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Effects of human BMP-2 on trans-differentiation of myoblast cells and human
rhabdomyosarcoma using an *in vitro* model system

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By

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Abstract

Fibrodysplasia Ossificans Progressiva (FOP) is an autosomal dominant disease that affects one in every two million persons. It is a disease that stimulates ossification in injured muscle cells. This mutation affects the bone morphogenetic protein receptor (BMPR), which is found on the surface of skeletal muscle cells. When bone morphogenetic proteins (BMP) come into contact with these receptors it causes a cascade of events to occur that transform skeletal muscle cells into bone cells. This mutation causes these receptors to remain constitutively activated in the presence of BMP. We compared the effects of human BMP2 on mouse myoblast cells (C2C12) and rhabdomyosarcoma (RD; human muscle cancer) cells. RD cells were used as the human muscle model for the BMPR. The results confirmed that C2C12 cells increased alkaline phosphatase (ALP) levels when BMP2 was added. ALP is one of the precursors for bone growth. The RD cells unexpectedly displayed relatively high ALP levels without the presence of BMP2. These results demonstrate trans-differentiation in C2C12 and RD cells, confirming that myoblast cells were reprogrammed to osteoblast cells by the addition of BMP2. Trans-differentiation is being initiated by the inflammatory response and molecules that are being delivered to these injured muscle cells. This experiment was preliminary work to find specific molecules that are coupling with BMP to transform injured myoblast cells into osteoblast cells.

Introduction

Fibrodysplasia Ossificans Progressiva (FOP) is an autosomal dominant disease caused by a sporadic mutation that produces erratic bone growth within muscle tissue [1]. Most patients

carry a single nucleotide change from guanine to adenosine on chromosome 2q23-24 [1]. FOP is not linked with gender or race and currently is estimated to affect one in every two million persons [2]. The onset of FOP can be triggered by minor injuries and even some viral infections, causing heterotrophic ossification (HO) [2]. However, FOP seems to impact only skeletal muscles and not cardiac or smooth muscles [2]. HO, in abnormal conditions, will cause non-skeletal tissues to ossify and turn into bone [3]. In normal conditions, HO contributes to embryonic bone formation as well as bone regeneration [3].

The FOP mutation lies on the bone morphogenetic protein (BMP) type-I receptor (BMPRIA) [4]. BMPs stimulate mesenchymal cells to differentiate into osteoblasts, therefore hindering muscle formation [4]. There are four different type-I BMP receptors, each of which has an affinity for a specific BMP isoform [4].

BMP signaling in a normal individual produces a heterodimeric complex of both type-I and type-II receptors, causing phosphorylation to occur at the glycine-serine (GS) domain [2]. This triggers the type-I receptor to turn on cytoplasmic *smad* 1/5/8 and p38MAPK signaling [2]. This activates inhibitors of differentiation which are proteins that can induce or inhibit cellular differentiation when they are overexpressed [5]. BMPRIA is critical in stopping BMP signaling by blocking the binding site with antagonists [5]. However, the FOP mutation is positioned on codon 206 in the GS domain, where BMPRIA is located [6]. Therefore, FOP individuals have a slightly activated, constitutive BMP type-I receptor [2]. Thus, downstream signaling is not controlled by the ligands and can generate unregulated bone and cartilage formation [2].

In FOP patients, BMP4 signaling is impaired, which may account for HO and the characteristic malformation of the big toe [7]. In a normal individual, there are autoregulatory negative feedback loops for BMP4 [7]. When presented with BMP4, certain cells will secrete

antagonists such as *Noggin*, gremlin, chordin, and follistatin [7]. These antagonists will bind to the BMP4 ligands and inhibit BMP4 [7]. High BMP4 activity in FOP individuals indicates that there are dysregulated negative feedback loops that are not caused from malfunctioning BMP antagonists [7].

One way to regulate intracellular signaling is through the use of *smad* proteins, which have the ability to stop myogenesis, but some *smad* proteins will induce BMP signaling and cause HO [8]. One of the markers related to bone growth is elevated by the level of alkaline phosphatase (ALP) activity [8]. *Smad 7* and *dorsomorphin* inhibit BMPRII activity and suppress bone formation by decreasing ALP activity [8].

Regulatory proteins such as *Noggin* and Sonic Hedgehog (SHH) are essential for muscle development, but myogenesis will not occur in the presence of BMPs [9]. The muscle precursors require Wnt signaling and the absence of BMPs in order to differentiate into myoblasts [10]. Different types of cellular signals are used in different regions of the body [11]. If the cells are located near the neural tube or surface epithelium, they will be regulated by Wnt signaling and BMPs [11]. If the cells are located closer to the notochord, the signaling is controlled by SHH and *Noggin* [11].

Wnt signaling is also important in osteoblast development because it causes a cascade of intracellular pathways in those cells [12]. Indian hedgehog (Ihh) proteins are present in early osteogenic progenitor cells that cause osteoblast differentiation, and these proteins are required for endochondral bone formation [12]. Endochondral ossification is the growth of bone tissue in cartilage [13]. Hedgehog-induced osteogenesis requires Wnt signaling, and Wnt7b is a crucial ligand for controlling osteogenesis [12]. Also, other precursors such as Tie2-Cre, which is an endothelial precursor, will contribute to osteogenic and chondrogenic stages [14].

Osteoprogenitor cells in the periosteum are initiated when muscle is injured [15]. These cells are also found with bone marrow stem cells (BMSC) [16]. Muscles affect bone growth by secreting growth factors that will aid in signaling osteogenesis [15]. This is why bone fractures that damage less muscle will regenerate faster whereas fractures with severe muscle trauma will regenerate much slower [15]. Growth factors such as transforming growth factors (e.g. TGF- β 1, TGF- β 2), vascular endothelial growth factors, and basic growth factors help regenerate tissue and bone [17]. Platelet-rich plasma stimulates bone generation due to its abundant growth factors [18].

Bone marrow stem cells (BMSC) are pluripotent and have the ability to transform into osteoblastic, chondrogenic, myogenic, and adipogenic lineages [16]. It was proposed that circulating BMSC aid in the development of bone [16]. Once osteoprogenitors express specific transcription factors such as Runx2, it was thought that the stem cell would follow an osteochondral lineage [16]. As this process continues, more distinct markers indicate bone differentiation [16]. One study found that muscle adjacent to bone that experienced trauma was able to trans-differentiate; the muscle cells and any non-osseous progenitors in the damaged region were reprogrammed to form bone [16]. Trans-differentiation is a process that occurs in injured myoblast cells. It is initiated by the inflammatory response and the presence of unidentified molecules. Identifying these molecules will inevitably lead to suppressing this transformation and helping FOP individuals. My experiment is setting the ground work by examining mouse muscle (C2C12) cells and rhabdomyosarcoma (RD; human muscle cancer) cells. This experiment is to test the presences of bone morphogenetic protein receptors on the muscle cells and their response to human BMP-2 stimulus.

Materials and Methods:

A human skeletal muscle-origin cancer cell line of rhabdomyosarcoma cells (RD) and a mouse myoblast cell line, C2C12, were used for this experiment. The C2C12 cell lines and RD cells lines were purchased from ATCC (Manassas, VA). A tissue culture media, fetal bovine serum, laminar flow hood, and a CO₂ incubator were used to keep the cells alive. Human BMP-2 and p-nitrophenyl phosphate stain were purchased from Thermo Fisher Scientific (Waltham, MA).

Setting up a welled plate with cover slides:

Four pieces of 22mm glass cover slips were sterilized in 70% ethanol for fifteen minutes. Each sterile cover slip was placed into one well of a six-well plate. The six-well plate took five minutes to air dry without the lid in the laminar flow hood. All of the slides in the wells were dry and all major drops of ethanol had evaporated before media and cells were added.

C2C12 and BMP:

The mouse muscle C2C12 cells were grown first. When the cells filled 90% of the flask the experiment could begin because enough cells were present. The growth media was removed from the flask and 1mL of .25% Trypsin was added. Trypsin is a protein that digests the extracellular matrix (ECM) of the cells causing them to detach from the flask. After trypsin was added, the flask was placed into an incubator at 37°C for two minutes to speed up the process. By using a microscope one could then see if the cells were suspended, and if not, the flask was tapped vigorously on its side. The cells needed to be freely suspended in order to distribute them into wells at the correct concentrations. Once the cells were all suspended, 5mL of regular medium (EMEM) was added and mixed. This makes the cells disassociate with each other for dispersal. The EMEM contains Ca²⁺ which is a molecule that binds to trypsin, allowing the cells

to attach themselves to the surface of the flask again, so the next couple of steps need to be done before that occurs. Ten microliters of cells were and placed on a haemocytometer to count the number of cells present in 1ml of solution. After counting the cells in each of the four chambers of the grid, an average was calculated to estimate the number of cells per milliliter. A volume of 180 μ L of cells in solution was placed on top of the cover slips in each of the four wells. Then one milliliters of fresh EMEM medium was added to each of the wells. The cells were then placed into the incubator. After two days, 20 μ L of human BMP-2 were added to two of the four wells and the plates were placed back into a CO₂ incubator for another seven days.

Fixing, staining, and mounting cells:

After the seven days, the six-welled plate was removed from the incubator and the media was removed from all the wells that were going to be fixed. One milliliter of 4% formalin (5.4mL of 37% formalin in 44.6mL of nonsterile PBS) was added to the wells and the cells were incubated at room temperature for fifteen minutes. Next, all of the 4% formalin was removed from the wells and 1mL of TBS (8g 1.37M NaCl, 0.2g of 0.027M KCl, 3g of 0.25M Tris/HCl, and 100mL of DI) was added. The cells were placed in TBS for ten minutes before staining them.

The stain used is an enzyme that detects alkaline phosphatase (ALK) activity [16]. When this enzyme comes into contact with ALK, it causes a color change to occur and stains the cells blue. The TBS was removed from all the wells that were being stained and had already been fixed. Then 1ml of stain was added to those wells. The plate was then placed into the CO₂ incubator at 37°C for 30 minutes. After 30 minutes, the stain was removed and 1mL of TBS was added. The stained cover slips were then ready to be mounted to slides.

The cover slips inside the wells were removed and placed on a paper towel, cell side facing upward, by using tweezers. The paper towel was labeled clearly to identify which cover slips they were. The cover slips were left to dry and two clean slides were used. The cover slips were mounted two per slide; one was a control and the other with a treatment. Both cover slips were mounted using mounting media and the slides were labeled accordingly.

RD and C2C12 experiment:

For this experiment, four wells each of two six-welled plates were used. Cover slips were prepared in the eight wells. RD and C2C12 cells were counted using a haemocytometer. Both flasks of RD and C2C12 cells were trypsinized and each of four wells on one plate received 180 μ L of C2C12. The remaining four wells on a different plate each received 220 μ L of RD cells. Both plates were labeled with cell type and treatment. Then 1mL of EMEM was added into each of the wells and left in the CO₂ incubator at 37°C for two days. Then 20 μ L of BMP were added to two RD wells and two C2C12 wells. The plates were then placed back into the incubator for seven days. The cells were then fixed, stained, and mounted onto slides.

RD media and RD cell experiment:

Two 6-welled plates were set up as previous described and cover slips were added to a total of eight wells. Only C2C12 cells were used in the wells first. The C2C12 cells were counted using a haemocytometer and 220 μ L of cells were added to each of the wells. The plates were then placed back into the CO₂ incubator at 37°C for two days. Next, 20 μ L of BMP were added to two of the wells. The two wells without BMP had their media removed and replaced with 1mL of RD-condition medium. Media was removed from another two wells (without BMP and without RD condition medium) and replaced with 1mL of RD cells. The RD cells were trypsinized and then diluted with 5mL of fresh EMEM media before being added to the wells.

The two remaining wells were used as controls and only were C2C12 cells and EMEM. The plates were placed in the incubator for seven days after which time the cells were fixed and stained. All of the wells were mounted except for one RD cell and one RD-condition medium cover slide.

Cartilage Stain:

The two wells that were not mounted (one RD-condition medium and one RD cell) were used to see if cartilage formation had occurred. It appeared that cells on both these cover slips were forming aggregates and looked similar to cartilage. The control was the C2C12 cells that were not treated with RD-condition medium or RD cells. Alcian green cartilage stain was used to detect cartilage by staining the cartilage green. All of the TBS was removed from both wells and 1mL of the stain was added. After thirty minutes, it was placed into the refrigerator for one day. Then both cover slips were mounted after looking at them under a dissecting microscope.

Results

As expected, when the C2C12 cells were introduced to BMP, they released alkaline phosphate, which was made apparent with staining (figure 1). The control cells (the C2C12 cells that did not have BMP added) stained very lightly (figure 1). The RD cells with BMP stained roughly fifty percent as well as the RD cells without BMP (figure 2). The RD cells were added to the C2C12 cells and exhibited ALT activity after staining (figure 3). The C2C12 cells with the addition of RD-condition media clumped together forming an aggregation of cells that stained darkly with ALT activity (figure 3). The RD-condition media was stained with Alcian Green and some of the aggregates stained green but it was inconclusive (figure 4). Also, the RD

media with the C2C12 cells showed more connections between the cells (figure 4). The control with C2C12 cells exhibited no cartilage staining.

Discussion:

Mouse muscle (C2C12) cells reacted as expected when introduced to human BMP-2; the cells exhibited large amounts of alkaline phosphatase activity. When the muscle cells were subjected to BMP-2, their formation changed. The C2C12 cells began to form clusters and the alkaline phosphatase activity appeared to form webs between the cells. The BMP-2 successfully caused trans-differentiation to occur in the C2C12 cells.

The human tumor (RD) cells have the same BMPR as the C2C12 cells. We expected the same results with the RD cells as with the mouse muscle cells, but surprisingly when the RD cells were subjected to BMP-2 the outcome was different. It appeared that the RD cells stained for alkaline phosphatase activity with and without the addition of BMP-2. Close to 80% of the C2C12 cells had improved alkaline phosphatase activity with BMP-2. It is possible that when the RD cells were harvested, some bone stem cells were accidentally collected. This would cause contamination of the sample by mixing the two types of cells. It is also possible that RD cells might have the ability to switch between lineages more easily because the tumor caused some genes to be turned on while other genes had been silenced. Another theory is that the RD cell is a type of stem cell that did not differentiate properly. This allows it to have the ability to become both muscle or bone cells.

These results promoted us to test whether the RD cells were secreting a molecule or producing a molecule within the cells. These molecules are causing increased levels of ALT which signify the initiation of trans-differentiation. The RD-condition media when added to the C2C12 cells caused the cells to aggregate. These cells appeared to be in the beginning stages of

differentiation. To further understand this reaction we stained the cells for cartilage, however the results were inconclusive. If cartilage was present, it would have indicated that cartilage is formed first instead of direct bone growth. This process is called endochondral ossification.

The C2C12 cells and RD cells had BMPR. The RD cells unexpectedly expressed increased levels of ALT without the addition of BMP-2. RD cells are therefore a bad human muscle model to use for future experiments. The next couple of experiments need to focus on identifying molecules that are coupling with the inflammatory response and the trans-differentiation process. The results will reveal new information about FOP signaling and help to discover a treatment that can suppress heterotrophic ossifications in FOP patients.

Figure 1:

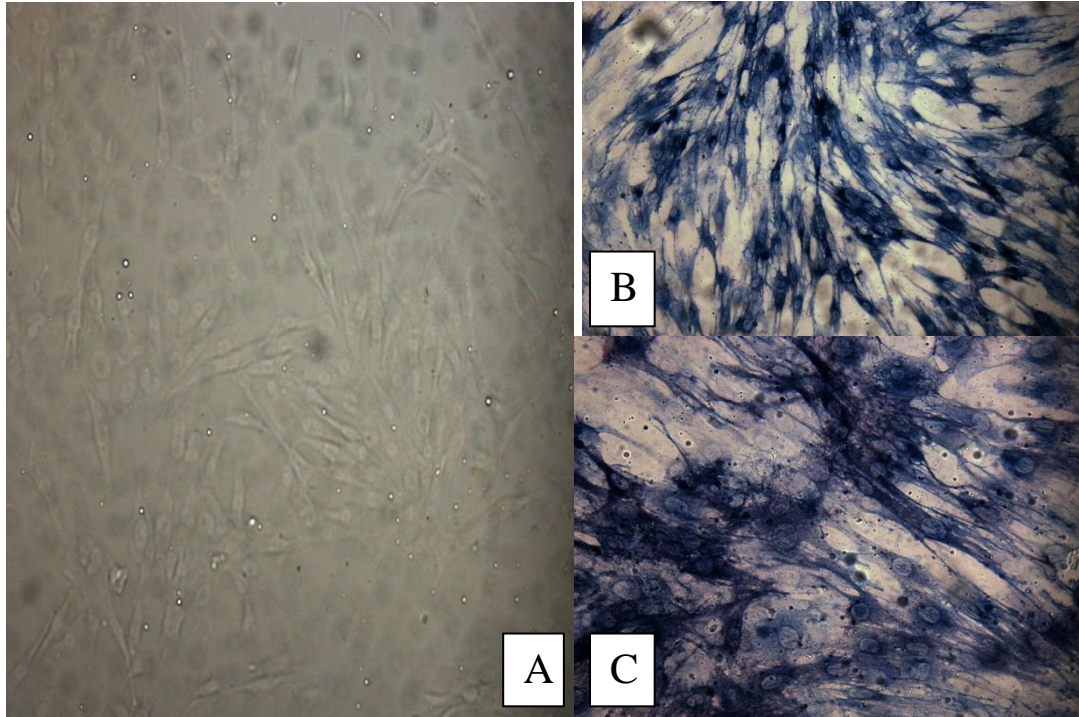


Figure 1: Mouse muscle cells (C2C12). **A:** control cells that were not given BMP-2. **B:** cells treated with BMP-2. **C:** higher magnification of the cells that were treated with BMP-2. Alkaline phosphate activity is revealed by the blue stain.

Figure 2:

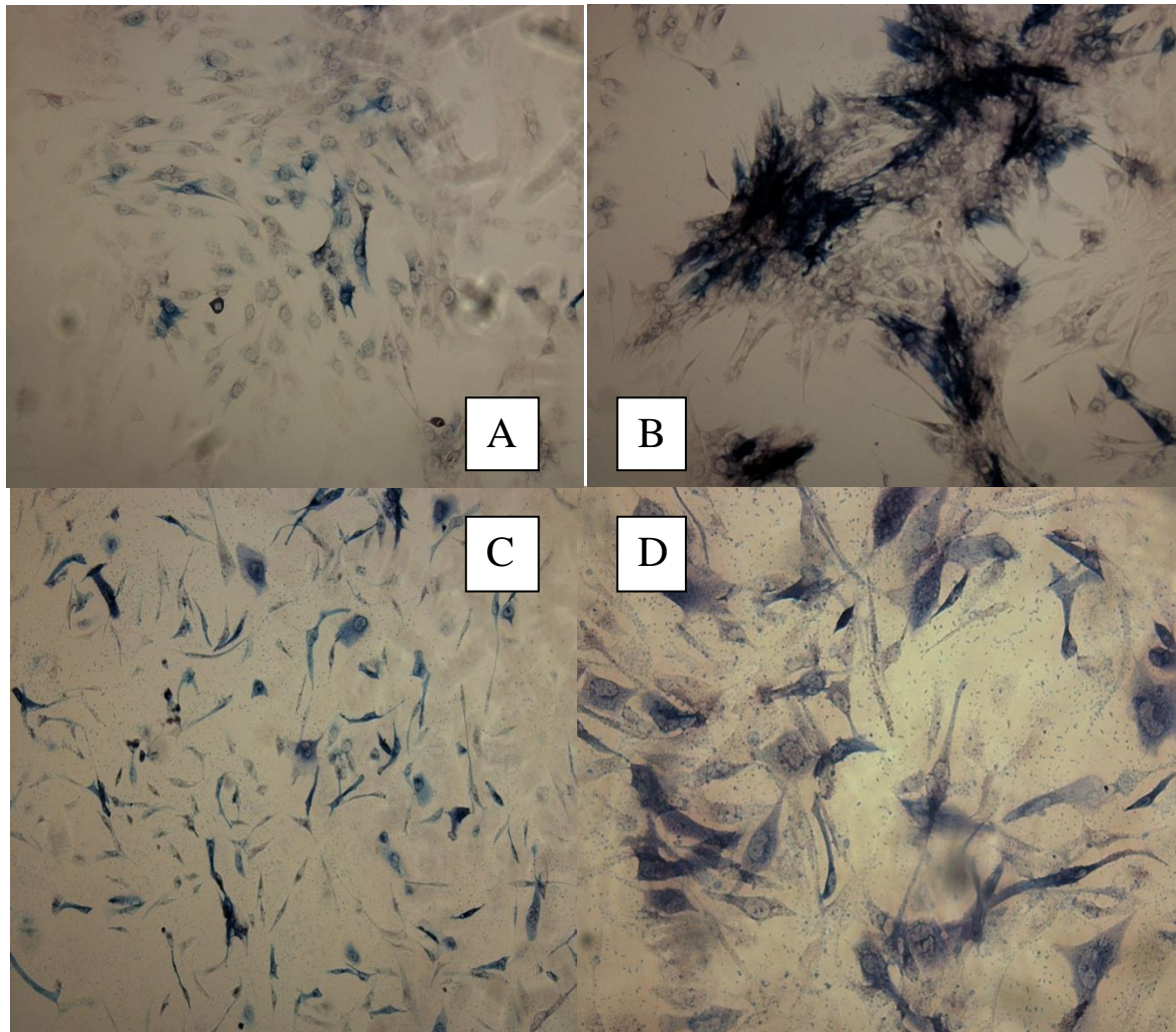


Figure 2: Mouse muscle cells (C2C12) cells and human tumor muscle (RD cells treated with or without BMP-2. **A:** C2C12 cells that were not treated with BMP-2. **B:** C2C12 cells treated with BMP-2. **C:** control RD cells **D:** RD cells subjected to BMP-2. Alkaline phosphatase activity stains blue.

Figure 3:

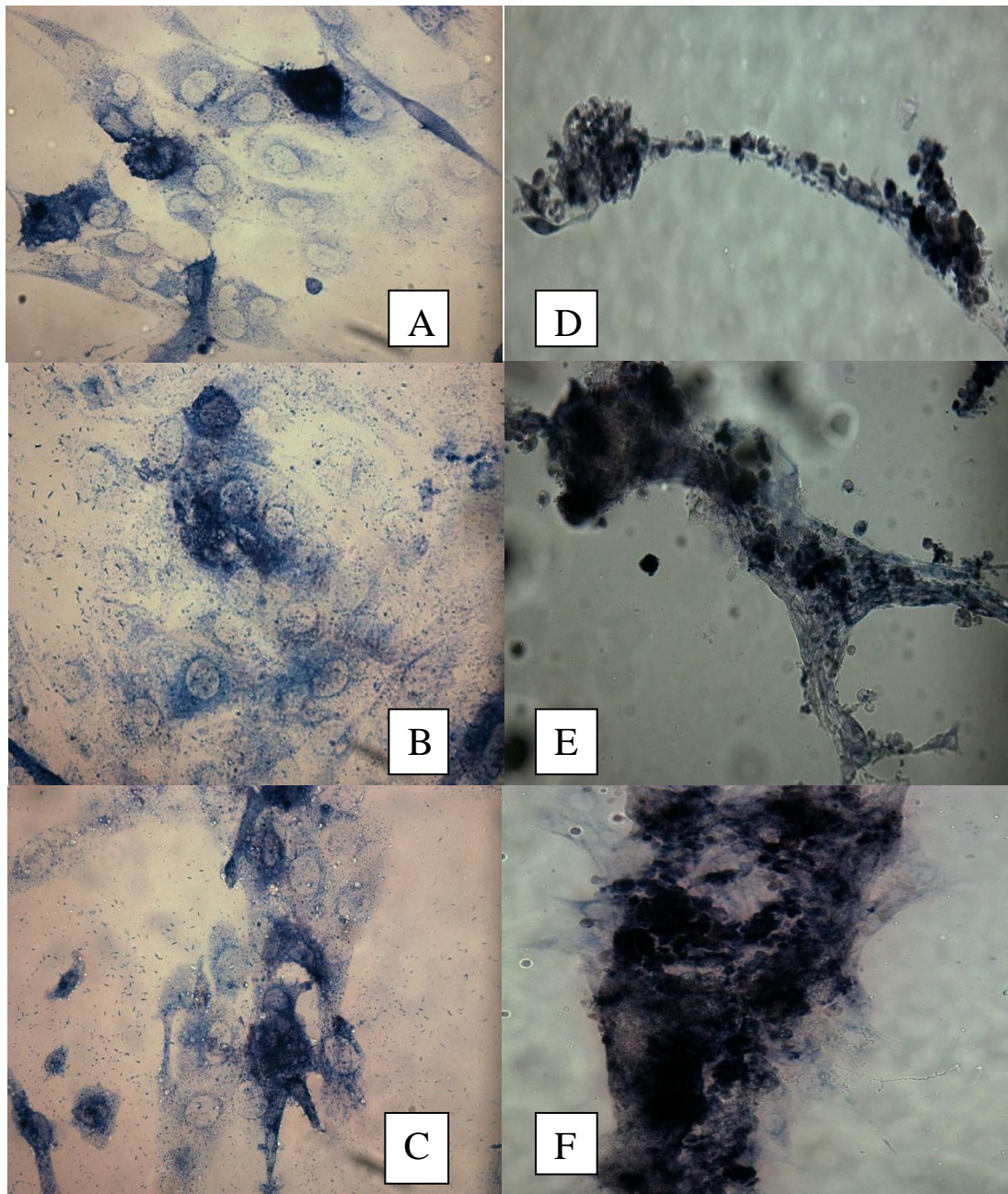


Figure 3: C2C12 cells treated with RD cells or RD media **A, B,** and **C:** C2C12 cells treated with RD cells. **D, E** and **F:** C2C12 cells treated with RD media; note the clumping cells. Blue staining indicates alkaline phosphatase activity.

Figure 4:

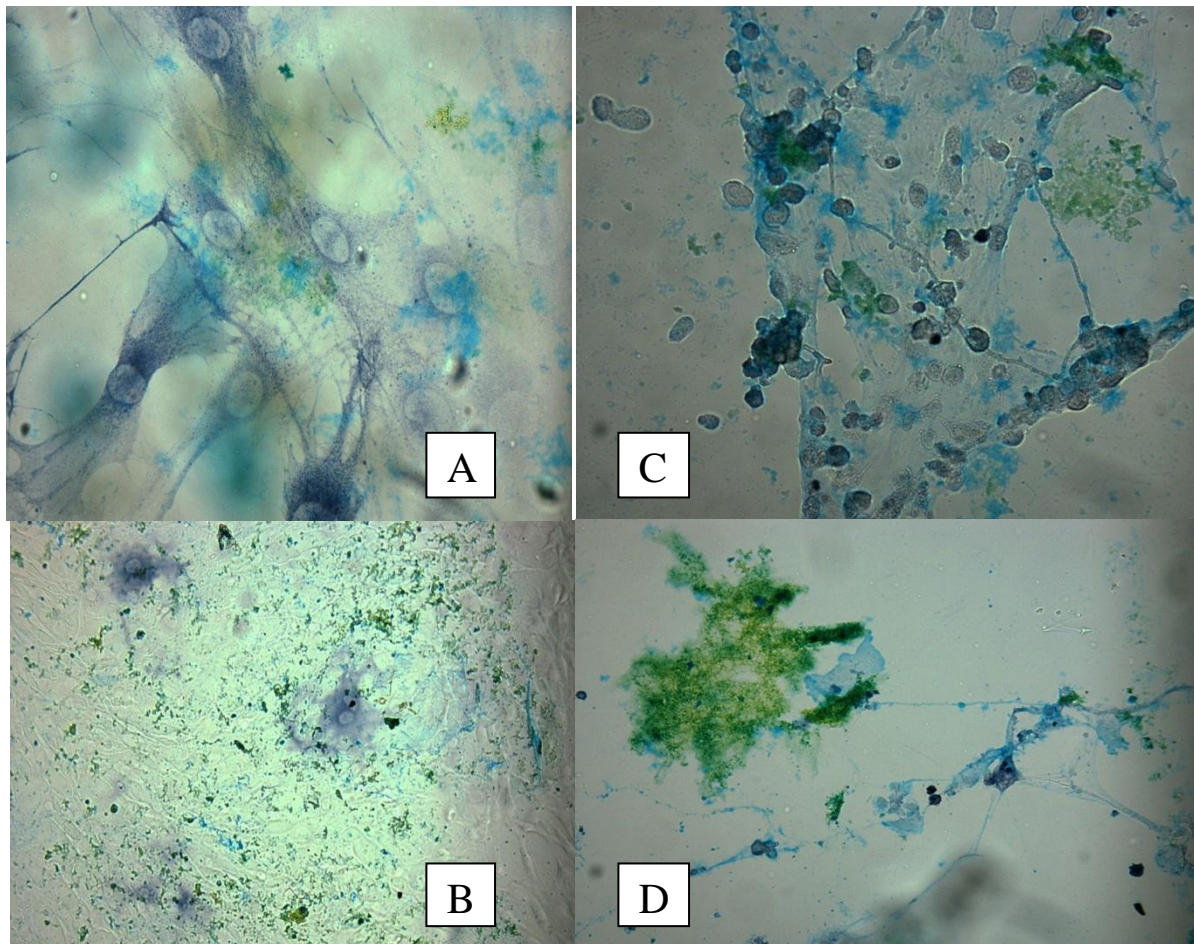


Figure 4: C2C12 cells treated with and without RD media. **A** and **B**: control C2C12 cells. **C** and **D**: C2C12 cells treated with RD media. Alkaline phosphatase activity stains blue. The cartilage stain (green) indicates trans-differentiation in the presence of RD media.

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