) GFP+ cells (brown) detected by DAB labeling throughout the jawbone in GPF+male mouse (positive control) (40×), presence of few cells in maternal jawbone defect by DAB labeling: B) Red arrows show fetal GFP+cell (100×), blach arrow pointed to the boundary of defect, whited arrow showed mice jawbone

Then, cDNA was created from 2 mg of total RNA via Superscript II reverse transcriptase (Invitrogen). Real-time PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems, Warrington, Cheshire, United Kingdom) according to manufacturer's guideline. Reaction conditions comprised 40 denaturation cycles (15 seconds) at 95°C following one minute of amplification at 60 °C. Cycle threshold (Ct) determined by Applied Biosystems 7500 Real-Time PCR System (Thermo Fisher scientific, USA). All reactions were performed in triplicate, and the expressions were normalized to that of the housekeeping gene beta actin. Assessed amplification curves for the reactions were represented by light cycler (Roche Molecular Biochemicals LightCycler Software®, Version 3.5). In this study we utilized the ensuing primers:
GFP-forward 5' -CATCGAGCTGAAGGGCATC-3',
GFP-reverse 5' -TGTTGTGGCGGATCTTGAAG-3'.

Results

The study design is shown in Figure 1. Seven pregnancies occurred in test and 2 in control groups and each resulted in production of 9-14 GFP+fetuses. All maternal mice were generally healthy and no clinical sign of infection was observed. A small number of inflammatory cells in loose connective tissue, without necrosis or steatosis were detected (Figure 2A). Immunoflurescent staining detected highly concentrated GFP+cells in the defect site (Figure 2B).

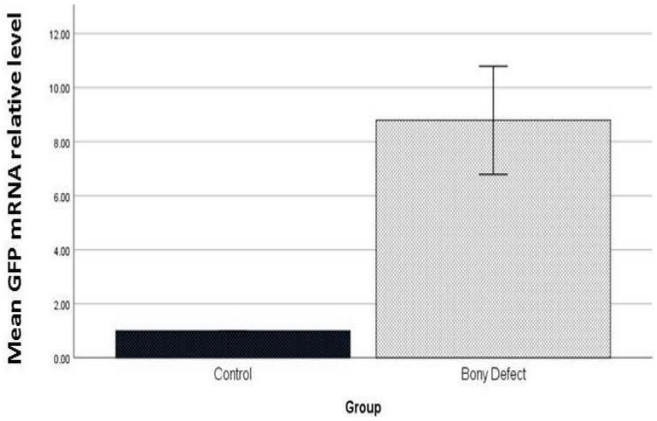

Figure 4. Green fluorescent protein's mRNA relative expression in the maternal jaw defect shows significant difference compared to control (* $P<0.05$ by independent t-test)

Immunohistochemical staining (IHC) showed that the fetal cells were present in 4 out of 7 maternal bone defects. Figure 3 shows the presence of a few isolated GFP+ cells in the jawbone. These cells were present at the defect margins and they were mononuclear. In contrast, the bone next to the injury site showed no evidence of GFP+cells. Also, in bone from negative control mice, no GFP-positive cells were detected. The GFP+cells were present throughout the tissues of the male mouse (positive control) showing the relative reliability of IHC for detecting GFP in bone (Figure 3B). Similarly, *qRT-PCR* also confirmed the presence of GFP+cells in maternal bone defects (Figure 4).

Discussion

Bone tissue has an immense regenerative capacity; however, healing of critical sized bone defects and non-union fractures is still a big challenge for scientists. Bone tissue engineering strategies for stem cell therapies have shown promising findings for bone regeneration (22-24), but these approaches are time and cost consuming and require special regulatory consideration (4). This has motivated the advancement of strategies to induce native stem cells for bone regeneration. In this regard, several lines of evidence support fetal cells as stem cells which responsible for micro chimerism. These cells showed a great ability to home in a maternal host organ or defect site during pregnancy (9) and even decades after pregnancy (10). They also have a multi-lineage capacity and stem cell like properties (10). Previous studies reported their high homing capacity and their involvement in skin wound healing and myocardium defects (25-27). To the best of authors' knowledge, the presence of maternal fetal cells in bone

tissue never investigated before. The presence of fetal cells in maternal tissues is a well-known phenomenon. The current study evaluated the presence of fetal GFP cells in iatrogenic jawbone defects of maternal mice. The fetal cells were present in maternal mandibular bone defects, while fetal cells were not observed in the normal mandibular bone as well as control group. When an injury occurs, progenitor cells migrate towards the defect and differentiate into mature cells and participate in tissue regeneration (28). However; their function in remained to be elucidated.

Since, previous studies have been shown the presence of fetal cells maternal circulation on day 10th post-impregnation (29) and increase as delivery nears (day 18th) (30). In current study, mandibular bone defect were created on the 12.5 day of pregnancy. Furthermore, by combining three detection methods, fluorescent microscopy, immunohistochemistry, and quantitative RT-PCR, we decreased the possibility of false positive detection in this study.

Fetal cells have been shown to pass the placenta and settle in maternal bone marrow (31, 32). Male cells have been detected in normal rib sections of women impregnated with males throughout life (31). In our experiment, however, no fetal cell was found in healthy mandibular bone. Cortical density of the mandible may be considered an inhibitory factor for fetal cells migration, whilst the surgical trauma to the mandible triggered fetal cells to migrate to the defect site.

Low amounts of detected GFP+cells (4 out of 7) in histological specimens may be due to heterogeneous distribution of fetal cells throughout the defect. Other studies also revealed that fetal cells were only detectable in some of the mothers during pregnancy or post-partum (33, 34).

The fetal GFP+cells at maternal bone defect were mononuclear. These cells were not perivascular and not like polymorphonuclear hematopoietic cells. Moreover, these cells were found along the margins of the lesion in row with other well-polarized cells. Considering the morphology and position of GFP+ cells in maternal bony defects, it is possible that these cells may have differentiated into osteoblasts and participated in regeneration of the defect by providing a reservoir of stem cells that can have effective role in bone regeneration (35-40).

The existence of fetal cells in maternal bone defects may change the interpretation of the cause, progression, and treatment of the bony lesions during pregnancy or maternal life. The effectiveness of fetal cells in improving bone healing may provide new insight in reconstruction and warrants further study.

Conclusion

The results revealed that fetal cells migrate through placenta and lodge in the margins of iatrogenic defects in the body of the lower jaw in pregnant C57Bl/6 mice. No GFP+ fetal cells were detected in normal mandibles and control groups. However, the type of fetal cells as well as their function is still unclear. Our findings open a new perspective in the future of the translational medicine of bone engineering that can stimulate fetal cell homing as osteoprogenitor cells in order to facilitate treatment of maternal large bony defects.

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Conflict of Interest: 'None declared'.

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