Impact of Fibroblast Growth Factor on Mesenchymal Stem Cell Osteogenic Differentiation and Migration

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Abstract

Adipose-derived Mesenchymal Stem Cells (MSCs) have proven to be highly useful in the therapeutic application of bone regeneration. Direct application in bone fractures and hip/joint replacements have yielded decrease in recovery time for patients. Further studies into how MSCs migrate, and what growth factors they are affected by could potentially pave way for further enhancement in bone regeneration therapy.

This study investigates the affect of basic Fibroblast Growth Factor (bFGF) on MSC differentiation and Migration. Type I Collagen was shown to enhance MSC proliferation and migration, thus was used as the initial coating when seeding the cells onto the wells. Our results indicate that bFGF did not statistically show to enhance nor impede osteoinduction of MSCs into Osteoblasts. We did not observe an earlier onset of ALP activity spike, which indicates preliminary phase of osteoinduction. We also did not observe any significantly noticeable increase in Calcium Phosphate deposition. Although the experiment design did not allow for the observation of the sign of chemo-attractant property of MSCs to FGF, we did observe a 3 fold increase in MSC migration when treated with FGF.

Introduction

Stem cell therapies are still in the early stages, however new therapies based upon MSCs are being pursued, due to their hypoimmunogenic nature and pluripotent capabilities. MSCs are being induced into bone cells that would be phenotypically and genetically similar to those in a patient's own body. Osteogenesis is a vital process in our body that creates bone tissue by osteoblast activity. This process is balanced by concurrent osteoclasts activity, the resorption of bone tissue. The imbalance of these activities can lead to degradation in the bone matrix and it's loss of structural integrity. The resulting fracture, or the need to replace the bone entirely require implanting synthetic materials such as ceramic or stainless steel implants. Although these implants use fixating "glue" such as Acrylic cement to secure the implant, ultimately it requires the infusion of the bone itself, to grow into the implant. This can be impeded when the patient is subject to old age, various disease treatments such as cancer, or graft versus host disease, when the Osteoblast precursor Mesenchymal Stem Cells (MSCs) lose their full functionality. The underlining issue of regaining that functionality is key to enhancing bone regeneration.

Taking advantage of widely available and relatively inexpensive adipose-derived MSCs, our team observed the effects of Fibroblast Growth Factor, basic (bFGF or FGF-2) on MSCs and on MSCs undergoing osteoinduction on initial coatings of type I collagen. bFGF has been previously shown to influence proliferation, morphogenesis, and vasculogenesis. To elucidate more specific effects of bFGF on osteogenesis, Alkaline Phosphatase (ALP) activity was monitored over the course of the experiment. Our previous work confirmed that MSCs differentiated and proliferated more highly on collagen type I coated plates than on uncoated plates (polystyrene). Furthermore, the investigaton of MSC migration will also be observed to determine the effects of bFGF using a Boyden Chamber and Scrath Assay. We hypothesize that bFGF will enhance extracellular ALP activity in MSCs undergoing osteoinduction and increase migrations rates, relative to MSCs in basal medium. Alizarin Red S staining will be used to confirm osteogenesis and osteoblast activity in mouse osteoblasts via positive staining of calcium deposits.

Materials and Methods

Plate preparation:
12-well polysterene cell culture plates (Costar) were prepared by coating each wellovernight at 5°C with either PBS (for uncoated wells) or a 5 µg/ml collagen containing solution. The PBS or collagen containing solution was removed and each well was washed twice with PBS prior to seeding cells.

Osteogenic Media:
Osteogenic media was prepared by combining the HyClone AdvanceSTEM Osteogenic Differentiation Kit medium and supplement according to the manufacturer's instructions.

Cell Seeding:
Human adipose-derived mesenchymal stem cells (MSCs) were obtained from ATCC. The cells were thawed according to the manufacturer's instructions and plated in 75 cm^2 cell culture flasks (BD Falcon) in regular human mesenchymal stem cell basal medium (HyClone) containing stem cell growth supplement (HyClone). Once incubated to 70-80% confluency the cells were harvested, trypsonized, and counted.

(Proliferation Assay & ALP Assay) **Each sample well was prepared to 10,000 cell/ml with a final media volume of 1mL . Conditions included: (1) Basal Media + 20ng/ml bFGF, (2) Basal Media – 20ng/ml bFGF, (3) Osteogenic Media + 20ng/ml bFGF, (4) Osteogenic Media – 20ng/ml bFGF. Each condition was plated onto their respective wells and their initial coatings of Fibronectin or Collagen. N=3. (Figure 4).**

(Boyden Chamber Assay) Each chamber was prepared with initial cell density of 5,000 cells/ml with a final volume of 200uL media in the chamber, and 500uL media in the well below. bFBF was supplemented at 20ng/mL final volume. Various combinations were used to create directional gradients (Figure 3).

Alizarin red staining and Alkaline Phosphatase Assay:
1% Alizarin red (Sigma-Aldrich) and Alkaline Phosphatase (abcam) assays were performed on Day 0, Day 7, Day 14, Day 21, and Day 28 according to the manufacturer's instructions.

Scratch Migration Assay:
Cells were plated onto a 6 well plate that were pre-coated with either Fibronectin or Collagen (Both at either 5 or 10 ug/ml). Initial Cell density was at 10,000 cell/ml. After 1 hour of cell adherence period, a 1cm scrape was made laterally across the well using a cell scraper. Over the course of 190 hours, measurements were made by referencing markers that were pre-edged below each well.

Results

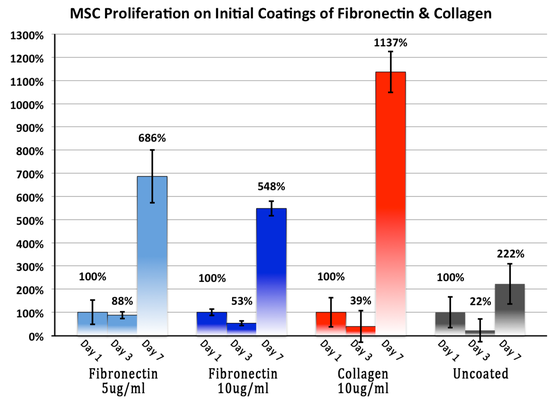
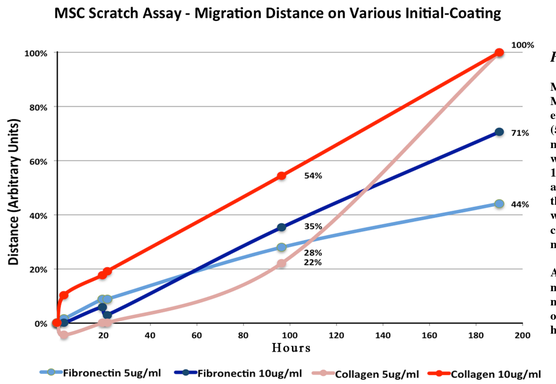


Figure 1:

Calcein Live/Dead Proliferation Assay determining the affect of initial coating of either Fibronectin (5 & 10ug/mL) or Collagen (10ug/mL). 10,000 cells were seeded per well and grown for a total of 7 days in Mtesenchymal Stem Cell Growth Medium. N=3.

Initial coating of Collagen 10ug/mL exhibited the greatest proliferation effect on Mesenchymal Stem Cells.



	Fibronectin				Collagen			
	5ug/ml		10ug/ml		5ug/ml		10ug/ml	
Hours	Average	95% Conf. Int.	Average	95% Conf. Int.	Average	95% Conf. Int.	Average	95% Conf. Int.
0	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
3	1.5%	8.6%	0.0%	0.0%	-4.4%	8.6%	10.3%	2.9%
20	8.8%	0.0%	5.9%	0.0%	0.0%	5.8%	17.6%	0.0%
22	8.8%	0.0%	2.9%	5.8%	0.0%	0.0%	19.1%	2.9%
97	27.9%	14.4%	35.3%	23.1%	22.1%	8.6%	54.4%	20.2%
190	44.1%	0.0%	70.6%	57.6%	100.0%	0.0%	100.0%	0.0%

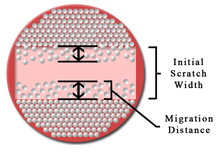


Figure 2:

Mesenchymal Stem Cell Scratch Migration Assay, determining the effect of initial coatings of Fibronectin (5&10 ug/ml) and Collagen (5&10ug/mL) on their migration rate. Cells were seeded at 10,000 cells per well. 1cm cell scraper was used to scrape across each well of the 6 well plate with their respective initial coatings. Cells were allowed to migrate over the course of 190 hours, with intermittent migration measures.

Although statistical significance could not be established due to low sample number, we observed a general trend of initial coating of Collagen 10ug/mL having the greatest rate of migration.

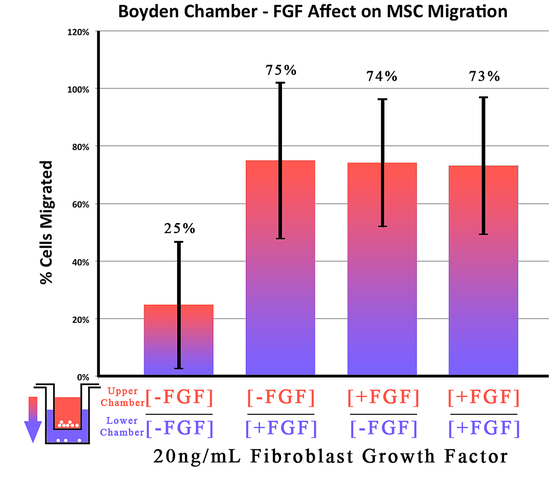


Figure 3:

Depicts the migration percentage of MSCs in Basal Media, quantified by the addition of the cells in the bottom of the well plus the cells on the bottom of the membrane divided by the total cells which include the cells in the top of the chamber. The cells migrated over a 7-day period. The addition of 20 ng/ml of bFGF was either put in the top chamber, bottom chamber, or both. N=3 for all conditions.

Regardless of which chamber bFGF was added to, the addition yielded roughly a 3 fold increase in cells that migrated across the membrane.

The lack of differentiation amongst the various combinations could suggest the membrane's inability to hold a FGF gradient long enough to yield any gradient specific effects.

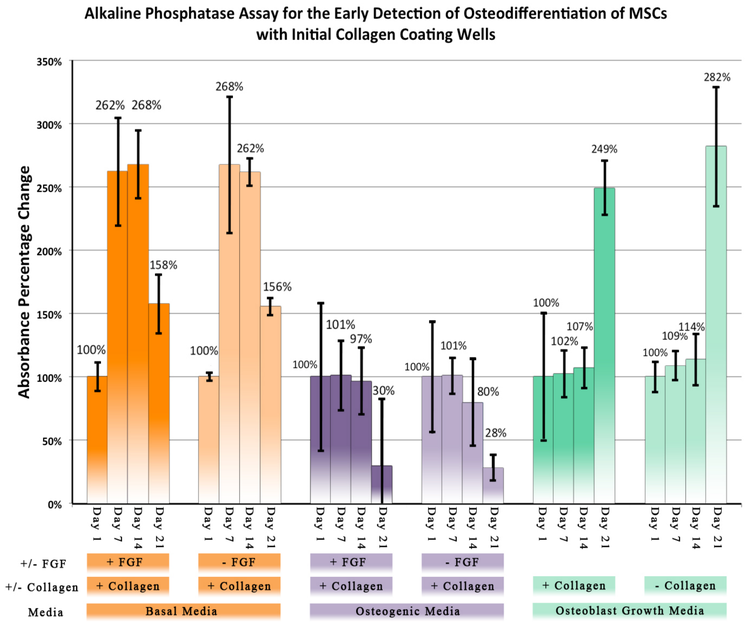


Figure 4:

ALP Assay was conducted on MSC cells that were initially seeded at 10,000 cells/well, cultured in either Basal Media or Osteogenic media. Osteoblasts were also included at the same initial cell seeding density, and were also measured for ALP activity in parallel with MSCs as a system control. Cells were grown for a total period of 21 days, with 1 week interval measurements. Variable conditions also included +/- bFGF and +/- Collagen.

Osteoblasts were used as system control, since ALP activity indicates the initial phase of Osteoblast differentiation Osteoblasts should not exhibit any sort of spike in ALP activity, and remain at a steady activity level throughout the experiment. However we observed a spike in the last ready (Day 21) for both +/- Initial Collagen coating, which is unexplained.

We observe no significant effect of bFGF on inducing osteogenic activity. The natural spike in ALP acivity in MSC grown in Basal Media at Day 7, seems to be unaffected by neither the initial coating of Collagen, or the addition of bFGF.

ALP spike expected to be seen in MSCs grown in Osteogenic Media was not observed in this analysis. However, the raw numbers indicate that the spike occurred immediately in the Day 1 readings, thus being normalized to the 100%.

Alizarin Red Staining of MSC and Osteoblasts treated with FGF and grown in various media

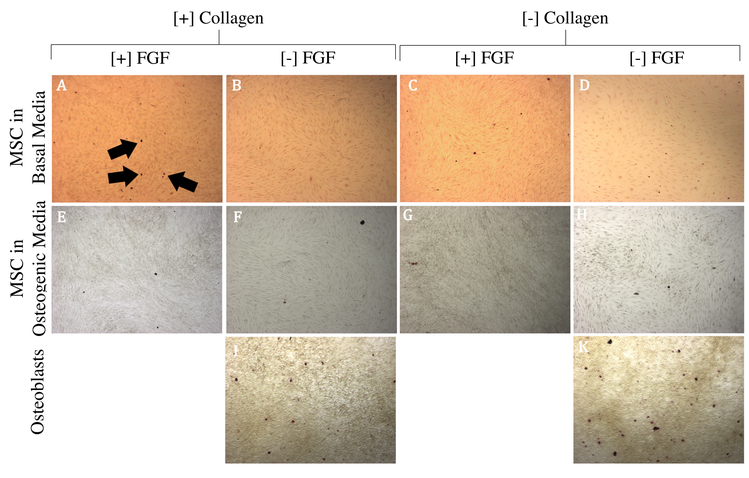


Figure 5

The images depicts the alizarin red stain results for days 1, 7, 14, and 21 for MSCs and Osteoblast cells (control). The conditions include an initial coating of Collagen of 5ug/ml ([+] Collagen), a lack of collagen coating ([-] Collagen), addition of FGF ([+] FGF), lack of FGF ([-] FGF), cultured in Basal Media, and cultured in Osteogenic Media. The Osteoblasts were cultured in Osteoblast Growth Media, and were treated with either [+] Collagen or [-] Collagen, but neither were treated with FGF. All images depict stains taken at Day 21.

(A) Arrows point to examples of Alizarin Red Staining of Calcium Phosphate deposits from Osteoblasts. There doesn't seem to be a definitive conclusion that can be pulled from visual inspection apart from the apparent lack of variance amongst various treatments of Collagen and FGF.

Conclusion

- **Collagen 10ug/ml facilitated the greatest amount in MSC Proliferation as well as Wound Filling.**
- **Basic Fibroblast Growth Factor (bFGF) increased the amount of cells that migrated by roughly 3 fold.**
- **Due to constraints of the experiment design, the chemo-attractant property of bFGF to MSCs were not established.**
- **bFGF did not induce Osteogenesis in MSCs.**