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Jakob T. Samsel
University of Tennessee, Knoxville, jsamsel@vols.utk.edu

Madhu Dhar
University of Tennessee - Knoxville, mdhar@utk.edu

Austin Bow
University of Tennessee, Knoxville, abow@vols.utk.edu

Tom Masi
University of Tennessee Medical Center

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Expansion, characterization, differentiation, and visualization of MC 3T3-E1 preosteoblast cells: an *in vitro* model to study bone healing and stem cell-mediated regeneration

Jakob Samsel[1]; Austin Bow, BS[2]; Tom Masi, PhD[3]; Madhu Dhar, PhD[2]

[1]University of Tennessee Biological Sciences; [2]University of Tennessee College of Veterinary Medicine; [3]University of Tennessee Medical Center

Abstract

By their nature, stem cells present a strong potential in regenerative medicine. Recent medical research is noticing this potential as stem cell therapies are becoming more and more commonplace. With new therapies being proposed and developed, questions are raised about the specific factors that play significant roles in affecting the biology of these cells. The research focus of our group is bone tissue regeneration, and we use the mouse MC 3T3-E1 preosteoblast cell line as a model to study changes during bone cell differentiation. These cells are studied to establish molecular and cellular assays to assess bone cell proliferation, differentiation, and visualization. The results from these cells will then be translated to adult stem cells undergoing bone formation. Cell proliferation assays were validated to obtain the optimal seeding density and optimal fetal bovine serum contained in the medium for growth and expansion of MC 3T3-E1 cells. Optimal concentrations of the biological factors dexamethasone, ascorbic acid, and betaglycerophosphate required for efficient bone cell differentiation have also been optimized. The differentiation process was evaluated microscopically and confirmed by a calcium-specific staining. Undifferentiated cells were used as controls. For clinical application, we did a dose curve to assess the concentration of tobramycin, an antibiotic that is administered subsequent to the addition of stem cells to damaged joints. Cells showed surprising tolerance to tobramycin between 0 to 360 μg/mL. The proliferation of cells suffered significantly at doses greater than 360 μg/mL. Further experiments are currently in progress to study and visualize MC 3T3-E1 cells. These include the development of a DNA plasmid to visualize bone cell differentiation consisting of a green fluorescent protein under the control of the osteocalcin promoter with a constitutively active m-cherry protein. Additionally, we are studying stem cell behavior on a novel polymer-hydroxyapatite – based biocomposite material with a long term goal of using it in bone defect regeneration.

Introduction

The discovery of stem cells has opened the door to many new therapies that were impossible a few decades ago. In developing zygotes and fetuses, stem cells are the first cells that mature into the many tissues of the body. In adults, there are stockpiles of stem cells that serve to replenish These cells throughout the life of the organism. Stem cells are often quick to divide and expand, and they have the ability to become many different cells (multipotency). By utilizing this natural process of cell replacement, stem cell therapies produce new tissues for a variety of maladies [1]. Stem

cells from a different individual can be given as an allograft transplant to provide some advantage that the patient's own tissues do not afford [2]. Alternatively, a patient's own stem cells (autograft) can be modified and replaced to produce therapeutic effects such as specific gene knockouts [3]. Finally, though not exhaustively, stem cells can be removed and expanded from an individual and then returned in a new location or in large number to provide a benefit to the patient [4].

Stem cells can generally be divided into four categories: embryonic stem cells, fetal stem cells, induced pluripotent stem cells (iPS), and somatic/adult stem cells. Embryonic and fetal stem cells are derived from developing organisms. While they represent many possible directions in medicine, these sources of stem cells carry the risk of hyperproliferation and cancer formation, and their collection in humans is often politicized and ethically debated. iPS cells are produced by treatment of lineage-restricted cells with transcription factors that result in pluripotency [1]. This is another promising route for stem cell therapy, but degrees of uncertainty in the methods for iPS creation and iPS biology represent challenges to their use in humans [5]. Considering the issues with the above methods, adult-derived somatic stem cells remain the best candidate for most stem cell treatments. Mesenchymal Stem Cells (MSC) are somatic stem cells that have the capacity to differentiate into tissues of the blood, cartilage, bone, and more. They can be isolated from bone marrow and fat tissues and serve as reasonably safe, reliable sources of stem cells [1].

The capability of MSCs to differentiate into the cells of the skeletal system make them ideal for use in bone tissue regeneration. For most bone defects, such as fractures, bone regenerates naturally. For larger defects, such as surgical removal of a large bone cyst, a bone graft may be needed to aid in healing. Historically, bone grafts have been taken from other parts of the body and shaped for the new defect. This method, autograft bone transplantation, has many drawbacks such as risk of donor and recipient site complications and inexact fit of the graft [6].

By using stem cell therapy to add MSCs to bone defect sites, the Dhar lab at the University of Tennessee, Knoxville College of Veterinary Medicine works to develop treatments that circumvent the risks associated with autograft bone tissue transplant. Further, the characteristic potential of stem cells makes them ideal for use in more novel therapies, which our lab endeavors to discover and expand upon.

The ultimate goal for our research is to develop medical procedures in humans. The road to human trials, however, is understandably long and difficult. Before this is a possibility, treatments must be explored in small and large animal models such as mice and goats. Our lab has shown promising success of several therapies in animal models, and we have begun to use these techniques on large animal veterinary patients [7] [8].

MC 3T3-E1 is a cell line of mouse (*Mus musculus*) calvaria preosteoblast cells. Commonly used for studies concerning bone differentiation and development, the textbook *Principles of Bone Biology* refers to them as "one of the most convenient and physiologically relevant systems for study of transcriptional control in calvarial osteoblasts." MC 3T3-E1 is a spontaneously-immortalized cell line that behaves as immature, committed osteoblast cells. The cell phenotype is very stable so long as stocks are rigorously maintained [9].

Science uses models to reliably simulate a system that would be too time-consuming, difficult, expensive, or otherwise inappropriate to test as a whole. Similarly, it would be inappropriate to test an entirely novel treatment method on a group of lab animals. Instead, treatments and new questions should first be explored *in vitro* with a suitable line of cells as a model. That starting point is the goal of this project – the establishment of an *in vitro* model for stem cell-mediated bone regeneration in our lab. Here, we use MC 3T3-E1 mouse preosteoblast cells towards this end. The cells are characterized to confirm and explore ideal conditions; they are studied to establish factors and time points in the process of differentiation; and they are used in ongoing projects to visualize them *in vitro* and test new therapeutic materials.

Characterization

Establishment

One million cryopreserved MC 3T3-E1 cells were purchased from American Type Culture Collection (ATCC) in August 2015. After returning the cells to liquid nitrogen for one month, we removed and quickly thawed them in a 37°C water bath. The thawed cells were added to 10mL of media, placed in a 75 cubic centimeter cell culture flask (T75), and incubated at 37°C with 5% CO_2. We changed the cell media every 48 to 72 hours.

Passaging

Once the cells reached confluency, we washed the cells with Hank's Balanced Salt Solution (HBSS), removed cells from the flask surface with with 5mL of 0.25% trypsin incubated at 37°C for two minutes, and then inactivated the trypsin by adding 5mL of media. Next, we pipetted the resulting 10mL into a 50 mL conical tube and centrifuged it at 3000 RPM for 10 minutes. We found that cells have a tendency to form aggregates after centrifugation, especially when centrifuged in smaller 15 mL tubes. The supernatant was removed following centrifugation, the pellet was disturbed and suspended in 2 mL of media, and the suspension was chilled on ice for 10 minutes. If cell aggregates persisted after thorough mixing, they were removed by micropipette.

Counting

We diluted cells 1:20 with trypan blue (20 μL cell suspension in 380 μL trypan blue) and counted by adding 10 μL of the dilution to a hemocytometer and counted 5 of the 1mm^2 grids which held 100 nL each. We calculated total cells by the formula

$$count * 20 * \frac{1}{5} * \frac{1}{100} * 10^6 = \frac{total\ cells}{mL}$$

Growing cells

The primary media used to grow cells was alpha Minimum Essential Medium (αMEM) with 10% US qualified, heat inactivated fetal bovine serum (FBS) and 1% penicillin/streptomycin antibiotic mixture (pen/strep; 10,000units/mL pen; 10,000 μg/mL strep). Media was stored at 4°C and warmed before addition to cells. Cells did not grow well when seeded at low density (below 5000 cells/cm^2) which suggests a dependence on cell-cell interactions for normal proliferation. Healthy cells maintained a relatively round morphology (**Figure 1.**) with few instances of cell death. The MC 3T3-E1 cells proved to grow very quickly. 300,000 cells plated in a 75mm flask reach numbers of 2.3 million (stdev 250,000) cells after incubation for 5 days.

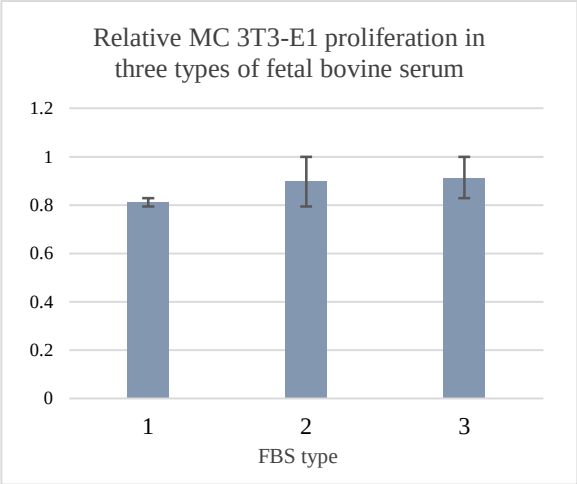

Figure 1. The cell counts from each replicate were divided by that replicate's maximum count to show relative proliferation. Cells were incubated for 5 days in αMEM with pen/strep and three different types of FBS. **1**. US qualified, heat inactivated FBS **2**. Non US qualified, heat inactivated FBS **3**. Non US qualified, Non heat inactivated FBS.

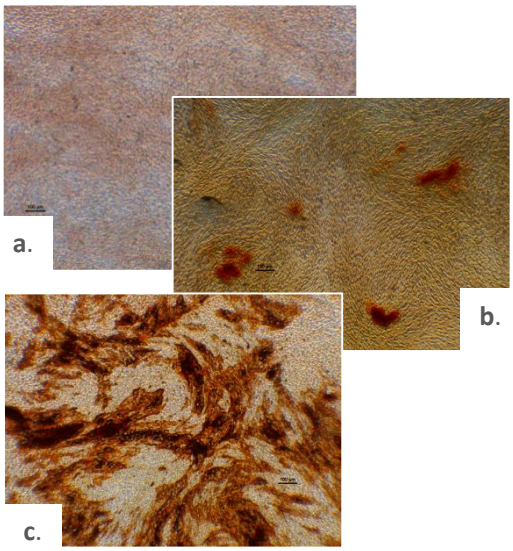

Figure 2. Images of cells incubated with and without differentiation factors after staining with Alizarin Red. **2a.** undifferentiated cells after 20 days of incubation in regular media. **2b.** cells after 14 days of incubation with differentiation factors. **2c.** Cells after 20 days of incubation with differentiation factors.

Serum Test

We wanted to know if the type of FBS used has an effect on MC 3T3-E1 proliferation. We tested three types of serum with αMEM and pen/strep:

1. US qualified, heat inactivated FBS
2. Non US qualified, heat inactivated FBS
3. Non US qualified, non heat inactivated FBS

We found slight variation in the relative proliferation, but we show no evidence to support preferential use of US qualified, heat inactivated FBS (#1), the most labor-intensive and expensive option. Consequently, we suggest the use of non US qualified, non heat inactivated FBS (#3) as it is the most cost effective (**figure 2.**).

Differentiation

Stem cell therapy of the skeletal system largely has the goal of using stem cells to regenerate healthy bone tissue in the patient. This differentiation process would happen naturally in the body by diffusion of factors that lead to differentiation into bone cells. To study this process and see how treatments affect bone differentiation, our lab must reliably simulate this development *in vitro*. We sought to determine a set of factors and a base timeline for bone formation in these cells.

Growth

Citing the work of Kanzaki et al., we found that the following concentrations of factors leads to ossification of MC 3T3-E1 cells [10]:

- 50 μg/mL ascorbic acid
- 10 mmol/L β-glycerophosphate
- 10 nmol/L dexamethasone

We tested for bone differentiation by growing MC 3T3-E1 cells in 6-well plates at initial density of 20,790 cells/cm^2. The 6-well, 9.62 cm^2 well plates were purchased from Thermofisher. Every 36 to 48 hours, half of the wells were fed differentiation media with the above concentrations of factors, and half were kept as controls and grown in normal media.

Staining

We fixed the cells for staining at time points between 7 and 24 days. For washing, fixing, and staining, approximately 1.5mL of each reagent was added by plastic pipette. We washed the cells twice with HBSS and then fixed them with 4% paraformaldehyde for 10 minutes at room temperature. Following this, we washed two additional times with HBSS and then added Alizarin Red stain. After cells were stained for 30 minutes, we removed Alizarin Red, washed twice with HBSS, and allowed the stained plates to dry. As per the manufacturer's directions, the Alizarin Red solution was prepared by adding 1.7 g of Alizarin Red to 100 mL of DI H_2O. The pH was then adjusted to 4.1 to 4.3 with ammonium hydroxide. The final volume was brought to 50 mL. Alizarin Red is a calcium specific stain. Adding it to cells that are becoming bone tissue allows us to visualize calcium deposition and infer ossification.

Results

After incubation in the aforementioned factors, we show that the MC 3T3-E1 cells begin the process of bone differentiation. By the 7^{th} day of growth in differentiation media, cell morphology noticeably changes, and the cell monolayer tends to develop into a swirling pattern compared to undifferentiated controls. Upon fixing and staining, we found that cells grown in the 6-well plates begin depositing calcium after approximately 14 days of incubation in the differentiation factors (**Figure 2b**.). By the 16^{th} day, the amount of calcium deposits significantly increases. By 20 days, the Alizarin Red calcium staining is very heavy (**Figure 2c**.). We found that specific culture conditions, such as initial density or culture surface, had a large impact on the timeline of cell differentiation. For example, cells grown at 7,074 cells/cm^2 in 60 mm, biolite individual tissue culture dishes purchased from Thermofisher took significantly longer to deposit calcium. (21 days compared to 14 days). For the sake of this experiment, results are shown from 6-well plates.

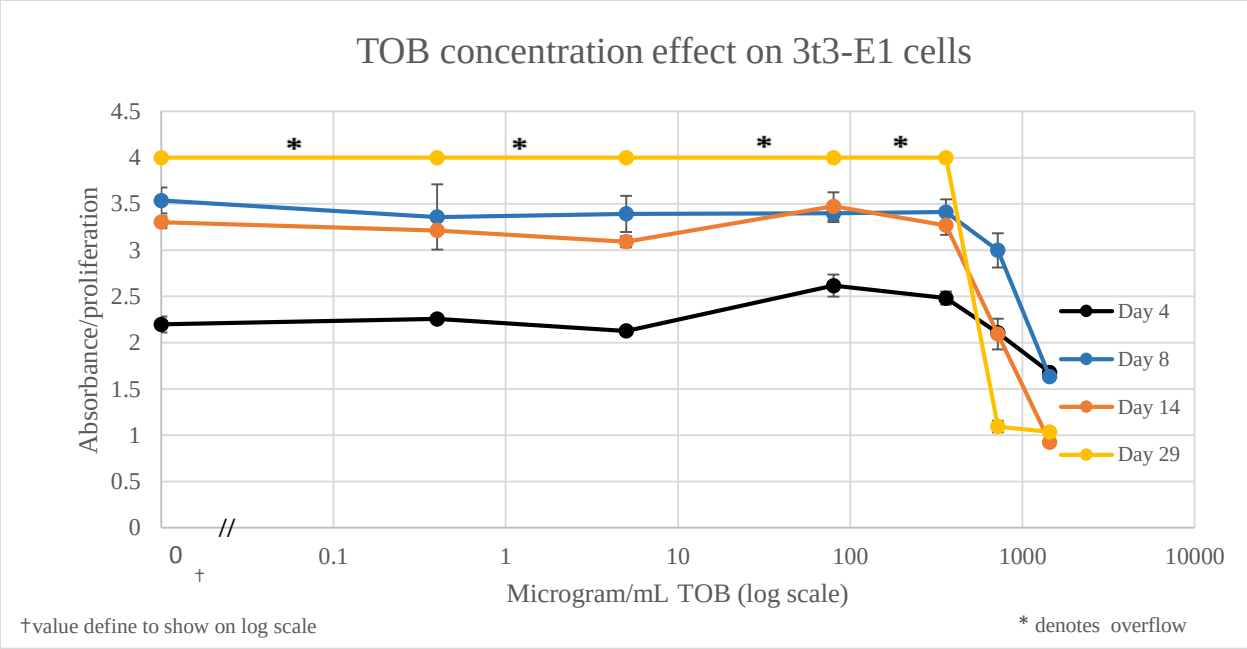

Figure 3. MTS assay results from cells grown in different concentrations of tobramycin. Tobramycin is toxic to cells above 360 μg/mL which is well above expected *in vivo* level; this suggests low toxicity to MC3T3-E1 cells.

Tobramycin dose curve

When surgeries are performed on animal models or in veterinary medicine for stem cell transplantation, these patients are often given antibiotics post operation to prevent infection. One of the most commonly used antibiotics for this end is Tobramycin, an aminoglycoside antibiotic. Tobramycin inhibits protein synthesis in gram-negative bacteria by irreversibly binding to the 30S ribosomal subunit [11]. As part of our goal to establish MC 3T3-E1 cells as a dependable model in our lab, we wanted to ensure that the drug to be used during subsequent *in vivo* models does not adversely affect MC3T3-E1 proliferation.

Methods

We seeded 96-well plates with 2200 cells/well at a density of 6,832 cells/cm^2. We fed the cells media with αMEM and 10% FBS, but without antibiotic to avoid error caused by drug interaction such as degradation of tobramycin by penicillin [12]. Recognizing the risk of growing cells without antibiotics, extra care was taken to avoid bacterial contamination. We waited 24 hours to ensure that cells attached before adding TOB; at that point, cells were given TOB at concentrations of 0, 0.4, 5, 80, 360, 720, and 1440 μg/mL. We quantified proliferation by MTS assay, a colorimetric method where MTS tetrazolium compound is reduced by NAD(P)H-dependent dehydrogenase enzymes in metabolically-active cells. This results in a change in absorbance at 490 nm corresponding to the

cell count [13]. We took readings in triplicate on days 4, 8, 14, and 28.

Results

Cells grown between 0 and 360 μg/mL of TOB showed no diminished proliferation. At concentrations of 720 and 1440 μg/mL, MC3T3-E1 proliferation suffered significantly. Even at the highest tested concentrations, the cells were less affected by TOB at earlier time points but greatly affected at later points (**Figure 3.**).

In vivo levels of TOB would be unlikely to exceed 10 μg/mL [14]. Even if an injection of TOB were given directly or near to the site of stem cell transplant, our data show that short-term exposure to TOB presents less toxicity compared to long-term exposure. These findings suggest little risk associated with *in vivo* use of TOB with MC3T3-E1 cells.

Future directions

Visualization

Working with Dr. Tom Masi at the University of Tennessee Medical Center, we are developing a DNA vector to allow visualization of bone cell differentiation in real time without fixing the cells i.e. without having to stop the experiment. The completed DNA vector will have two fluorescent protein genes, an m-cherry gene (Excitation wavelength: 587 nm, Emission: 610 nm) and a green fluorescent protein gene (GFP) (Excitation: 395 nm, Emission: 509 nm). The m-cherry gene will be constitutively on so that undifferentiated cells fluoresce red. The GFP gene will be under the control of the osteocalcin promoter. Osteocalcin is a protein synthesized by osteoblasts and is

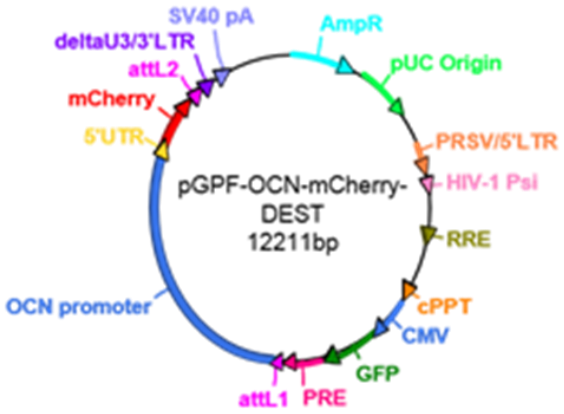

Figure 4. DNA vector makeup for 3T3-E1 bone differentiation visualization. As transfected cells differentiate, the fluorescence should change from red to green.

associated with bone mineralization [15]. **Figure 4**. Shows the DNA vector makeup. When the 3T3-E1 cells begin functioning as osteoblasts, the cell fluorescence should change. This will allow us to visualize the process of bone differentiation in living cells.

We have optimized a procedure for the transfection of a positive control plasmid with a constitutively active m-cherry gene (**Figure 5.**). We used polyethylenimine (PEI) as a transfection reagent. We experimented with different ratios of DNA to PEI (1:1, 1:2, 1:3) and found that the 1:3 ratio provides the best degree of transfection. Additionally, we tested to see the ideal time of incubation with DNA and PEI (2 hours, 6 hours, 24 hours), and found 6 hours to be the most successful. To transfect cells in 24 well plates seeded at 25,000 cells/ cm^2, we changed all cell media to antibiotic-free. For 1:3 ratio, we added 24uL of 1mg/mL PEI solution to 50 uL αMEM to vial 1.

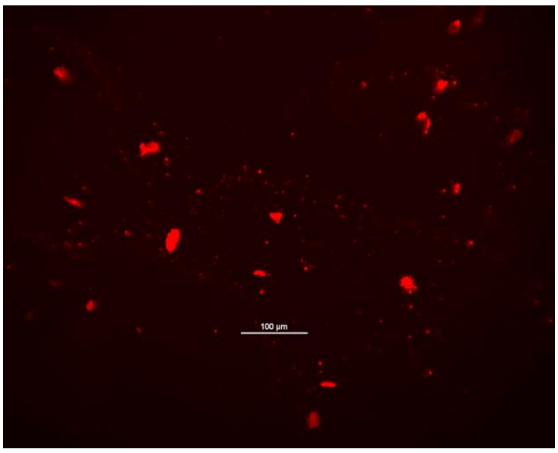

Figure 5. Preliminary experiments show successful transfection of the positive control plasmid with only m-cherry using PEI

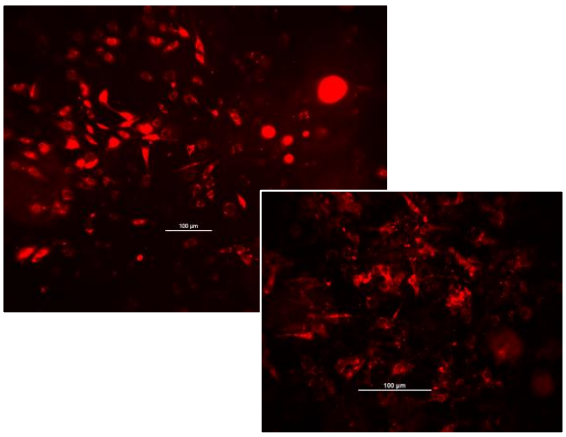

Figure 6. Cytoplasmic staining of MC 3T3-E1 cells grown on the polymer-hydroxyapatite biomaterial.

In vial 2, we added 8uL of 1ug/mL DNA to 50 uL αMEM. We then combined vials 1 and 2 and allowed them to rest at RT for 10 minutes. 33 uL of this mixture was added to each well and removed after 6 hours.

Biomaterial

Our lab has begun testing novel treatments using 3T3-E1 cells as a model. With the Nanotechnology Center at University of Arkansas at Little Rock, we are testing the potential use of a polymer-hydroxyapatite biomaterial for use in stem cell-mediated bone regeneration. In this collaboration, our labs hope to develop a material that can be transplanted into bone defects along with mesenchymal stem cells. Ideally, the biomaterial would provide a slow-degrading scaffold onto which new bone could grow. By adding stem cells to the material, this process could be expedited for quicker and better healing.

We are assessing the cytotoxicity of the material, and we are determining the ideal ratios of polymer and hydroxyapatite for ideal cell infiltration (**Figure 6**.).

Conclusion

Stem cell therapies continue to revolutionize many fields of medicine. To build that momentum, research must continue to be done into this promising field. Central to any biomedical research is the use of models to simulate how a particular therapy of interest behaves *in vivo*. A model should be relatively simple and well-characterized. By having well-defined models, one is able to answer important questions without risking resources or lives in later trials.

The research into many details concerning MC 3T3-E1 cells has given that crucial definition to the Dhar lab. We have shown the characteristics and ideal conditions of these cells; we have shown inductive factors and timelines for their differentiation into bone tissues; we have shown how they respond to drugs that will be used in subsequent *in vivo* trials; and we are using these findings to discover new ways of visualizing them and of using them in regenerative medicine. The use of the MC 3T3-E1 cell line will serve as a crucial tool to explore many new therapies in the Dhar lab

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