Mechanical Stiffness in 3D Embryonic Stem Cell Aggregates Undergoing Osteochondral Differentiation

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Mechanical Stiffness in 3D Embryonic Stem Cell Aggregates Undergoing Osteochondral Differentiation

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS AND ABBREVIATIONS

1.	**RT-PCR**	**Real Time Polymerase Chain Reaction**
2.	**ESC**	**Embryonic Stem Cell**
3.	**3D**	**Three Dimensional**
4.	**D#**	**Day (number of day)**
5.	**RNA**	**Ribonucleic Acid**
6.	**DNA**	**Deoxyribonucleic Acid**
7.	**cDNA**	**Complementary Deoxyribonucleic Acid**
8.	**sGAG**	**Sulfated Glycosaminoglycan**
9.	**BM**	**Basal Media**
10.	**DM**	**Differentiation Media**
11.	**P**	**p-value**

SUMMARY

This study aimed to investigate the effect of osteochondral differentiation on mechanical stiffness of 3D mouse embryonic stem cell (ESC) aggregates. Both soluble osteoinductive cues and mineral coated microparticles were used to induce osteochondral differentiation. Uniform 3D ESC aggregates were formed by forced aggregation of mouse D3 ESCs in AggreWell™. MPs were incorporated into ESC aggregates by mixing with ESCs prior to aggregate formation at either 1:3 or 1:1 MP to cell ratio. ESC aggregates were cultured in either basal media (BM) or differentiation media (DM) which contained β-glycerophosphate and ascorbic acid to induce osteochondral differentiation. The mechanical stiffness of aggregates was determined from the creep tests performed via the MicroSquisher. The DM groups were significantly stiffer ($P<0.05$) than the BM groups, but there was no significant difference between the concentrations of mineral particles within the treatment groups. The gene expression of osteogenic and chondrogenic markers was evaluated at D14 using RT-PCR. Osteogenic and chondrogenic markers expression in DM groups was significantly higher than in BM groups at D14 ($P<0.05$). MP incorporation also increased the expression of chondrogenic markers in BM and osteogenic marker expressions in DM compared to No MP groups ($P<0.05$). Alizarin Red and Safranin O/Fast Green stains were performed to assess the change of ECM composition of ESC aggregates on D14. In addition to the different glycosaminoglycan staining patterns between soluble treatments and MP incorporation, significant increase in mineralization was observed in DM culture in comparison to BM groups, which was further increased in the presence of MPs. It was evident that osteochondral differentiation occurred in the DM groups and increased stiffness. Together, these results suggested that osteoconductive cues alone or in combination with MPs

can effectively promote osteochondral differentiation and enhance mineralization, which may contribute to the increase in stiffness of ESC aggregates.

CHAPTER ONE

INTRODUCTION

Embryonic stem cells (ESCs) are one of the greatest tools to understanding early development of tissues due to their ability to differentiate into all cell types(Czyz & Wobus, 2001). ESCs show promise in furthering the understanding of cell function as well as provide a medium to manipulate for future medical treatments. ESC aggregates are a mass of embryonic stem cells that have formed a single three dimensional colony. ESC aggregates differentiate towards cell phenotypes in all three germ layers – endoderm, mesoderm, and ectoderm (Kurosawa, 2007) . Engineering these aggregates creates adept tools for modeling cellular and molecular activity in early development (Zhang & Xia, 2012). By using microwell (AggreWell®) technology, 3D aggregates can be formed without scaffolds and the amount of cells per aggregate can be controlled (Kinney, Saeed, & McDevitt, 2012)– allowing for uniformity and homogeneity of cell type throughout the aggregate. 3D aggregates can develop into viable tissues (Nakano et al., 2012) and can be used to model the development of tissues. The cells derived from 3D aggregates can be implanted in vivo to regulate local microenvironment and to aid tissue regeneration.

By subjecting 3D ESC aggregates to a microenvironment conducive to osteochondrogenesis, we aimed to induce cellular differentiation toward osteochondroal lineages and potentially to form cartilage or bone after implantation in vivo to replace or fortify the damaged tissues. Both biochemical cues and biomaterials have been shown to modulate the process of osteochondral development in mesenchymal stem cells (Sundelacruz & Kaplan, 2009). Microparticles have been incorporated into cell aggregates with success, and have proven to be an effective method of morphogen delivery (Bratt-Leal, Nguyen, Hammersmith, Singh, &

McDevitt, 2013). Mineral coated hydroxyapatite particles have a high content of calcium and phosphate and can bind and sequester growth factors, which have been used to promote osteochondrogenesis of bone marrow-derived mesenchymal stem cells(Suárez-González et al., 2012).

In this work, we aimed to systematically study the effects of various osteochondrogenic inductive factors and/or mineral particles on osteochondrogenic differentiation of 3D ESC aggregates at the molecular, protein, and biomechanical levels. The specific goal of our study was to investigate the changes in the biomechanical properties of ESC aggregates during the course of osteochondral differentiation. Cartilage and bone have specific mechanical properties associated with their maturation status(Pek, Wan, & Ying, 2010). Characterizing the ESC aggregates makes it possible to compare them to bone and cartilage. Mechanical stiffness can be found by calculating the apparent modulus of the ESC aggregates using a parallel plate micro compression platform(Koay et al., 2009). Changes in osteochondrogenic markers and morphological analysis of the ESC aggregates were used to indicate the stages of osteochondrogenesis and provide data that can be correlated to the mechanical testing data. The findings of our study have yielded new insight into the osteochondral development of differentiating ESC aggregates.

CHAPTER TWO

METHODOLOGY

Mouse ESC culture

Mouse embryonic stem cells (mESCs) (D3) were cultured on 0.1% gelatin coated tissue culture dishes in ESC media composed of DMEM (Mediatech Inc., Herndon, VA) supplemented with 15% FBS (Hyclone, Logan, UT), 1x non-essential amino acids (Mediatech), 2mM L-glutamine (Mediatech), 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin (Mediatech), 0.1 mM 2-mercaptoethanol (Fisher Scientific, Fair-lawn NJ), and 10^3 U/ml leukemia inhibitory factor (LIF) (Millipore, Billerica, MA).

ESC Culture and ESC Aggregate Formation

Uniform 3D ESC aggregates were formed by forced aggregation of mouse D3 ESCs in 400x400 µm AggreWell™ microwells. In order to incorporate mineral coated hydroxyapatite microparticles (MPs), MPs were mixed with ESCs prior to aggregate formation. They were instituted in either a 1:3 MP to cell ratio or a 1:1. The aggregates were removed from the inserts after a 24 hour period and suspended in serum-containing cell culture media (DMEM+15% FBS) on rotary orbital shakers (45 rpm) for 5 days of pre-mesoderm differentiation. The cell aggregates were cultured for 14 additional days in the presence or absence of the following soluble factors in order to induce osteochondral differentiation: 5 mM β-glycerophosphate (BGP), 0.2 mM ascorbic acid (VitC). The experimental group and culture conditions are listed in Table 1. The ESC aggregates were harvested at either D2 and D14 for mechanical test, real-time PCR or histology, respectively.

Table 1. Experimental groups and culture conditions

	Day 0-4	Day 5-14		
Group	Basal	Basal	5mM BGP	0.2mM VitC
No MP BM	x	x		
No MP DM	x	x	x	x
1:3 MP BM	x	x		
1:3 MP DM	x	x	x	x
1:1 MP BM	x	x		
1:1 MP DM	x	x	x	x

Mechanical Testing

The bulk stiffness of the ESC aggregates was measured using the parallel plate micro-compression system CellScale Microsquisher. The Microsquisher is prepared by filling the bath with PBS, placing the glass prism against the glass wall, and inserting a microbeam of appropriate stiffness. The microbeams used had either a diameter of 0.1016 or 0.1524 μm. The microbeam's length was recorded after insertion. The Microsquisher operates by lowering a microbeam onto the cell aggregate and deforming it. There are beams of varying thicknesses; an appropriate thickness much be chosen in order to obtain the best data from the samples. Tissue deformation ratios are automatically calculated after the compression and factor into the selection of the beam diameter. When the microbeam deforms the sample, it also measures the forces it applies. That force is back-calculated in real-time via the following equation (CellScale, 2011):

$$Deflection = \frac{Force \times Beam\ Length^3}{3 \times Beam\ Modulus \times Beam\ Area\ Moment\ of\ Inertia}$$

An ESC aggregate was placed on the prism using a pipet and the plate moved on top of the aggregate to start the compression (Fig. 1). The initial length of the aggregate was determined by selecting the top and bottom of it and a tracking point was placed on the plate. The ESC aggregate was compressed and the deflection measured via image tracking. 3 aggregates were tested to determine how much force is required to deform the aggregate to 40% of its initial length. The averaged force was applied to a new set of aggregates (n=8) from each experimental group for 2 minutes per aggregate. The creep curve were recorded and used to determine the apparent modulus for each sample using a custom MatLab programs established by Melissa Kinney that calculates the equations below.

$$u(t) = \frac{4\sigma R_o}{3E_\infty}\left[1+\left(\frac{\tau_\varepsilon}{\tau_\sigma}-1\right)e^{\frac{-t}{\tau_\sigma}}\right]H(t) \quad (1)$$

$$E_o = \frac{\tau_\sigma}{\tau_\varepsilon}E_\infty \quad (2)$$

$$\mu = \tau_\varepsilon\left(E_o - E_\infty\right) \quad (3)$$

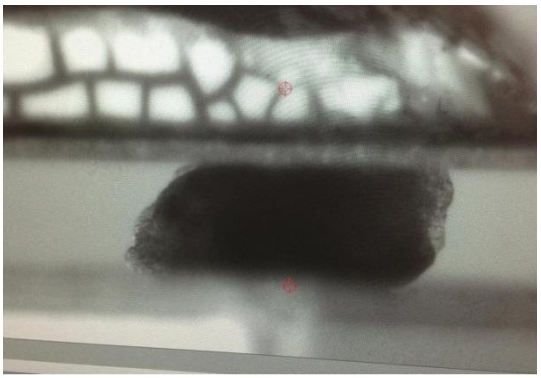

Figure 1. ESC Aggregate compression during Microsquisher testing

Real Time PCR Analysis of Gene Expression of Osteochondral Markers

The total RNA from the ESC aggregate samples was isolated via RNase Easy Kit. The

purified RNA were quantified using NanoDrop – the concentration of RNA were found and the purity given by the ratio of A260/A280. The RNA was converted into cDNA using iScript cDNA Synthesis Kit. Gene expression of selected osteogenic markers (Runt-related transcription factor 2, osterix, osteopontin, collagen type 1, bone sialoprotein, osteocalcin) and chondrogenic markers (SRY (sex determining region Y)-box 9, collagen type 2, Aggrecan, collagen type 10) were amplified via Syber Green-based Real Time PCR. The PCR primers have been previously designed and optimized and their optimal temperature calculated (Table 2). The expression level was normalized to the housekeeping gene, ribosomal protein S18.

Table 2. Optimal Temperature for PCR primers.

Gene	SOX9	COL2	BSP	OPN	OCN	OSX	RUNX2	COL1	RPS18
T(C)	59	63.3	65	65	61.4	63.3	65	64.5	59

Histology

The samples were prepared by fixing in 10% formalin for 90 minutes and encapsulated in Histogel® prior to histological processing (ThermoScientific).Then, samples were embedded in paraffin and then cut into 5 micron sections. They were subjected to Safranin O/Fast Green and Alizarin Red staining to highlight sulfated GAG content and mineralization (calcification), respectively. The Safranin O/Fast Green stain was performed to protocol (IHCWorld), as was the Alizarin Red stain (IHCWorld). The stained slides were imaged at 4X using a microscope.

Statistical Analysis

The data was analyzed as the mean±standard error. Two-way ANOVA tests and Bonferroni Post Hoc tests were used to determine statistical significance ($p<0.05$).

CHAPTER THREE

RESULTS

Mechanical Stiffness

The D14 groups were all significantly stiffer than the D2 groups. The DM D14 groups were all significantly stiffer than the D14 BM groups. The varying mineral particle concentrations did not significantly affect stiffness within the same media types.

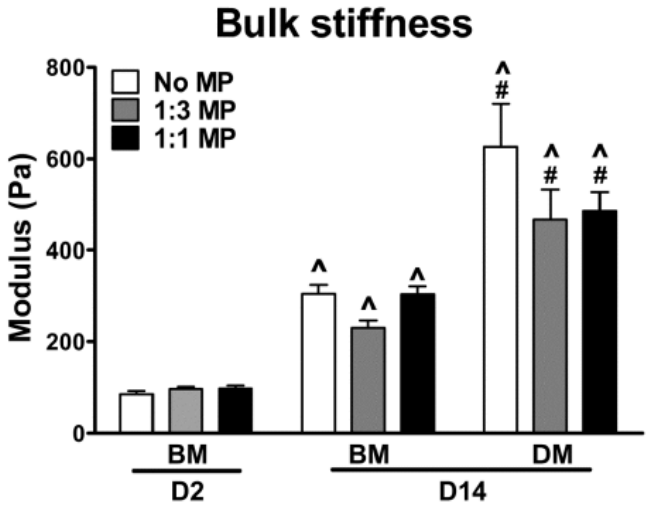

Figure 2. Mechanical Stiffness of 3D ESC Aggregates

3D ESC aggregates were harvested and subjected to mechanical tests on D2 and D14. Aggregates were first tested to determine the force to achieve 40% deformation (n=3) and then underwent creep testing (n=8). The stiffness was calculated from data extrapolated from the creep testing. The Young's Moduli are presented as mean±standard error. ^ = P <0.05 vs D2 BM, # = P<0.05 vs D14 BM.

Osteochondrogenic Gene Expression

The expression levels of the chondrogenic markers SOX9, COL2, ACAN, and COL10a1, and the osteogenic markers OSX, OPN, OCN, RUNX2, BSP, and COL1a1 were measured via RT-PCR. DM groups were significantly different from BM groups for osteogenic and chondrogenic

markers at D14 (P<0.05). 1:1 MP DM and 1:3 MP DM groups had higher osteogenic marker expressions over NO MP DM and the BM groups (P<0.05). In BM, the mineral particle groups experienced higher chondrogenic marker expression. Osteogenic markers expression increased but not across the board. In DM, the chondrogenic markers were more frequently down-regulated as mineral particle concentration increased. Almost all osteogenic markers were up-regulated for the mineral particle groups.

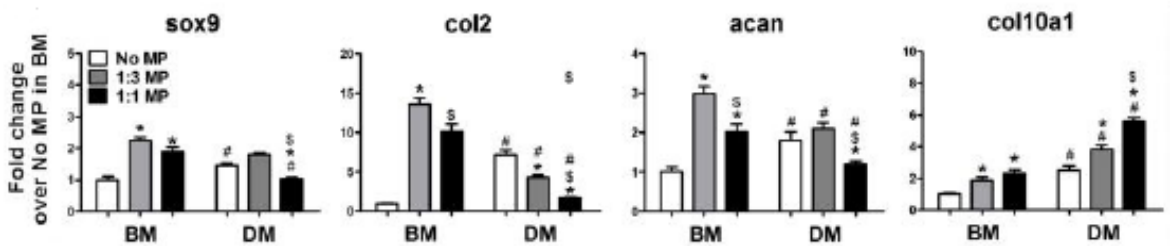

Figure 3. Gene Expression of Chondrogenic Markers in 3D ESC Aggregates

The 3D ESC aggregates were harvested at D14. RT-PCR testing was performed to protocol. Expression levels are presented as the fold-change over the No MP in BM mean±standard error. * = P < 0.05 vs No MP under same culture, $ = P<0.05 vs 1:3MP under same culture, and # = P<0.05 vs BM.

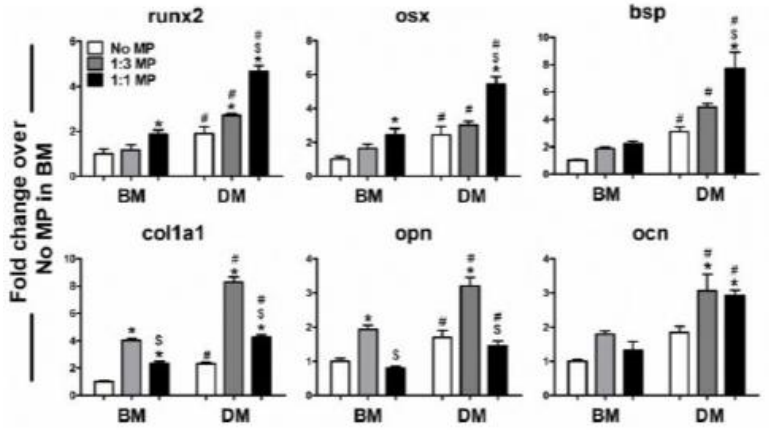

Figure 4. Gene Expression of Osteogenic Markers in 3D ESC Aggregates

The 3D ESC aggregates were harvested at D14. RT-PCR testing was performed to protocol. Expression levels are presented as the fold-change over the No MP in BM mean±standard error. All markers but OCN were significantly different based on media types. * = $P < 0.05$ vs No MP under same culture, $ = $P<0.05$ vs 1:3MP under same culture, and # = $P<0.05$ vs BM.

Histology

The Safranin O/Fast Green and Alizarin Red stains were performed in order to investigate the morphological changes and ECM compositions of the ESC stem cell aggregates. The Safranin O/Fast Green stain showed heterogeneous sGAG production in the DM groups. The Alizarin Red stain showed increased mineralization in DM compared to BM and with increasing mineral particle concentration in DM.

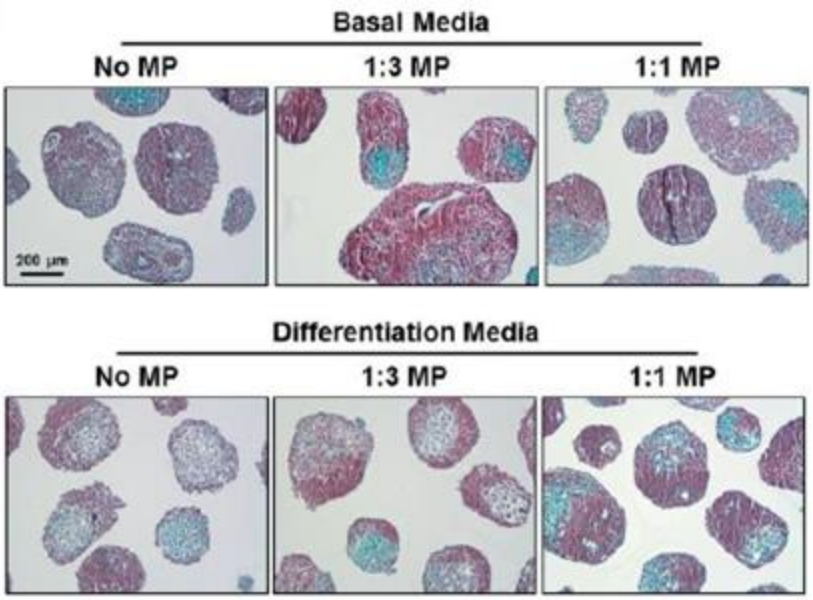

Figure 5. Safranin O/Fast Green Stains of 3D ESC Aggregate Sections

Cells were harvested on Day 14 of culture fixed in 10% formalin and processed in Histogel. The blocks were cut into 5 micron sections, baked, and then stained according to Safranin O/Fast Green protocol.

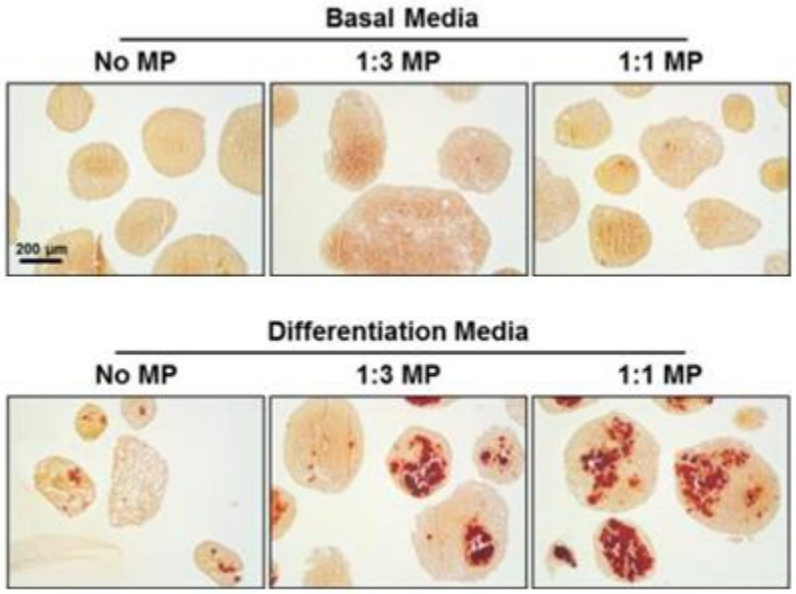

Figure 6. Alizarin Red Stains of 3D ESC Aggregate Sections.

Cells were harvested on Day 14 of culture fixed in 10% formalin and processed in Histogel. The blocks were cut into 5 micron sections, baked, and then stained according to Alizarin Red protocol.

CHAPTER FOUR

DISCUSSION

Influence of Differentiation on Mechanical Stiffness

The mechanical properties of stem cell aggregates changed significantly throughout osteochondral differentiation. The addition of the soluble factors β-glycerophosphate and ascorbic acid into the media increased the stiffness of the ESC aggregates. The analysis of osteogenic and chondrogenic markers strongly indicate that osteochondral differentiation occurred within the stem cell aggregates cultured in the differentiation media. Furthermore, the Safranin O/Fast Green and Alizarin Red stains showed the greater sGAG content and mineralization of the groups cultured in the differentiation media, further indicating osteochondral differentiation. It is evident that significant osteochondrogenesis occurred within the DM groups and increased stiffness as compared to the BM group by day 14. It was expected that the mechanical stiffness would be higher in the DM groups compared to the BM groups. The applied concentrations of bGP and ascorbic acid have proven to induce osteochondrogenesis. The combination of genetic marker expression, mechanical stiffness, and histology demonstrates that the aforementioned osteoinductive cues drove the mechanical changes of the extracellular matrix.

Influence of Mineral Particles on Mechanical Stiffness

The mineral particles produced no significant change in stiffness. There was a possibility the addition of the mineral particles would influence the osteochondral differentiation of the ESC aggregates and in turn increase the stiffness of the aggregates. The combination of the mineral particles with the differentiation media was thought to potentially augment the effects of the

individual components of the DM groups. It was theorized that the content and material properties of the mineral particles would be responsible. Despite the lack of significant change in mechanical stiffness, the mineral particle incorporated aggregates did, however, experience significant genetic marker changes. In the basal media, the mineral particles increased the expression of early osteochondrogenic markers but left late osteochondrogenic markers relatively unchanged. The accompanying Alizarin Red stains showed no remarkable increase in mineralization. Early chondrogenic markers were down-regulated and supporting histology showed low sGAG content. However, all osteogenic markers were up-regulated in all mineral particle groups and the accompanying Alizarin Red stains showed greater mineralization of the samples. The combination of the DM conditions and the mineral particles promoted greater osteogenic differentiation in the aggregates - including cells that were already chondrogenically differentiating. Yet, the greater expression of osteogenic markers did not correlate to a significant increase in the mechanical stiffness of the aggregates. It is possible that the mineral particles induced genetic change via several pathways. The physical presence of the mineral particles may have sent biomechanical cues to the cells, signaling for osteogenic differentiation. Alternatively, the high content of calcium and phosphate in the mineral coated hydroxyapatite particles may have bound and accumulated growth factors within the aggregates, sending biochemical cues for osteogenic differentiation. The increase of osteogenic markers did not significantly affect the mineralization of the extracellular matrix or significant change in stiffness would have been present. With the change demonstrated through histology and RT-PCR and the lack of significant mechanical stiffness change, the examination of additional mechanical properties may better describe the overall effect of the varying concentrations of mineral particles on the aggregates.

CHAPTER FIVE

CONCLUSIONS

The characterization of the mechanical properties of ESC aggregates grants greater insight into their structure and phenotypes. Mechanical stiffness is an important metric to investigate as it grants insight into the extracellular matrix development and phenotype of the cells. It is clear from this study that the exposure to osteoinductive cues stiffens the extra cellular matrix of the ESC aggregates as they osteochondrogenically differentiate. With further study, comparison between the stem cell aggregates and the native tissue can be used to determine efficacy of the stem cell microenvironment manipulation. As the power to control stem cell differentiation grows, the ability to compare the laboratory grown tissue to natively grown tissue and to establish metrics for tissue definition becomes increasingly important.

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VITA

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