THE USE OF SMALL INTESTINAL SUBMUCOSA AS A BIOSCAFFOLD FOR HUMAN EMBRYONIC STEM CELLS

by

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(Under the Direction of Steven L. Stice)

ABSTRACT

Porcine derived Small Intestinal Submucosa (SIS) is one of the most widely used biomaterials available for tissue engineering. Human embryonic stem cells (hESC) could potentially provide an unlimited source of cells suitable for combination with biomaterials for use in regenerative medicine applications. To explore the nature of hESC/SIS interaction, undifferentiated hESCs were cultured in the presence of the SIS matrix, and cellular behavior was characterized using comparative expression profiling of 44 early differentiation genes using quantitative RT-PCR. Results indicate that the SIS matrix is able to influence the differentiation of hESCs towards a vascular endothelial related phenotype. The application of hESCs with the SIS matrix provides a mechanism by which an ESC derived endothelial biomatrix may be produced and may offer support in the development of an engineered artificial tissue with endothelial-like properties for therapeutic purposes.

INDEX WORDS: Embryonic Stem Cell (ESC); Small Intestinal Submucosa (SIS); Matrigel; Mouse Embryonic Fibroblast (MEF); Endothelial Differentiation; Tissue Engineering; Gene Expression

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CHAPTER 1

INTRODUCTION

Introduction to Stem Cells

The isolation and cultivation of Embryonic Stem Cells has the potential to be one of the most significant advancements in medical science since the discovery of Penicillin. If utilized properly, their ability to advance our understanding of biological and medical science will rival that of the human genome project. And when combined with such knowledge, has the potential to exceed the greatest advancements known to date. The use of embryonic stem cells as a means of cell based therapy, combined with genetic modification, could potentially bring an end to many diseases for which there is currently no known treatment or cure.

In the United States alone, 128.4 million people are afflicted with diseases or injuries that could potentially be cured or treated with stem cell technology [1]. More than 3,000 people die each day in the U.S. that could have been treated with stem cell based therapies [2]. In addition to this figure, more than 90,000 patients are currently awaiting organ transplants in the U.S. according to the Organ Procurement and Transplantation Network (OPTN), a contract organization under the U.S. department of Health and Human Resources. It is estimated that 17 people die each day due to a shortage of organs and tissues available for transplant. Many of these patients could also be saved due to the regenerative properties of stem cells and the benefits of stem cell technology.

The introduction of stem cells into medicine represents a new era of medical science. It lays the foundation for the genesis of a new field of medicine, *Regenerative Medicine* [3]. The

principal of regenerative medicine is simplistically straight forward, replace diseased or damaged cells with ones that are healthy and functional. This revolutionary form of treatment is appropriately termed *Cell Replacement Therapy*, and it is this basic technique that provides the fundamental construct for a number of innovative cell based therapies. The role of embryonic stem cells in regenerative medicine and cell based therapies is absolutely essential as they will provide the basis for an unlimited supply of transplantable cells that may be tailored to a patient's specific needs.

The application of embryonic stem cells to regenerative medicine is not the only benefit of stem cell research. There are in fact, several other major employments for embryonic stem cells in science and research that warrant their continued study. One such employment is the use of human embryonic stem cells as a model for early human development [4, 5]. It has always been difficult to map the sequence of events governing early human development due to the moral and ethical constraints regarding research with human embryos. It has thus been necessary to find a replacement. Due to the overlapping similarities between all mammalian species, the mouse has always served as a model for mammalian development. Therefore, much of what we currently know about human embryonic development has come from studies utilizing the mouse as a model. Since the isolation of human embryonic stem cell lines in 1998 [6], there have emerged several key differences between mouse embryonic stem cells and human embryonic stem cells [7]. This raises the question of exactly what other differences of scientific and clinical importance are there between these two species and propels forward the argument for using human embryonic stem cells as the authoritative model for early human development.

Yet another major employment for embryonic stem cells is their potential usefulness in the realm of pharmacology and toxicology [8]. It is sometimes difficult to predict the deleterious

effects of certain novel drugs even when animal testing is present. This holds especially true for embryotoxicology studies. Due to variances between species, assessing levels of embryo toxicity in standard animal models presents somewhat of a challenge. The use of embryonic stem cells in toxicity studies allows for an effective and harmless means of screening novel drugs *in vitro*. This in turn will lead to an increased ability to gauge potential toxicity in humans and lead to safer and more effective drug design. It will also reduce the amount of testing necessary in animal models and may help relieve some of the moral and ethical controversy surrounding animal testing.

Perhaps one of the more innovative uses for embryonic stem cells is their incorporation into bioscaffolds for use in *Tissue Engineering* applications [9]. Biomedical engineering is a relatively new technological field that is on the cutting edge of transplant medicine. It incorporates multiple disciplines of science including medicine, cell biology, materials science, and engineering. Tissue engineering is one of the few specialized areas of biomedical engineering in which stem cells may have a profound effect on the success of the science. The principals of tissue engineering are divided into three major parts: Take cells from a suitable host and expand them in culture until the necessary numbers are attained, seed these cells onto some form of biodegradable extracellular matrix, then transplant the entire construct into a recipient for reconstruction of a mechanical or physiological defect to a diseased or damaged tissue or organ. There has been much success in transplanting bioscaffolds seeded with mature and fetal cell types into recipients needing reconstructive type therapies [9-11]. There are however, still some major limitations that restrict the use of cell-seeded bioscaffolds in transplant medicine. The ability to expand certain cell types *in vitro* and immunoincompatibility issues are but a few of the challenges still facing tissue engineers [11]. It is hoped that the use of

embryonic and/or adult stem cells will offer an alternative solution to these issues and allow patients to forgo the use of immunosuppressive pharmaceuticals that typically accompany transplant surgeries.

It is clear what the potential of embryonic stem cells, as well as other forms of stem cells, has to offer the world. The benefits that stem cells offer to science and medicine for both basic science and practical application technologies are overwhelming. It is thus necessary and prudent to progress forward with research focusing on the development of stem cell based technologies.

History and Development of Stem Cell Biology: A Brief Timeline of Events

The history of stem cells is a long and controversial tale spanning nearly 50 years in the making. Until recently, stem cell biology was virtually unknown to the general public. It remained a specialized field of knowledge limited to scientists and researchers in developmental biology and medicine. This however, all changed in 1998 when a team led by Jamie Thomson at The Regional Primate Research Center at The University of Wisconsin-Madison became the first group to successfully develop an embryonic stem cell line from discarded human blastocysts [6]. This in turn, created a surge of controversy throughout medical, religious, political and ethical faucets, and a debate that exploded into the global media spotlight.

Before the isolation of human stem cells, there was considerable research with stem cells from other species. Even before research with true stem cells, there was research on stem cell-like cells. It was this research that laid the foundation for much of the present day work. Although opinions vary as to which experiments are most significant in shaping the current field of stem cell biology, it appears relevant to begin somewhere around the mid 20th century.

In 1953, a young scientist named Leroy C. Stevens went to work at the Jackson Laboratory in Bar Harbor, Maine. Stevens was working on a project with Clarence C. Little, the founder of the laboratory, to investigate the relationship between smoking tobacco and lung cancer. The project was funded by a major tobacco company hoping to show it was the paper used in the manufacture of cigarettes instead of the tobacco that contributed to cancer [12]. Stevens was using the inbred mice strain 129 in his experiments when he noticed that around 1% of the male mice had unusually large scrotums. Dissection revealed a mass of cells that appeared to contain an undifferentiated portion of embryonic-like cells amongst partially differentiated cell types of various morphologies including skin, bone, muscle and nerves [13].

The type of malignant tumor Stevens had observed is known as a teratocarcinoma or teratoma, (Greek - *teratos* "monster") and until this point, its description in males had only been observed in humans [14-17] and horses [18]. The undifferentiated cells contained within this particular type of tumor are referred to as *Embryonal Carcinoma* (EC) cells because of their resemblance to early embryonic cells. Comparison of tumors between human and horse revealed similar differentiation of adult tissue types with the main difference being that human teratomas often contained EC cells making them malignant, while the horse teratoma fully differentiated into adult tissues rendering it benign. Mouse teratomas show characteristics of both human and horse types [19]. Study of the human form of teratoma provided limited information outside of anatomical and morphological descriptions, and studies in the horse proved to be difficult since teratomas in the horse are rarely malignant, making the isolation of an active form of the tumor elusive. Thus, the discovery in mice created an opportunity to provide an animal model of teratoma that could go on to yield valuable data for oncologist and physicians. Excited with his new found discovery, Stevens applied for a grant from the American Cancer Society to begin an

investigation of teratoma biology. Later, he received a grant from the National Cancer Institute that would go on to fund years of dedicated research to understanding this oddity of nature.

Upon investigation, Stevens learned that if he took sections from teratomas and transplanted them subcutaneously back into strain 129 mice, the transplants would give rise to new teratomas. While most transplants completely differentiated upon transplantation, one in particular flourished. When Stevens isolated this teratoma and examined its morphology, he noticed it was composed largely of undifferentiated EC cells. When Stevens transplanted sections of this teratoma into the intraperitoneal cavities of strain 129 mice, the transplants continued to flourish and give rise to more teratomas. Stevens realized that as long as the undifferentiated portion of EC cells persisted in the transplant, the transplants would continue to flourish and give rise to new teratomas that could be serially transplanted through multiple generations. This effectively established the first known transplantable teratocarcinoma cell line originating from the testes. Serial transplantation studies involving ovarian teratomas in mice [20, 21] alluded to the existence of pluripotent embryonic-like cells being responsible for the progressive growth of serially transplanted ovarian tumors. In addition, these cells appear to be responsible for the continued self-renewal of undifferentiated EC cells as well as the other more differentiated cell types found throughout the tumor. Stevens concurred with these findings by suggesting that the undifferentiated and differentiated cell types found within testicular teratomas "stem from pluripotent embryonic-like cells" [13].

Stevens began an in depth examination of the growth characteristics of this newly discovered transplantable cell line. By mincing up sections of teratoma containing the EC cells and then transplanting them back into the intraperitoneal cavities of strain 129 mice, he produced 15 different sublines using over 100 mice and 1400 different grafts. One subline in particular

showed a high rate of progressive growth and was further subjected to subline derivation. For reasons unknown, some lines produced teratomas that had developmental preferences towards particular tissue types while others showed no preference. With further inspection, Stevens made an observation that would prove to be monumental in directing his future research for the next 20 years. Examination of the ultra-structure of teratomas from one subline in particular revealed a structure with a striking similarity to early stage developing embryos. Stevens was aware that earlier documentations by Peyron and Lewis [15, 16] described blastocyst-like structures in teratomas of humans, and it seemed now that mouse teratomas followed suit as well. Stevens coined the term *Embryoid Bodies* (EB) and attributed the formation of these structures to pluripotent stem cells contained within the grafts [22].

Intrigued, and realizing the potential significance of this new development, Stevens begin studies to trace the origin and biology of these enigmatic cells and the embryo-like structures they produced. This led to research demonstrating that the sequence of tissue differentiation in mouse teratomas follows closely to that of normal mouse embryos. Under certain conditions, such as suspended growth in a fluid microenvironment, pluripotent stem cells of teratomatous origin from both human and mouse will form EBs that mimic the formation of normal early embryos [15, 23]. In 1964, Kleinsmith and Pierce demonstrated the multipotentiality of individual EC cells by clonally isolating individual cells and subjecting them to differentiation and EB formation by grafting them into the intraperitoneal cavities of mice [24]. The differentiation capacity of clonal EC cells *in vitro* was demonstrated as well by Martin and Evans one year later [25]. Further studies conclusively showed that not only did these "undifferentiated pluripotent embryonic-like stem cells" give rise to many different cell and tissue types, but that the resulting EBs also contained the same developmental potential as normal embryos [23, 26].

The exact origin of these cells were elusive, but Stevens began to suspect they were congenital in nature when dissections of testicles from newborn mice revealed teratomas composed almost entirely of undifferentiated embryonal cells mixed in with normal germ cells [23]. This led to a "germ cell" theory of origin that, if proven, would help explain the vast developmental potential of these newly discovered cells. Realizing the value of what he had discovered, Stevens set out to develop a line of mice highly susceptible to this form of tumor. Through selective breeding, Stevens learned that certain genetic and environmental factors could raise the incidence of teratomas from 1 to 10 percent [27]. Now having a model for teratoma formation, Stevens could begin experiments to trace the origins of these embryonic-like tumors more thoroughly. He began dissecting the testes of fetal mice from various developmental stages to discover teratoma formation within the seminiferous tubules as early as embryonic day 15 (E15) [28]. Suspecting Primordial Germ Cells (PGC) as the origin, he decided to graft the genital ridges of E12.5 – E13.5 fetuses into the testes of strain 129 adult mice. Grafts utilizing E12.5 genital ridges showed high incidence of teratoma formation while E13.5 grafts showed little or no teratoma formation [29, 30]. Noting the significant morphological and developmental changes that occur in PGCs between days E12.5 and E13.5, this lent strong evidence to support the germ cell theory of origin. This theory was confirmed in a follow up experiment in which E12.5 genital ridges from fetal mice lacking PGCs were transplanted into strain 129 adult testes and showed little or no incidence of teratoma formation [31].

Surprisingly, grafts from genital ridges of females did not produce teratomas [31]. This led to the idea that there may be an undetermined precursor cell that could give rise to teratomas before sex specification of PGCs in the genital ridges occurred. To test this theory, and in and attempt to produce teratomas from cells that were genetically female, Stevens decided to graft

normal whole mouse embryos into the testes of strain 129 mice. He found that these grafts developed into teratomas that were similar to spontaneous natural teratomas regardless of the sex of the embryos [32, 33]. The developmental similarities between spontaneous or experimentally induced teratomas and normal mouse embryos are undeniably similar. In an effort to better understand the origin and development of teratoma formation, it seemed ideal to study the developmental fate of normal mouse embryos. Previous experiments that showed early embryos could produce teratomas that contained cells which remained undifferentiated through multiple transplant generations prompted the question of whether or not the undifferentiated embryonic stem cells of the early embryo could also be made to retain their pluripotent nature indefinitely through the generation of a progressively growing serially transplantable teratocarcinoma cell line. Thus, the quest to isolate true embryonic stem cells had begun.

In 1970, Stevens transplanted pre- and post-implantation mouse embryos into the testes of adult mice [34]. Some of the resulting teratomas retained their proliferative capacities and the undifferentiated stem cells could be serially transplanted intraperitoneally to other mice. These transplants produced EBs nearly identical to normal early mouse embryos and indistinguishable from spontaneous natural teratomas. Grafts varying from E1 through E9 revealed a strict window of development, from E3 to E6, in producing teratomas with optimal proliferative capabilities [33]. This concurred within the window of development during which pluripotent embryonic stem cells are most prominent. Experiments by Solter [35] narrowed the location of pluripotent stem cells in the embryo to the egg cylinder (embryo proper), and experiments by Stevens, Grobstein, and Levak-Svajger [36-38] zeroed in specifically to primitive ectoderm by grafting dissected sections of developing egg cylinders to various locations in mice. The ectoderm was the only section to form EBs that differentiated into multiple tissue types as well

as maintain a population of undifferentiated pluripotent cells. It seemed that the developmental capacity of pluripotent embryonic stem cells had been found within the "monster" of a mouse.

These experiments provided evidence of a direct link between EC cells of teratomas and Inter Cell Mass (ICM) cells of a blastocyst stage embryo. Experiments aimed at comparing stem cells from the ICM to EC cells from the teratoma demonstrated that the two cell types were essentially homologous [39, 40]. In 1974, Brinster provided evidence of this link by inserting pluripotent EC cells from mouse teratomas into the ICM of blastocyst stage mouse embryos [41]. He was able to show it was possible to produce viable live born chimaeric mice where the EC cells significantly contributed to the development of the embryo. This was significant because it justified and allowed for the use of human EC cells to serve as a loose model of early human development [42, 43]. Because most EC cells are aneuploid, they show little or no integration into the germ line [7].

Now that a direct link had been established between EC cells and cells of the ICM, as well as providing strong evidence showing the ICM was responsible for giving rise to the embryo proper, efforts began to directly isolate a line of "true" pluripotent *Embryonic Stem Cells* (ESC) from the ICM of early mouse embryos. In 1981, this feat was accomplished by two different groups using slightly different isolation methods. In July of 1981, Evans & Kaufman reported in the journal *Nature* they had successfully isolated a line of pluripotent mouse ESCs (mESC) from mouse blastocysts that were capable of being maintained and expanded in culture [44]. This feat was again announced a few months later by Gail Martin in December of 1981 in *PNAS* [45]. These articles marked the first establishment of a true pluripotent ESC line that could be maintained in culture while retaining full developmental capacity. This capacity for differentiation was tested and confirmed in 1984 by Bradley, Evans, and Kaufman when these

mESCs were subjected to the ultimate test of pluripotency by reintroducing them back into the ICM of mouse blastocysts [46]. When these embryos were transferred to surrogate mothers, they produced normal viable offspring that had shown mESC integration into all tissue types including the germ line. Because of earlier work linking ESCs to EC cells, and EC origin to PGCs, attempts to isolate a pluripotent form of stem cell from PGC precursors came to fruition in 1992 when a team lead by Hogan developed a line of *Embryonic Germ* (EG) cells originating from the genital ridges of mouse fetuses [47]. This stem cell type is similar to ESCs from the ICM, but has some behavioral differences that will be discussed later.

In 1995, stem cell biology took another leap forward when a team lead by Jamie Thomson at The Regional Primate Research Center at The University of Wisconsin-Madison established the first primate ESC line from a Rhesus Monkey [48]. This ultimately led to one of the most significant events in science and medicine in recent history. Just three years later in 1998, Thomson's team isolated the first human ESC (hESC) line from discarded human blastocysts [6]. This was complemented only weeks earlier by the derivation of human EG (hEG) cells by John Gearhart's team at John Hopkins University Medical School [49]. These human and non-human primate ESCs show full developmental capacity as demonstrated by their ability to differentiate into tissue types of all three embryonic germ layers. They form EBs both *in vitro* and *in vivo* when transplanted subcutaneously and intraperitoneally into mice [6, 48, 49]. ESC lines have also been established from various other species including but not limited to: Rat [50], Pig [51], Sheep [51], Chicken [52], Mink [53], Hamster [54], Rabbit [55], Cow [56], Marmoset [57], and most recently Dog [58].

As the future of stem cell science progresses into the new era, there awaits still a multitude of discovery. The science is, at best, still in its infancy as compared to other fields of

biology. There are still several key areas that must be refined and explored in order for stem cell research to succeed in reaching its full potential. One of which is mastering ESC derivation of specific cell types *in vitro*. There are numerous examples of this already [59], but a higher efficiency and greater variety of cell types is still necessary before the full therapeutic potential is reached. The use of stem cells as a form of cellular transplant therapy also faces the same obstacles as traditional transplant therapies such as immunorejection of transplanted tissues. Production of patient specific stem cells through *Somatic Cell Nuclear Transfer* (SCNT) remains a controversial but realistic solution to such dilemmas. The isolation of an mESC line from a cloned mouse embryo has already been accomplished and is paving the way for future research utilizing this technology [60]. The establishment of patient specific hESC lines is undoubtedly an eagerly anticipated event. Once this feat is accomplished, we will witness a new era in medical science when these same hESCs will be used to perform autologous transplants back into the donor to cure or treat illnesses of all kinds.

Stem Cell Biology: A Short Review

Properties of Stem Cells

Stem cells hold great promise as both a resource for understanding the basic elements of cell and developmental biology, and as a tool for providing limitless therapeutic potential for medical science. They are unique in that they have the ability to develop into a multitude of various cell and tissue types, and under appropriate conditions, are capable of continued self-renewal. But not all stem cells share equal properties. Stem cells from different sources display different characteristics giving each its own set of benefits…stem cells are available in five major varieties: *Embryonic, Embryonic Germ, Adult, Germ, and Fetal*.

Embryonic Stem Cells (ESC) are defined by two major properties [7]. The first is their ability to differentiate into all cell and tissue types of the body. This property is referred to as *pluripotency*. The second is their ability for continued *self-renewal*. This allows for an unlimited copy of cells under appropriate culture conditions. It is these properties that make ESCs such an attractive and powerful tool for medical research. In human embryos, ESCs make up the ICM of a 5-7 day old blastocyst. Under normal conditions, the ICM will eventually go on to form the embryo proper and give rise to a developing fetus. It is within this time frame that cells from the ICM are harvested to produce ESCs for therapeutic or research purposes [6, 7]. Because ESCs are naturally only available for a short period of time in a developing embryo, the immortalization of ESCs through continued self-renewal is an artifact of the culture system.

Embryonic Germ (EG) stem cells are derived from primordial germ cells found within the genital ridges of developing fetuses. In human, this occurs at around eight and a half weeks [7, 49]. They are referred to as EG cells to denote their cell of origin. EG cells behave *in vitro* similarly to ESCs in that they are pluripotent and self-renewing, but they also have key differences that set them apart from true ESCs. Because EG cells are derived from PGCs, they have already gone through genome reprogramming cascades [61], a property ESCs do not display. It is unsure what effects this will have on the applications of EG cells at this time.

Adult Stem cells (ASC) are derived from a self-renewing population of stem cells contained within a specialized niche located within the differentiated tissues of mature organs [62]. Adult stem cells are *multipotent*, meaning they have the capacity to give rise to all cell types contained within the organ or tissue in which they reside. There is also evidence adult stem cells show some degree of *plasticity*, meaning they have the ability to give rise to cell types outside of their organ of residence, or *in vitro*, to differentiate into cell types originating from a

different embryonic germ layer from that of their native organ. It is suspected that most or all organs contain one or more populations of adult stem cells that are responsible for maintaining the health and viability of the organ throughout the life span of the organism. *In vitro*, adult stem cells seem to show greater stability against unwanted differentiation as compared to ESCs, but have limited self-renewing capabilities.

Germ Stem cells, also called Germline Stem cells, are a type of adult stem cell specifically found within the gonads of mature organisms and are responsible for the production of gametes [63, 64]. Germline stem cells show long term self-renewal capacity *in vitro* but their differentiation capacities have not been thoroughly tested [65].

Fetal Stem cells (FSC) are cells present in the circulation during the fetal stages of development [66]. There are two main types of fetal stem cells found in circulation: Fetal Hematopoietic and Fetal Mesenchymal stem cells. Fetal stem cells are multipotent, but are slightly different from their adult counterparts in that they seem to have better proliferative capacity *in vitro* and are capable of differentiating into a greater variety of cell types. Stem cells harvested from the placenta and umbilical cord of newborns contain predominately hematopoietic fetal stem cells [67].

While all the different types of stem cells have the potential to be therapeutic, it is the ESC that holds so much promise. It is this type of stem cell that may hold the key to unlocking the regenerative potential of this new found science and push forward the boundaries of human medicine. The attractiveness of ESCs is that they are a theoretical blank slate, a snapshot of neutrality that contains within them the genetic potential to derive all cell types of the body and some extra-embryonic tissues as well. It is this property that makes them such a valuable resource for medical science.

Morphology & Characterization

ESCs are round, nearly transparent, and typically between 15-20μm in diameter. The nucleus takes up nearly 90% of the cytoplasmic area with two or more prominent nucleoli usually visible [6, 44, 45, 48, 57]. The cytoplasm is composed almost entirely of free ribosomes and mitochondria, indicative of the high rate of cellular protein production and the energetically expensive processes necessary for continued self-replication. The cell cycle for hESCs is typically around 24-36 hours [68]. ESCs in culture grow in circular, loosely compacted, mound shaped colonies consisting of anywhere between 100 to 1000 cells per colony before passaging is necessary. Because early differentiating ESCs are difficult to distinguish from pluripotent ESCs, a set of glyco-conjugate surface markers and intracellular transcription factors are used to characterize the state of ESCs. The POU transcription factor OCT-3\4 (hereafter referred to as OCT-4) is the most widely accepted marker of ESC pluripotency. While OCT-4 is a strong indicator of pluripotency, its combined expression with additional markers is important for characterization of the pluripotent state. The expression of OCT-4 in combination with selective surface markers SSEA-4 and SSEA-3 (Stage Specific Embryonic Antigen) are typically characteristic of pluripotent hESCs [6, 69, 70]. The markers TRA-1-60 and TRA-1-81 (Tumor Rejection Antigen) are also heavily used markers to identify hESCs [6, 70]. In addition, hESCs test negative for SSEA-1 and show high Alkaline Phosphatase (AP) activity [6]. All primate ESCs to date share the same marker expression patterns. hEG cells on the other hand, test positive for SSEA-1 while also testing positive for OCT-4, SSEA-3\4, TRA-1-60\81, and AP [49, 71]. Mouse ESCs and EG cells in contrast, are positive for OCT-4, SSEA-1, and AP, but negative for SSEA-3\4 and TRA-1-60\81 [44, 45, 47, 60]. It is note worthy to add that a lot of the early work that led to the identification of these markers began with mouse EC cells from

teratomas. Thus, ESCs and EC cells share a nearly identical set of marker expression patterns in both mouse and human [7].

Isolation and Maintenance of Stem Cells in Culture

The isolation of ESCs can be accomplished by several methods. The most common method, and the one used for the isolation of mouse and human ESC lines [6, 45], is a technique developed by Solter in 1975 known as *immunosurgery* [72]. In this technique, blastocysts are exposed to antibodies specific to antigens present on the trophectoderm cells surrounding the ICM. The blastocyst is then subjected to complement serum and the surrounding trophectoderm cells are effectively destroyed leaving only the ICM. The ICM is then briefly treated with a trypsin digest to achieve a single cell solution before immediately transferring to a culture system. Culture of ESCs most commonly involves co-culture with inactivated Mouse Embryonic Fibroblasts (MEF), either by irradiation or chemical treatment with a compound such as Mitomycin C, which intercalates itself into the DNA inhibiting transcription and DNA synthesis [7]. ESCs are considered an anchorage dependent cell type, and thus need something to attach to in order to achieve optimal proliferation in the undifferentiated state. ESCs that are not allowed to attach and are grown in suspension will differentiate more quickly and form EBs sooner than ESCs allowed to adhere to a matrix such as that produced by MEFs. Aside from producing an extracellular matrix rich in protein and biologically active compounds, it is also believed that MEFs produce factors that are highly beneficial to maintaining ESCs in an undifferentiated state [73]. This earned MEFs the nickname *feeder cells* because it is believed that MEFs are providing factors that "feed" ESCs to promote high levels of proliferation and self-renewal while maintaining pluripotency. In 1987, it was found that MEF feeder cells could be forgone under specialized culture and media conditions [74]. This finding now allowed

culture systems to exist in a feeder free format, and MEFs were replaced with the purified extracts of collagen and other protein rich mixtures that mimicked the natural ECM produced by MEFs. Products such as Matrigel, a basement membrane ECM, were shown to successfully culture ESCs in an undifferentiated state for extended periods of time [75, 76]. Early work involving cultures with synthetic matrices however, still required the use of unknown tropic factors thought to be secreted by MEFs, so ESCs grown on Matrigel had to be cultured in media that had been conditioned by MEFs or other cell types [75, 76]. ESC culture media generally consists of a basic formulation of necessary proteins, sugars, fats, vitamins and minerals supplemented with 10-20% Fetal Bovine Serum (FBS). L-glutamine, Basic Fibroblast Growth Factor (bFGF), and β-Mercaptoethanol (BME) are also commonly added to ESC media [7]. mESC media incorporates the use of Leukemia Inhibitory Factor (LIF) which has been shown to be a key factor in promoting growth of mESCs in the undifferentiated state [77, 78]. LIF has shown little or no effect on human or primate ESCs [6, 48]. Today, ESC culture has evolved into almost a completely feeder and serum free system. By using synthetic matrices and serum replacement products, ESCs can now be successfully cultured under nearly defined conditions [79, 80]. In 2005, it was reported that an hESC line had been derived completely under feeder and serum free conditions [81]. This accomplishment represents an important step in the use of hESCs as a form of cellular transplant therapy. The use of MEFs in hESC culture is an issue that must be over come as hESCs co-cultured with MEFs are considered xenotransplants. The use of xenotransplants in medicine is highly regulated and hESCs that fall under this category may not be suitable for transplantation therapies to humans. Thus, the establishment of a feeder and serum free culture system, as well as the derivation of hESCs under these conditions, is essential if hESCs are to be used as a means of cellular transplant therapy in humans.

Molecular Biology of Pluripotency and Self-Renewal

Maintenance of pluripotency and self-renewal involves a series of signal transduction cascades that must coexist in a delicate balance. The continued state of self-renewal is dependent upon the continued state of pluripotency and vice versa. When one of these properties is interrupted, the other follows suit shortly after. The intricacies of this dance involve several key performers and are still somewhat cryptic. However, certain advancements have been made that are beginning to illuminate the mechanisms of this balance.

In 1987, it was discovered that media conditioned by Buffalo Rat Liver cells was sufficient to maintain mESCs in an undifferentiated state without the use of MEF feeder cells [74]. It was found shortly after that the polypeptide responsible for this action was nearly identical to a recently discovered hematopoietic regulatory factor [77]. A single cytokine, Leukemia Inhibitory Factor, was found to be sufficient in maintaining both pluripotency and self-renewal in mESCs [77, 78]. LIF works by acting on the LIF specific receptor subunit and the gp130 subunit, which is also responsive to a selective group of related IL-6 cytokines [82]. When activated, these two subunits come together to form the functional LIF receptor and activate the JAK/STAT (Janus Associated Kinase/Signal Transducing Activator of Transcription) pathway [83]. Upon activation, the tyrosine kinase JAK5 recruits and phosphorylates the transcription factor STAT3. STAT3 activation forms STAT3 homodimers which then migrate into the nucleus to activate or suppress genes important for pluripotency and self-renewal. STAT3 activation alone is sufficient for maintaining self-renewal of mESCs [84] and when STAT3 activation is suppressed, differentiation ensues [83, 85].

Stimulation of the gp130 subunit by LIF also activates the Ras/Mitogen Activated Protein Kinase (MAPK) pathway [86]. In this pathway, the tyrosine phosphatase SHP-2 binds with

Gab1 and RAS at the gp130 receptor to activate Raf and MAPK kinases (MEK). MEKs in turn activate serine/threonine MAP kinases which phosphorylate other proteins that move into the nucleus to activate various transcription factors that promote differentiation [87]. The addition of MEK inhibitors to mESC cultures thus increases self-renewal and enhances STAT3 effects [88].

Stimulation of two different pathways that drive ESCs in opposite directions may seem counter productive, but LIF is not the only compound that has this effect on ESCs. Bone Morphogenic Protein (BMP) has similar effects on ESCs in that it initiates signal transduction cascades that promote both self-renewal and differentiation at the same time [89-91]. BMP binding activates type I and type II serine/threonine kinase receptors to form a heterodimer in much the same fashion as the LIF receptor [92]. Activation of the BMP receptor recruits and phosphorylates SMAD proteins which then combine with a co-SMAD protein such as SMAD4, forming a heterodimer which moves into the nucleus to interact with various transcription factors to promote either self-renewal or differentiation. BMPs are part of the TGFb Superfamily of growth factors, and it has been shown that activation of the TGFb/Activin/Nodal pathways also contribute to either differentiation or pluripotency through the activation of various SMAD complexes [93]. One of the targets of the SMAD complex is the Id (Inhibitor of Differentiation) set of genes [90]. It has been shown that Id proteins will bind with pro-differentiation proteins, neutralizing their differentiating effects and promoting pluripotency and self-renewal [90, 94].

In culture, mESCs grown in a feeder free system will maintain self-renewal and pluripotency if supplemented with LIF and FBS [77, 78]. If FBS is removed but LIF is maintained, mESCs will still differentiate towards a neuroectoderm fate, despite the presence of LIF [73]. If FBS is retained but LIF is removed, mESCs will differentiate towards a mesoderm

or endoderm fate [73]. Since BMP4 has been shown to have an anti-neural effect in developing embryos [95], and BMP4 and FBS have been shown to induce expression of Id proteins in much the same manor [90], mESC cultures supplemented with both LIF and BMP4 in a feeder free system are able to maintain pluripotency and self-renewal even in the absence of FBS [90]. It seems then, that LIF and BMP4 occupy two sides of the same coin. LIF acts to inhibit differentiation towards a mesodermal or endodermal lineage while BMP4 acts to inhibit differentiation towards an ectodermal lineage. Because mESCs cultured in the absence of both of these two compounds naturally differentiate into cell types of all three embryonic germ layers, LIF and BMP4 seem to work synergistically to inhibit the default pathways of differentiation.

While STAT3 expression and BMP4 activation together have been shown to be sufficient in maintaining pluripotency and self-renewal, they may not be entirely necessary. In 2003, Chambers and Mitsui reported in the journal *Cell* the discovery of a homeobox protein transcription factor known as Nanog [96, 97]. Nanog has been shown to be capable of maintaining pluripotency, self-renewal, and epiblast formation [96, 97], and is expressed specifically in ESCs, EC, and EG cells, but not in adult stem cells or differentiated cells [96, 97]. Nanog has been shown to induce expression of Id proteins in the same manner as BMP4 [90], and to bind with SMAD proteins inhibiting SMAD activated differentiation [98, 99]. ESCs that overexpress Nanog could be able to maintain pluripotency and self-renewal in a feeder and serum free culture system even in the absence of both LIF and BMP4 [90, 96, 97]. Nanog appears during the morula stage of development and is retained throughout the ICM of the blastocyst as well as by the early PGCs [97, 100]. In mice deficient for Nanog, the ICM spontaneously and prematurely differentiates into visceral and parietal endoderm [100]. It is unclear how Nanog is activated but studies indicate it is downstream from OCT-4 [73].

Like Nanog, OCT-4 is expressed in pluripotent ESCs, EC, and EG cells [101]. Its expression is critical for proper formation of the ICM and ESCs, and a loss in OCT-4 expression results in a loss of self-renewal and pluripotency [102]. Its expression must be maintained within specified levels for self-renewal to continue, and even a small increase or decrease will initiate differentiation events [103]. While OCT-4 remains to be one of the most important and crucial factors in maintaining pluripotency and self-renewal of ESCs, it alone is not capable of maintaining the pluripotent state [103]. OCT-4 must therefore work in combination with other factors such as STAT3, SMADs, and Nanog to maintain the pluripotent state. OCT-4 works as a transcriptional co-factor by binding with other transcription factors such as REX1, SOX2, and FOXD3. All have been shown to act cooperatively with OCT-4 to regulate pluripotency and self-renewal of ESCs [104-107]. It seems then, that there are several key pathways that act cooperatively to achieve the same end result, maintenance of the pluripotent state and self-renewal.

Tissue Engineering Applications of Embryonic Stem cells

Introduction to Tissue Engineering

The use of stem cells in Regenerative Medicine falls into two major categories: Cell Replacement Therapy and Tissue Engineering. In cell replacement therapy, stem cells are used to replace diseased or damaged cells of a defective tissue or organ with ones that are healthy and functional. This would incorporate the use of stem cells in a single cell solution that could be administered as a systemic injection or into the organ or tissue directly. Cell replacement therapy can also apply to cells in a simple tissue format. This includes cells engineered to form simple homogeneous layers of a specific cell type, or small aggregates of heterogeneous cell

types that function together as a cohesive unit. In tissue engineering, cells are used to construct three dimensional tissue replacement transplants with the aid of a bioscaffold or biomaterial carrier [9]. The entire construct is then transplanted or grafted into the recipient for the correction of a structural, mechanical, or physiological defect of a diseased or damaged tissue or organ. Tissue engineering is a multidisciplinary field that brings together cell biology, medicine, materials science, and engineering to form a constructive unit of functional science. Difficulties with traditional tissue engineering procedures are mostly at the cellular level and are similar to issues seen with traditional transplant therapies such as cellular availability and immunotolerance [9]. It is hoped that stem cells will alleviate these issues and move the field into its next stage of evolution.

Principles of Tissue Engineering

The philosophy of tissue engineering directs reconstruction from two main approaches. The first involves the use of cell-seeded bioscaffolds as a tool for reconstructive surgical therapy [9], while the second involves the use of acellular biomaterials as a source for regenerative initiation by the body's own resources [108]. Traditionally, tissue engineering has held strict to the first approach. In this method, an artificial tissue-like construct is generated through the combination of cells and an acellular bioscaffold. First, cells are taken from a reliable source, usually the patient, and cultured *in vitro* to achieve the number of cells necessary for therapy. These cells are then seeded onto some form of bioscaffold, or incorporated with a biomaterial known as a carrier, that will allow for the continued proliferation and survival of the seeded cells *in vitro* and *in vivo* once transplanted [9]. The last step involves the utilization of this cell-seeded bioscaffold or biomaterial to be surgically implanted into the patient for the correction of a physiological or mechanical defect. The use of biomaterial carriers such as hydroxyapatite and

tricalcium phosphates together with bone marrow stromal stem cells to aid in the regeneration of bone tissue [109], or the use of cultured adult stem cell keratinocytes combined with a biocompatible dermis-like substrate to produce skin grafts [110], are prime examples of how biomaterials combined with cellular materials can produce a tissue engineered product.

The second method of tissue engineering takes on a slightly different approach. It relies on the use of acellular biomaterials or bioscaffolds to assist the body with its own natural regenerative properties. For various reasons, sometimes the body is just not able to cope with the demands required for tissue remodeling following traumatic injury. In other cases, factors such as genetic or age related issues hamper the success of natural regeneration. The aim of tissue engineering in this respect is to use transplanted biomaterials either conditioned with or containing biomolecules that will initiate regenerative processes to start that would normally not be able to [108]. This is accomplished by naturally recruiting native cells to the site of injury and using the bioscaffold as a template for tissue remodeling. The use of porcine Small Intestinal Submucosa (SIS) in the regeneration of the bladder wall after trauma is an example of an acellular bioscaffold naturally laced with growth factors that can be implanted to a site of injury and initiate natural regenerative process [111]. The use of gelatin microspheres containing bFGF or VEGF can also be transplanted with, or incorporated into, bioscaffolds to aid in angiogenesis of transplanted tissues [112]. The same technique using Nerve Growth Factor has been utilized to promote the growth of nerve cells [113]. The use of biocompatible beads or capsules that have been coated or filled with growth factors like TGFb or other cytokins could be transplanted directly into soft tissue wounds to speed recovery and reduce scaring. This method is used regularly in developmental biology to examine the effects growth factors on local developmental processes and could easily be adapted for therapeutic use.

Properties of Bioscaffolds & Biomaterials

An ideal biomaterial should demonstrate certain basic properties if it is to be successful as a therapeutic tool. The first of these is cell-material compatibility. A good biomaterial should always be compatible with the selected or target cell type. How different cell types react when seeded onto a biomaterial is predominately influenced by the composition of the material. Bioscaffolds can be either completely synthetic, derived from natural tissues, or a combination of both [9]. The scaffold may have certain beneficial or adverse effects on the cell type used so proper matching of cell type with material is essential. Some materials can change the physiology or even influence the phenotype of certain cells when seeded onto them [114]. The materials must be conducive to continued growth and proliferation of seeded cells as the product transcends from an *in vitro* to an *in vivo* environment [11].

The next major consideration is how the acellular or cell-seeded bioscaffold will interact with the target environment. If the product is to be used in soft tissue reconstruction, then a natural biodegradable material is preferred. If the product is to be used for correction of a mechanical or structural defect, such as tendon, joint or bone reconstruction, then a much more durable and longer lasting material such as a synthetic polymer or ceramic type material may be preferred. If biomaterials are to be transplanted in an acellular format and are relying on the natural engraftment of cells once transplanted, then the chemical nature of the material must agree with the chemical nature of the environment. It is important that the transplanted biomaterial will not simply degrade, but will also successfully recruit the target cell type for that environment [115]. Physical stress on the construct must also be taken into account. Transplants to areas subject to high levels of friction and mechanical or structural stress, such as a tendon, bone, or joint must utilize materials with high tensile strengths and durability.

All biomaterials should ideally be biodegradable over time. However, depending on the area of transplant, time of expected regeneration, and physical stresses placed on the implant, not all biomaterials are equal for every application. Biodegradation of various materials range from a few weeks for hydrogels, to a few years for ceramics [9]. Natural materials such as the SIS matrix can vary in weeks to months depending on if it is used as an acellular graft, or a cell-seeded graft, and can also vary by location and cell type used. [111, 116-118]. As tissue remodeling takes place, transplanted cells within the bioscaffold, or naturally recruited native cells, begin to lay down their own extracellular matrix. Eventually the artificial matrix of the biomaterial should be slowly broken down, replaced, and reabsorbed as the surrounding tissue is regenerated and remodeled [119].

Once transplanted, adequate blood supply and vascularization must be established quickly or cells could die [10]. By incorporating angiogenic factors into scaffolds and biomaterials prior to transplant, or by the co-transplant of angiogenic inducing microshperes, angiogenesis can be greatly accelerated [112]. Certain natural biomaterials, such as the SIS matrix, contain growth factors to help with cell proliferation and angiogenesis naturally [120].

To ensure success after transplantation, biomaterials must be biocompatible with the host immune system. It is important that there be no host immune response to avoid immunorejection of the transplant. Some acellular materials can be encapsulated with semipermeable membranes which add additional immune protection to lessen the response [121]. Hopefully, this will eventually be overcome by the use of custom designed biomaterials specific for each tissue environment [9]. The use of natural materials, such as the SIS matrix, show little or no immune reaction making it an ideal candidate for soft tissue reconstruction in either an acellular or autologous cell-seeded format [122-124].

Tissue Engineering and Embryonic Stem Cells

One of the major issues surrounding early tissue engineering was the generation of sufficient quantities of specific cell types *in vitro* for combination with biomaterials [11]. Cell culture of defined cell types in recent years has become sufficient for *in vitro* studies, but not for large scale tissue engineering applications [9]. For tissue engineering to move into the next phase, a more readily available source of cells is still needed. The use of ESCs hope to alleviate this problem by providing a potentially unlimited source of cells available for differentiation and subsequent transplantation [7]. ESCs will provide tissue engineers with a resource for the derivation of any cell type needed in large enough quantities for transplant. They will enable scientist to custom engineer cells with specific traits, through genetic modification, that will be necessary for specialized applications. Stem cells could be engineered to function optimally with various types of biomaterials and bioscaffolds, or be free to let the inherent properties of natural biomaterials influence their phenotype directly. Research with biomaterials containing plasmid vectors capable of genetically modifying cells *in vivo* is redefining the way materials alter cell behavior and physiology [125]. This method, in combination with biomaterials laced with specific growth factors and cytokins, could provide a powerful tool for producing high levels of specific cell types directly from ESCs simply by seeding them onto the appropriate bioscaffold.

While current strategies focus mainly on the production of living tissues, the future of tissue engineering will no doubt progress towards whole organ generation. The use of stem cells to culture functional units of organs or develop organs suitable for transplant may not be too far off. Chondrocytes embedded in biocompatible gels cultured around a moldable scaffold have produced artificial ears *in vitro* [9]. In 2006, the first tissue engineered bladder was transplanted into human patients by taking urothelial and bladder smooth muscle cells from the recipient,

expanding them *in vitro*, then seeding them over a bladder shaped bioscaffold to produce the first fully tissue engineered organ for human transplant [126]. ESCs could be used to generate the appropriate cell types or functional units of organs *in vitro*, then seeded onto the appropriate scaffolds to take the shape of natural organs. Once these organ replacement cell-seeded bioscaffolds are dense enough for transplant, they could be placed in profusion chambers to initiate vasculogenesis and infiltration of the construct. Once the proper network of vasculature is created, these products could be transplanted into patients to replace failing organs. If patient specific stem cells could be derived through SCNT, then custom designed, genetically matched, biological constructs specific to each patient's individual needs could be met. The use of immunosuppressive pharmacotherapy would be unnecessary as the cells would be a perfect genetic match to the patient.

As modern medicine moves into the 21st century, so does the need for the advancement of medicine. Better healthcare means people are living longer and better lives, which results in more age related disorders, which in turn increases the demand for even better healthcare solutions. It is anticipated that advances in tissue engineering and stem cell biology will rise to meet these challenges by providing a method for which therapeutic levels of cellular material may be produced for combination with biomaterials for the establishment of cell based therapies.

Small Intestinal Submucosa: A Bioscaffold for Tissue Engineering

Introduction to the SIS matrix

Small Intestinal Submucosa, or SIS, is a type of natural acellular biopolymer that has been used extensively in numerous tissue engineering applications. SIS represents, in many ways, the ideal bioscaffold. It is exemplary of every characteristic desired in a multifunctional

diverse biomaterial. It has been shown to be extremely conducive to growth of multiple cell types *in vitro* [120, 127], and may be transplanted into patients to numerous locations in either a cell-seeded format [128-130] or an acellular format [111, 131, 132]. SIS naturally recruits multiple cell types and maintains cell function *in vivo* [111, 132, 133], is able to partake in extensive tissue remodeling [111, 132, 133], and is capable of inducing angiogenesis upon transplantation [111, 128, 132]. SIS is also completely biodegradable [111, 128, 132] and shows no signs of immunorejection [123, 124, 128]. SIS is FDA approved and has been used in numerous clinical applications such as vascular reconstruction [134], vascular flap replacement [135], myocardial wall repair [132], bladder wall repair [111], stomach wall repair [136], intestinal wall repair [131], urethral repair [137], body wall and pelvic floor repair [129], hernia repair [138], corneal repair [139], ear cartilage regeneration [140], neural dura reconstruction [141], tendonous repair [142] and enhanced bone growth [143], just to name a few. To date, it is estimated that over 200,000 patients have been treated using the SIS matrix for soft tissue remodeling and wound repair [144]. SIS is one of the best selling and most widely used bioscaffolds on the market today. It was designed in 1988 by Stephen Badylak at Purdue University in an effort to find a suitable material in which soft tissue remodeling could take place in a three dimensional environment. Until its innovation, available materials feel short of expected performance and caused several problems when transplanted *in vivo* [134]. It was first proven as a superior material in 1989 when it was used as a vascular graft in the aorta of dogs [134]. Since then, the number of clinical applications that have been developed utilizing this extraordinary biomaterial is growing steadily. The production of SIS for commercial use is overseen by Cook® Biotech Incorporated (www.cookbiotech.com). Different treatments of SIS have yielded several products with specific properties optimized for use in specialized

applications. For example, Surgisis® can be applied to thoracic, colon, plastic, and general surgeries as well as hernia and body wall defects. Stratasis® is optimized for urinary treatments, and Oasis® Wound Matrix is used for partial and full thickness wound management. The use of SIS in tissue engineering applications follows the same principles as outlined in the previous tissue engineering sections. Briefly, appropriate cells would be taken from a patient through a soft tissue biopsy. They would be cultured *in vitro* until adequate cell numbers were obtained, then seeded onto the SIS matrix for further proliferation. The cell-seeded SIS would then be transplanted back into the patient for reconstruction of a specific defect. The SIS matrix could also be directly transplanted into a patient in the acellular format. In this example, the SIS matrix would be secured into position over a defect such as a hernia or soft tissue perforation using sutures or some other means. In either case, after transplant the matrix will naturally engraft the appropriate cell types and promote angiogenesis of new vasculature. Over a period of weeks to months, extensive tissue remodeling occurs and the SIS matrix is slowly degraded and replaced with the extracellular matrix of the endogenous cell types. This allows new tissue to be effectively remodeled and physiological function to be restored without scar tissue formation.

Preparation of the SIS matrix

While there may be slightly different methods for preparing SIS, they all follow the same basic protocol [145]. The SIS matrix originates from porcine small intestine. The small intestine consists of three sections. Starting from the stomach, there is the duodenum, which is the shortest of the three sections, the jejunum in the middle, and the longest part, the ileum, at the end connecting to the colon. In cross section, the layers from outside to inside are: Serosa, External Muscularis, Submucosa (Tunica Submucosa, Muscularis Mucosa, and Stratum Compactum), and Mucosa (Figure 1.1A). Sections of the jejunum are harvested and the serosal

and external muscularis layers from the outside, along with the superficial mucosal layers from the inside, are removed mechanically within four hours post mortem. This leaves the submucosa, which consists of the tunica submucosa making up the abluminal side of SIS, and the basilar layers, muscualris mucosa and stratum compactum, making up luminal side of SIS. The luminal (mucosal) side of SIS is smooth (Figure 1.2A) and cells seeded onto this side have a tendency to grow in mono layers and remain along the surface [120]. The abluminal (serosal) surface is more porous and rough (Figure 1.2B) and cells seeded onto this side have a tendency to migrate into the matrix [120]. Once the submucosa layer is isolated, it is cut longitudinally to create a flat sheet which is then treated with dH_2O to lyse all cellular material. Next, 0.1% peracetic acid, 20% ethanol, and gamma irradiation or ethylene oxide are used to disinfect and sterilize the material [120, 145]. Once the material is processed (Figure 1.1B), it can be stored in either sterile PBS or dH_2O at 4° C, or can be dehydrated for long term storage [145]. The dehydrated form of SIS gives it a shelf life of roughly 18 months and rehydration consists of simply incubating the material in PBS or culture media for approximately 10 minutes.

Structural Properties of the SIS matrix

One of the properties that make the SIS matrix such an ideal biomaterial is its natural three-dimensional architecture which allows cells to cultivate in an environment similar to their own natural extracellular environment. The extracellular matrix (ECM) plays an important role in maintaining the health and structural stability of living tissues [144]. It provides a structural framework on which cells can proliferate and perform tissue specific task. The type of ECM in a given tissue is specific to the cell type present, and can be influenced by various forces such as mechanical stress, biochemical mediums, pH, oxygen content, or hormonal and protein constituents [144]. The ECM and residing cell types thus form a symbiotic relationship with

each influencing the other. The ECM consists of various proteins, glycoproteins, glycosaminoglycans, and numerous growth factors and cytokins. The most abundant proteins found in the ECM are the collagens. With more than 20 different types currently known, they make up more than 90% of the dry weight of the ECM, with collagen Type I being the most common [144]. Glycoproteins such as Fibronectin and Laminin come in second, and are important adhesion molecules between cells and the ECM [146]. Both play critical roles in vascular formation and proper organ and tissue development [144]. Glycosaminoglycans, such as the Chondroitin Sulfates, Heparin, Heparan Sulfates, and Hyaluronic acid, are important for binding growth factors and cytokins and maintaining proper osmotic balance [144]. Growth factors and cytokines are important components of all ECMs and allow for the proper growth, proliferation, and differentiation of new cells during times of tissue remodeling and wound repair [110]. Cytokines commonly present in some, but not all, ECMs are: Vascular Endothelial Growth Factor (VEGF), Fibroblast Growth Factor (FGF), Transforming Growth Factor-Beta (TGFb), Bone Morphogenic Protein (BMP), Epithelial cell Growth Factor (EGF), Keratinocyte Growth Factor (KGF), Hepatocyte Growth Factor (HGF), and Platelet cell Derived Growth Factor (PDGF).

More than 90% of the dry weight of the SIS matrix is composed of collagens Type I and III, with an overwhelming majority (>90%) being Type I [145]. Other collagens include Types IV, V, and VI [127]. Fibronectin is present in small amounts, around 0.08% [146], but is greatly increased by a variety of cell types during tissue remodeling [147]. The glycosaminoglycans Chondroitin Sulfate A and B, as well as Heparin, Heparan Sulfate, and Hyaluronic acid, are also all present in the SIS matrix [148]. Primary growth factors found within the SIS matrix include VEGF [149], FGF-2, and TGFb1 [150].

The SIS matrix is approximately 80um thick after processing [120] and shows a high degree of polarity as evidenced by the difference in the multiple layers that compose the matrix, the difference in cellular behavior in respect to each side [120], and the directional permeability of liquids through the matrix [151]. Permeability, as measured by static water passage through the matrix at 120mmHg, is four times higher in the serosal to mucosal direction than from mucosal to serosal. Permeability is termed porosity, and in terms of biomaterials, higher is better. Natural, unprocessed SIS has a porosity of 2.99ml/min cm^2 and increases to 8.33ml/min cm^2 after processing [151].

The fiber architecture of the SIS matrix is predominately parallel with the long axis of the small intestine with cross fibers running approximately ±28° perpendicular to the longitudinal axis [152]. Compliance, elasticity, tensile strength, and load stress tolerance have all been discussed elsewhere [152-154]. Briefly, the stress load tolerance, as measured by a biaxial stress test of ball burst pressure, and compared to competitive biomaterials, shows SIS to be as strong as porcine Urinary Bladder Matrix (UBM) and Urinary Bladder Submucosa (UBS), but not as strong as UBM+UBS or canine Stomach Submucosa (SS). The stress load capacity of SIS can be adjusted to increase based on need by applying multiple layers of the SIS matrix together instead of using it as a single ply material. Compliance (stiffness) and elasticity (ability to return to the initial state after deformation) are important properties in the evaluation of the SIS matrix as a tool for applications such as vascular grafts and bladder wall reconstruction where compliance and elasticity can determine the success of proper regeneration. Studies indicate SIS compliance is identical to that of carotid arteries in a number of species including human. Elasticity is also highly similar across multiple species and thus underscores its use as an ideal tool for vascular repair and regeneration [153]. These properties have been shown to be location

dependent over the length of the small intestine. The use of the jejunum as the source for the SIS matrix is due to its optimal porosity, elasticity, and compliance as compared to other proximal (duodenal) and distal (ileum) sections of the small intestine [155].

Biological Properties of the SIS matrix

The biological properties of the SIS matrix are a combination of both cytokines and other proteins such as the collagens and Fibronectin, but revolve mostly around the growth factors and cytokines endogenous to the matrix. These are VEGF [149], TGFb1, [156], and FGF-2 [150].

VEGF

Vascular Endothelial Growth Factor (VEGF) is a homodimeric glycoprotein available in four different isoforms [157] whose primary role is the establishment of vascular networks [158, 159]. VEGF acts specifically on endothelial cell proliferation and differentiation through the FLK1 (KDR) and FLT1 receptors, both of which are expressed almost exclusively in endothelial cells. It has also been shown however that FLK1 is also expressed in hESCs and may be responsible for the early initiation of vasculogenesis in developing embryos [160]. Mice deficient for VEGF, or either of its receptors FLK1 or FLT1, show a sever reduction in vasculogenesis and is lethal at mid gestation [158, 159]. ESCs lacking the VEGF gene also show significant reduction in teratoma formation in nude mice [158]. VEGF is present at approximately 770 pg/g of dry weight SIS [149], and is linked to the ECM through Heparan Sulfate glycosaminoglycans [157].

TGF-β1

Transforming Growth Factor-Beta 1 (TGFb1) is part of the TGFb Superfamily of growth factors. There are three isoforms of TGFb in mammals (TGFb-1, 2, & 3) and all are highly conserved between species. TGFb1 is a homodimeric glycoprotein whose physiological

functions are too numerous to list here but are reviewed elsewhere [161]. TGFb1 is produced by multiple cell types and is an essential ECM regulator having both proliferative and inhibitory effects on a variety of cell types throughout all stages of development and differentiation. In respect to its role in the SIS matrix, it has been shown to promote accelerated wound healing and tissue remodeling and stimulates proliferation of vascular endothelial cells in the early stages of angiogenesis [162]. TGFb1 is present at approximately 768 pg/g of dry weight SIS [156].

FGF-2

Fibroblast Growth Factor-2 (FGF-2) is a monomeric protein available in three isoforms [163] whose physiological functions consists predominately of angiogenesis [164, 165], neural development [166-168], and wound repair [169-171]. It is linked to the ECM through Heparan Sulfate [172], and has been shown to act synergistically with PDGF to produce effects similar to VEGF to stimulate angiogenesis and tissue regeneration [173]. FGF-2 knockout mice show abnormal, but not lethal, development suggesting it is not essential for gestation. However, FGF-2 knockouts show sever frontal cortex underdevelopment and dramatically reduced open wound healing time [163].

The distribution of these factors, as observed by immunofluorescence of antibodies specific to each factor, show VEGF to be localized primarily around native vascular paths, while TGFb and FGF-2 are evenly dispersed throughout the matrix with FGF-2 strongly associated with collagen architecture [174]. The ability of these three primary growth factors to initiate angiogenesis and accelerate wound healing is well established [110]. These factors probably do not work independently but rely on a combined effort for efficient tissue remodeling [173]. The presence of such strong mitogenic factors in a biomaterial such as the SIS matrix gives precedence to its success as a bioscaffold for numerous tissue engineering applications.

The biodegradation of SIS is dependent on location of transplant and the cell types present, either pre-seeded or recruited post graft [117, 134]. In general, ^{14}C-labeled SIS is shown to be degraded to less than 10% by three months when used as a graft for bladder wall reconstruction [116]. At 12 months, ^{14}C-labeled SIS was undetectable in the bladder wall showing complete biodegradation and neogenesis of bladder wall tissue. Analysis of ^{14}C in tissue and fluid samples indicate SIS is rapidly broken down and excreted in the urine while small amounts are recycled into lung, kidney, and liver, with only lung retaining any detectable levels at 12 months.

Immunocompatibility of SIS with recipient tissues is another important element of its success as a biomaterial. The lack of an immune mediated inflammatory response seems to be the result of an immunosuppressive effect SIS has on helper T (Th) cell maturation and activation by interfering with interleukin 12 secretion through the combined action of TGFb and other unknown factors present in the matrix [123]. Additionally, suppression of Th activation in response to SIS transplants appears to be a local effect and does not compromise general systemic immunity to other pathogens [124], nor does it compromise protection against infection at the site of transplant, even in the presence of contaminating bacteria [175, 176].

Another crucial property owing to the success of SIS is its ability to naturally recruit the necessary cell types for proper tissue remodeling. Studies indicate that a majority of cells occupying the SIS matrix during tissue regeneration are marrow derived cells, although the specific type of marrow derived cells are not known [133, 177]. The importance of marrow derived cells in regeneration has been demonstrated for a variety of tissue types [133, 177-179]. Studies showing the phenotypic influence of the SIS matrix on adult stem cells have been documented suggesting the SIS matrix is able to differentiate recruited cell types to the desired

phenotype necessary for proper tissue remodeling. In one such study, the culture of muscle derived stem cells on SIS developed into contracting myotubules that were Ca^{2+} sensitive [180].

In summary, the SIS matrix as a tool for tissue engineering has numerous benefits over other similar biomaterials. Its flexibility for use as either an acellular or cell-seeded bioscaffold allows for a dynamic range of applications in various tissues and organs. The retention of certain growth factors and cytokins after processing enhances its ability to initiate soft tissue remodeling and stimulates rapid angiogenesis. Its durable and biodegradable structure is ideal for providing temporary structural support until the body's own natural regenerative processes take over, and it is rapidly degraded and excreted within weeks to months leaving fully functional regenerated tissue. The SIS matrix is immunocompatible and even shows signs of antimicrobial effects. These combined properties make the SIS matrix a highly valuable tool for regenerative medicine and tissue engineering technologies. The SIS matrix has already been shown to be successful in thousands of patients and its potential for future tissue engineering and regenerative medicine applications is steadily increasing.

Importance of Research and Future Impact on Tissue Engineering

One of the major problems in tissue engineering lies with the generation of sufficient quantities of cells *in vitro* for combination with bioscaffolds and biomaterials [11]. This is mainly due to the limited proliferation capacities of defined or adult cell types [11]. While most applications of SIS involve successful transplant as an acellular material, there are some examples where cell-seeded SIS provides better regenerative effects than acellular SIS [118, 130, 181]. As applications of SIS expand, so does the need for an available cell source with high proliferation potential able to generate therapeutic levels of cellular material. This issue does not

fall upon the SIS matrix alone, but upon all biomaterials and bioscaffolds where combination with living cells is needed. The use of ESCs in tissue engineering and regenerative medicine hopes to alleviate these problems by providing a potentially unlimited source of cellular material. ESCs have been shown to have high proliferative capabilities *in vitro*, and may provide an adequate source of cells suitable for transplant therapy and tissue engineering applications [3, 11, 182]. Most applications of ESCs would involve differentiation towards a more defined cell type, such as an adult stem cell or adult tissue cell, before transplant. Because the SIS matrix has been shown to influence the phenotype of primary adult cells [120], as well as certain adult and fetal stem cells [118, 180], it is plausible that the SIS matrix may also influence the phenotype of undifferentiated pluripotent ESCs as well.

The cytokins contained within the SIS matrix are ideal for the natural engraftment and proliferation of cells *in vivo*. It is suspected that these same cytokines will also prove beneficial in the support and proliferation of ESCs *in vitro*, and may be responsible for phenotypic influence of ESCs cultured on SIS. As knowledge of factors responsible for driving differentiation of ESCs in culture expands, perhaps the addition of these factors to bioscaffolds by incorporating them directly into the matrix will provide cell type specific matrices. This would provide a tool in which ESCs could be directly applied to bioscaffolds, and the matrix itself could drive differentiation. If combined with specific derivation media, this effect could be enhanced to yield extremely high quantities of type specific ESC derived cells in a an artificial tissue format that could be readily transplanted, eliminating time sensitive problems that arise from traditional expansion and seeding protocols.

The use of SIS in tissue engineering applications is representative of the very principles upon which tissue engineering is built. ESCs are capable of multiplying indefinitely and may

provide a potentially unlimited supply of cells available for therapy. It seems then, that the combination of the most potentially beneficial resource available in medicine, be combined with one of the most extensively used and diverse biomaterials known. The use of ESCs with the SIS matrix will greatly advance tissue engineering applications to levels previously unattainable. This is the key to providing unparallel treatment for current medical conditions in which conventional medicine has failed.

It will be the goal of this thesis to investigate the dynamic interactions between hESCs and the porcine derived SIS matrix to explore the potential of hESCs as a source of cellular material in tissue engineering applications. This work will examine the ability of the SIS matrix to support the culture of hESCs *in vitro*, and to explore the ability of the SIS matrix to influence the differentiation of hESCs directly. If differentiation via the SIS matrix does in fact occur, this work will also investigate the nature of this differentiation and examine how the SIS matrix may influence the differentiation of hESCs differently than hESCs cultured on two commonly used platforms such as Mouse Embryonic Fibroblasts or the acellular ECM chondrosarcoma extract Matrigel, both of which have been suggested to allow random differentiation [183, 184]. Due to the specific structural and biological composition of the SIS matrix, specifically the presence of VEGF, we believe the SIS matrix may push hESCs to differentiate towards a vascular or endothelial related phenotype. This hypothesis will be tested by examining the expression profiles of various early differentiation markers representative of the three embryonic germ layers as well as markers for pluripotency and general differentiation.

While the research presented hereafter is only a starting point, it should provide a glimpse of where the future direction of tissue engineering and medicine are headed: the union of two fields, stem cell biology and biomaterials science, for the engineering of living tissues.

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Figures for CHAPTER 1

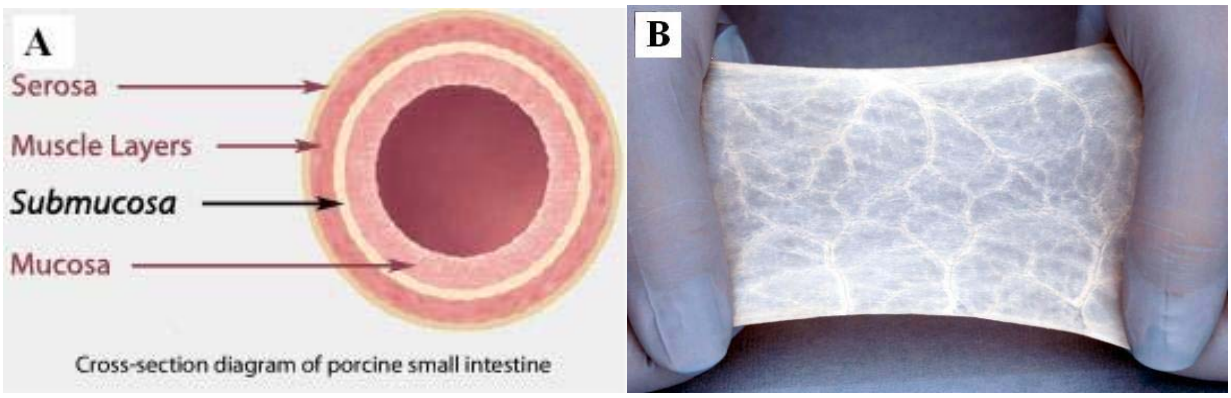

Figure 1.1 – (A) Cross sectional area of the Small Intestine indicating the major layers. (B) Small Intestinal Submucosa (SIS) after processing. (www.cookbiotech.com)

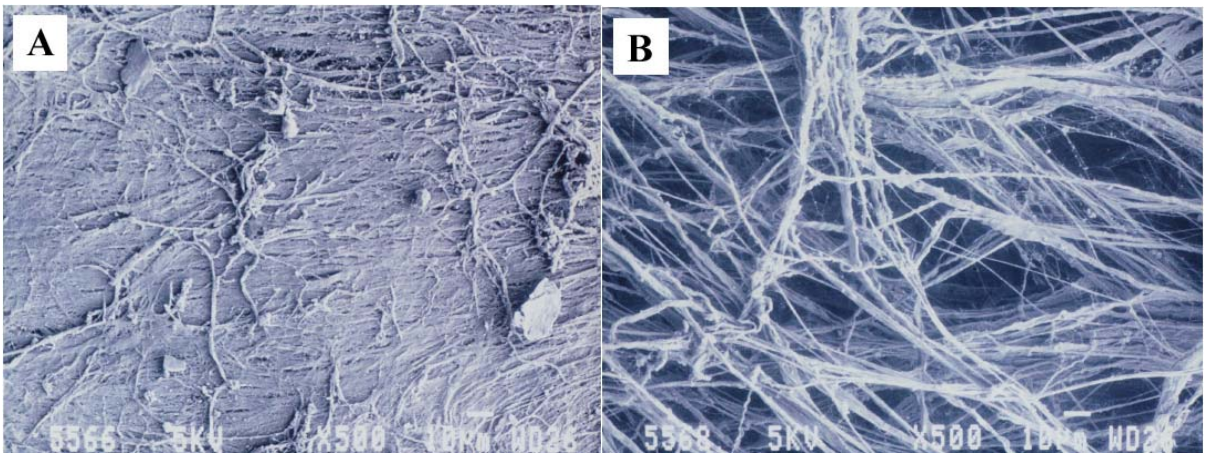

Figure 1.2 – (A) Scanning EM of the luminal (mucosal) side of SIS matrix (500X). (B) Scanning EM of the abluminal (serosal) side of SIS matrix (500X). Lindberg K, Badylak SF. 2001. *Burns* 27: 254-66.

CHAPTER 2

THE USE OF SMALL INTESTINAL SUBMUCOSA AS A BIOSCAFFOLD FOR HUMAN EMBRYONIC STEM CELLS

Introduction

Tissue Engineering seeks to restore the structural, mechanical, or physiological function to diseased or damaged tissues and organs. It encompasses the use of natural or synthetic biomaterials which can be utilized as either an acellular scaffold, or in conjunction with living cells to produce a cell-seeded bioscaffold with artificial tissue-like properties. These materials can then be transplanted to patients to stimulate or aid in the bodies own natural regenerative processes that would otherwise have difficulty initiating.

One of the major problems facing tissue engineers however, lies with the generation of sufficient quantities of specific cell types *in vitro* for combination with biomaterials [1]. This is mainly due to the limited proliferation capacities of defined or adult cell types when subjected to *in vitro* conditions [1]. Embryonic stem cells may offer a partial solution to this problem.

Human Embryonic Stem Cells (hESC) are pluripotent, self-renewing cells derived from the Inner Cell Mass of blastocyst stage embryos [2]. They have a high proliferative potential *in vitro* and the ability to differentiate into all cell and tissue types of the body. hESCs have been shown to maintain pluripotency and self-renewal over long term culture periods while retaining their ability to differentiate into multiple cell types representing all three embryonic germ layers [3]. hESCs may therefore prove to be an adequate source of cellular material suitable for generating therapeutic quantities necessary for use in tissue engineering and regenerative medicine applications.

One of the most widely used biomaterials available for tissue engineering applications is the porcine derived matrix Small Intestinal Submucosa (SIS) [4]. The SIS matrix is a natural, acellular biopolymer derived from the submucosal layer of porcine small intestine. Processing of the submucosa produces a strong, durable, extracellular matrix-like biomaterial abundant in collagen Types I and III [5], and growth factors VEGF [6], FGF-2, and TGFb1 [7]. Other collagens present include Types IV, V, and VI [8] and the glycoprotein Fibronectin [5, 9]. The SIS matrix also contains various proteoglycans and glycosaminoglycans such as Chondroitin Sulfate A and B, Heparin, Heparan Sulfate, and Hyaluronic acid [10]. It has been shown to be extremely conducive to the growth and support of various cell types *in vitro* [5, 8], and may be transplanted into patients to numerous locations in either a cell-seeded [11-13] or acellular format [14-16]. As a transplant material, the SIS matrix naturally recruits and engrafts multiple cell types *in vivo* and participates in extensive tissue remodeling [15-17]. It is capable of inducing angiogenesis upon transplantation [11, 15, 16], and is completely biodegradable [11, 15, 16] with no immunorejection and minimal inflammatory response [11, 18, 19]. SIS is FDA approved and has been used in numerous clinical applications including vascular graft replacement [20], vascular flap replacement [21], myocardial wall repair [15], bladder wall repair [16], stomach wall repair [22], intestinal wall repair [14], urethral reconstruction [23], body wall and pelvic floor repair [12], and corneal repair [24], just to name a few.

While most applications of the SIS matrix involve successful transplant as an acellular material, there are some examples where cell-seeded SIS provides superior regenerative effects over acellular SIS. Studies involving transplant of the SIS matrix pre-seeded with various cell types including urothelial and bladder smooth muscle cells [13], bone marrow derived adult stem cells [25], and modified embryonic germ cells [26], all performed considerably better than

acellular SIS for the regeneration of bladder wall defects. The use of pre-seeded SIS demonstrated superior tissue remodeling and regeneration, recovery time, and reduced scar and artifact formation as compared to its acellular counterpart. These results provide high intellectual merit for exploring the use of pre-seeded SIS in regenerative medicine and tissue engineering applications. These endeavors may be hindered however, due to the limited availability of a suitable cell source.

The use of hESCs in conjunction with the SIS matrix could thus allow for the production of a high density cell-seeded bioscaffold for use in multiple tissue engineering and regenerative medicine applications [1, 27, 28]. Because the SIS matrix has been shown to influence the behavior of primary adult cell types [5], as well as influence the phenotype of certain adult and fetal stem cells [25, 26, 29], it is plausible that the SIS matrix may also influence the behavior and phenotype of undifferentiated pluripotent hESCs as well.

To explore the dynamic interactions between hESCs and the porcine derived SIS matrix, we will examine the ability of the SIS matrix to support the culture of hESCs *in vitro*, and determine if the SIS matrix has the ability to influence the differentiation of hESCs directly. If differentiation via the SIS matrix does in fact occur, we will also investigate the nature of this differentiation and examine how the SIS matrix may influence differentiation of hESCs differently than other commonly used platforms such as Mouse Embryonic Fibroblasts (MEF) or the acellular ECM chondrosarcoma extract Matrigel, both of which have been suggested to allow random differentiation [30, 31]. Due to the specific structural and biological composition of the SIS matrix, specifically the presence of VEGF, we believe the SIS matrix may push hESCs to differentiate towards a vascular or endothelial related phenotype. This hypothesis will be tested by examining the expression profiles of various early differentiation markers representative of

the three embryonic germ layers as well as markers for pluripotency and general differentiation. This study will also incorporate basic histology to further characterize the behavior of hESCs on the SIS matrix under *in vitro* conditions.

Materials and Methods

hESC Culture

All hESCs used in this study were of the line WA09 (WiCell). On going cultures of hESC stocks were maintained via coculture on Mitomycin C (Sigma) inactivated Mouse Embryonic Fibroblasts in hESC culture media consisting of DMEM/F12 (Gibco) supplemented with 15% Fetal Bovine Serum (Hyclone) and 5% KnockOut Serum Replacement (Gibco), 1% Non-Essential Amino Acids (Gibco), 1% Penicillin and Streptomycin (Gibco), 1mM L-Glutamine (Gibco), 4ng/ml basic Fibroblast Growth Factor (Sigma), and 0.1mM β-Mercaptoethanol (Sigma). Cells were cultured in 35mm cell culture dishes (BD Falcon) on inactivated MEFs in 5% CO_2 and air at 37°C with daily media changes. Colonies were mechanically dissociated and replated onto fresh inactivated MEFs for expansion and maintenance as needed. Experimental sets of hESCs on MEFs were cultured under identical conditions as maintenance stocks without passaging.

Preparation of Matrigel

Stock solutions of Matrigel (BD Biosciences) were diluted 1:30 in DMEM/KO media (Gibco). Working solutions of Matrigel were diluted and kept at 4°C until plating. Two milliliters of working solution was pipetted into 35mm cell culture dishes and allowed to congeal at room temperature for two hours. Excess Matrigel was removed and Matrigel coated dishes were washed three times in DMEM/KO media. hESCs in hESC culture media were immediately

plated onto Matrigel coated dishes after the last wash. Cells were cultured in 5% CO_2 and air at 37°C with daily media changes.

Preparation of the SIS matrix

The SIS matrix (Cook® Biotech) was received dehydrated in sterile packaging. Under sterile conditions, the SIS matrix was cut to conform to circular wells in 4-well culture plates (BD Falcon) and placed abluminal side up. SIS was then rehydrated with hESC culture media for approximately 10 minutes at room temperature. Since hydrated SIS in media has a tendency to float, autoclaved surgical stainless steel rings were placed on top of the SIS matrix to secure SIS to the bottom of the culture well in preparation for seeding cells. hESCs in hESC culture media were then seeded onto the abluminal side of the SIS matrix and allowed to sit undisturbed for two days to allow for sufficient attachment of hESCs to the SIS matrix. After two days, the steel rings were removed and the SIS matrix was relocated to 35mm cell culture dishes and allowed to float freely in hESC culture media for the duration of the experiment. Cells were cultured in 5% CO_2 and air at 37°C with daily media changes.

RNA Isolation

RNA isolation from hESCs was achieved using the Qiagen RNeasy mini kit, and was carried out according to the Qiagen protocol handbook. Briefly, hESCs were manually collected from either MEFs or Matrigel, then centrifuged and washed three times in Phosphate Buffered Saline with Ca^{+2} and Mg^{+2} (PBS^{++}) (Hyclone). The cell pellet was resuspended in 500µl of cell lysis buffer. For hESCs on SIS, the cell-seeded bioscaffold was washed by submergence in PBS^{++} then submerged in 1ml of cell lysis buffer in a 1.5ml tube and mechanically agitated by vortexing intermittently for five minutes. The residual SIS matrix was then removed from the cell lysis buffer and discarded. Cell lysis solutions generated from hESCs on SIS, MEFs, and

Matrigel all followed the same protocol as outlined in the Qiagen protocol handbook from this point forward. Briefly, samples were centrifuged through a Qiashredder spin column at maximal g's for two minutes. This step was repeated one additional time for cell lysis solutions containing hESCs grown on the SIS matrix due to the high protein content of the lysate solution. For all samples, an equal volume of 70% EtOH was added to the filtrate and the entire contents were centrifuged through an RNeasy mini spin column at maximal g's for 15s. The RNeasy mini spin columns were washed with wash reagents contained within the kit two times at maximal g's for 15s, then one additional time for two minutes. Next, 30µl of sterile RNase free water was added to the spin columns and allowed to refrigerate at 4°C for 30 minutes. The RNeasy mini spin columns were then centrifuged at maximal g's for one minute and the flow through containing RNA and RNase free water was collected in 1.5ml eppendorf tubes. RNA quantification was carried out using a 1:40 dilution of RNA in RNase free water and quantified using the µQuant plate reader by Biotek instruments.

cDNA Production

cDNA production was carried out using the ABI High Capacity cDNA Archive Kit and followed the protocol as outlined in the ABI manual. Briefly, 750ng of RNA was combined with Archive Kit components including: RT buffer, RT random primers, dNTPs, MultiScribe RTase, and RNase free water in concentrations as outlined in the ABI manual for a 50µl RXN volume. Contents were mixed in 0.2ml microfuge tubes and ran under the following thermocycling conditions: 25°C for 10 minutes; 37°C for 120 minutes; 4°C hold.

Preparation of cDNA for use in Real Time-Polymerase Chain Reaction

cDNA was prepared for use in the ABI 7900HT RT-PCR Sequence Detection System according to ABI protocol as outlined in the ABI 7900HT manual. Briefly, 8µl of stock cDNA

was combined with 42µl of RNase free water and 50µl of ABI Universal PCR Master Mix-2x in 0.2ml microfuge tubes. Each sample was then loaded into the appropriate well of an ABI Microfluidics Low Density Taqman Array. The array was centrifuged at 1200 rpms for one minute two separate times, sealed using the sealing apparatus from ABI, then loaded into the ABI 7900HT and ran under the following thermocycling conditions: 97°C for 10 minutes for hot start; 97°C for 30 seconds; 59.7°C for 60 seconds. Steps 2 and 3 were repeated for 40 cycles. Results were analyzed using ABI Sequence Detection Software 2.21.

Immunocytochemistry

hESCs for immunocytochemistry were cocultured on Mitomycin-C inactivated MEFs in four well glass chamber slides (Falcon) under identical conditions as hESC experimental sets. Cells were fixed in 2% formaldehyde for 20 minutes, washed three times in PBS^{++}, then incubated in blocking solution for 45 minutes (for extracellular markers: 6% Goat Serum in PBS^{++}; for intracellular markers: 6% Goat Serum, 1% Polyvinyl Pyrrolidone, and 0.3% Triton X-100 in High Salt Buffer (250mM NaCl, and 50mM Tris buffer, pH 7.4 in dH_2O)). Cells were then incubated in primary antibody solution (Chemicon, Mouse monoclonal, diluted 1:750 in Block solution) for one hour at room temperature then washed four times in wash buffer (intracellular wash: Three times in 0.05% Tween 20 in High Salt Buffer and one time in High Salt Buffer; extracellular wash: PBS^{++}). Cells were then incubated in secondary antibody solution (Alexa Fluor Molecular Probes, Goat anti-Mouse, diluted 1:1,000 in Block solution) for one hour at room temperature then washed four times as before. DAPI (1:10,000 in dH_2O) was applied for five minutes and all wells were then washed three times in PBS^{++}. VectaShield coverslip adhesive was applied to each slide before adding a cover slip and sealing with nail varnish. Immunofluorescence analysis was carried out using the Nikon T-2500 at x100.

Histology and Staining of the SIS Matrix

hESCs on the SIS matrix were cultured for seven and fourteen days under identical conditions as experimental sets of hESCs on SIS. Cell-seeded SIS was fixed in 10% formaldehyde for approximately two hours, then infused with paraffin and sectioned at 5 microns. SIS sections were plated onto glass slides and stained with Hematoxalyn and Eosin. Analysis of hESC-seeded SIS matrix was carried out using phase contrast light microscopy on the Nikon T-2500 at x400.

Experimental Design

hESCs were cultured in the presence of Small Intestinal Submucosa, Mouse Embryonic Fibroblast, and Matrigel for seven and fourteen days. At the end of each time point, cells were harvested, and RNA from each treatment group was isolated and cDNA produced for comparative expression profiling of 44 early differentiation genes in cells from each treatment group against a calibrator sample of known pluripotent hESCs. Expression profiling was carried out through quantitative Real Time-Polymerase Chain Reaction (qt-PCR) utilizing the ABI Microfluidics Low Density Taqman Array. The microfluidics array for this study was formatted into eight replicate sample lanes with each lane accessing 44 individual primer/probe sets. To verify the undifferentiated state of the starting population of day zero hESCs on MEFs, immunocytochemistry was performed with the intracellular pluripotent marker OCT-4, and the extracellular pluripotent marker SSEA-4. The early differentiation marker SSEA-1 was also tested for in starting populations of hESCs. Controls for immunocytochemistry consisted of hESCs incubated in secondary antibody without primary antibody. hESCs cultured on the SIS matrix at seven and fourteen days were also subjected histological examination by staining with Hematoxalyn and Eosin. Controls for SIS histology consisted of the acellular SIS matrix stained

with Hematoxalyn and Eosin. Negative controls for qt-PCR consisted of samples containing mRNA isolated from MEFs only, and an RNA isolation procedure of the acellular SIS matrix. Neither negative control sample produced any reliable or detectable amplification signal when subjected to qt-PCR. All qt-PCR experiments were performed with three completely independent biological replicates.

Statistical Analysis of qt-PCR Data

Relative Quantification (RQ) of gene expression from qt-PCR data was determined using the comparative Ct method: where $RQ = 2^{-(\Delta\Delta Ct)}$, and $\Delta\Delta Ct = (\Delta Ct_{Treatment}) - (\Delta Ct_{Calibrator})$, and $\Delta Ct = (Ct_{Target\ Gene}) - (Ct_{Endogenous\ Control})$ [32]. Statistically significant differences in relative gene expression were evaluated using a one tailed Student's t-test to make pair-wise comparisons between RQ values for each gene in a treatment group to the same gene in a calibrator group. The calibrator group was day zero hESCs on MEFs and the endogenous control was 18s rRNA. Values are considered significant at $P < 0.05$.

Results

Immunocytochemistry

To verify the undifferentiated state of the starting population of day zero hESCs on MEFs, immunocytochemistry was performed using antibodies against known pluripotent markers for hESCs. hESCs are shown to stain positive for the pluripotent markers OCT-4, SSEA-4 and Alkaline Phosphatase, and negative the early differentiation marker SSEA-1 [2, 33]. Our results here show the initial starting population of day zero hESCs on MEFs stained positive for the intracellular transcription factor OCT-4, and the extracellular marker SSEA-4, while staining negative for the extracellular marker SSEA-1 (Figure 2.1). These cells also stained

positive for Alkaline Phosphatase activity and exhibited a normal karyotype at the beginning of this study (Data not shown). These results support the presumption that the starting population of day zero hESCs on MEFs are pluripotent undifferentiated stem cells.

hESC Culture on the SIS matrix

To evaluate the effects of SIS matrix on undifferentiated hESCs in culture, cells were seeded onto the abluminal side of the SIS matrix and allowed to culture for seven and fourteen days. After seven days, cells had begun to migrate from the surface of the matrix down into the center of the matrix with some cells reaching the bottom of the matrix (Figure 2.2A-C). After fourteen days, cells occupied all levels of the SIS matrix from top to bottom (Figure 2.2D-F).

The gross anatomical structure of the SIS matrix consists of bundles of collagen fibrils running throughout the matrix with a thin collagen mesh occupying the spaces in-between. The morphology of hESCs throughout the matrix was considerably different within the collagen bundles than within the collagen mesh surrounding the bundles. hESCs within the collagen mesh showed a more rounded morphology and appeared randomly dispersed throughout the mesh (Figure 2.2A-F, H), while hESCs within the collagen bundles showed a more flat and elongated type morphology (Figure 2.2G, H). In addition, hESCs within the collagen bundles appeared to orient themselves perpendicular to the long axis of the bundle. The differences in morphology of cells occupying the collagen bundles from cells occupying the collagen mesh were consistent throughout the entire thickness of the matrix.

Analysis of qt-PCR Data

To evaluate the ability of the SIS matrix to influence the differentiation of hESCs directly, and to determine if the SIS matrix will influence hESCs to differentiate differently than other matrices, qt-PCR was performed to assess the changes in gene expression of 44 early

differentiation genes in hESCs cultured on SIS, MEFs, or Matrigel for seven and fourteen days. qt-PCR provides a measure of gene expression relative to a calibrator sample where values are a ratio of expression between a treatment group and a calibrator group, and are presented as a relative quantification (RQ) that is indicative of fold change. Since RQ values are a ratio, RQ values representing the calibrator group are normalized to a starting value of one. RQ values above one indicate an increase in relative expression while values below one indicate a decrease in relative expression. RQ values in this study are relative to a calibrator group of day zero hESCs on MEFs. The 44 genes evaluated consists of markers that represent various lineages of differentiation including the three embryonic germ layers: ectoderm, endoderm, and mesoderm, as well as markers for pluripotency and general differentiation. A list of the 44 genes evaluated in this study is provided in Table 2.1.

Of the 44 genes evaluated, 10 represented the pluripotent state. After seven days in culture, hESCs on MEFs showed a significant decrease in relative expression in only two of the 10 pluripotent markers while hESCs on Matrigel showed a significant decrease in six of 10, and hESCs on SIS showed a significant decrease in seven of 10, all relative to day zero (Figure 2.3). In addition, some genes that showed significant differences in expression relative to day zero also showed significant differences in expression relative to different treatment groups. For example, while OCT-4 expression was significantly decreased in hESCs on both SIS and Matrigel relative to day zero, OCT-4 expression in hESCs on SIS was down regulated significantly more than OCT-4 expression in hESCs on Matrigel (Figure 2.3). FGFR4 and EBAF both showed similar results to OCT-4 with FGFR4 being down regulated significantly more in hESCs on SIS than on Matrigel and EBAF being down regulated significantly more in hESCs on SIS than on MEFs. After fourteen days in culture, hESCs on MEFs and Matrigel

showed a significant decrease in relative expression in eight of the 10 pluripotent markers while hESCs on SIS showed a significant decrease in all 10 pluripotent markers (Figure 2.3). Additionally, OCT-4, DNMT3B, FST, and SOX2 were shown to be down regulated significantly more in hESCs on SIS and Matrigel than in hESCs on MEFs (Figure 2.3). Relative expression of OCT-4 was down regulated 41.7 fold in hESCs on SIS after fourteen days, making it the most down regulated marker in this study. Relative expression of OCT-4 in hESCs on MEFs and Matrigel was down regulated 2.2 fold and 14.3 fold, respectively. In addition, only DNMT3B and FGF4 showed any statistically significant reduction in relative expression from day seven to day fourteen in hESCs on MEFs, while all markers except EBAF and FGFR4 showed a statistically significant reduction in relative expression from day seven to day fourteen in hESCs on both Matrigel and SIS.

There were three genes that represented the ectoderm lineage and four genes that represented the endoderm lineage that were used in this study. After seven days in culture, there was no significant change in relative expression for any of the ectoderm markers in hESCs on MEFs. Only increases in MSI1 in the Matrigel group and NES in the SIS group showed any significant change after seven days (Figure 2.4). After fourteen days in culture, hESCs on MEFs and SIS both showed a significant decrease in relative expression of NES and NEFH, while hESCs on Matrigel showed a significant decrease in all three ectoderm markers: MSI1, NES, and NEFH (Figure 2.4). NES was also down regulated significantly more in hESCs on Matrigel than on MEFs. Relative expression of endoderm markers after seven days showed FOXA2, NODAL, and CER1 decreased significantly in hESCs on MEFs, while HNF4A, FOXA2, and CER1 decreased significantly in hESCs on SIS. hESCs on Matrigel showed only one significant decrease, FOXA2, and one significant increase, NODAL, in relative expression at day seven

(Figure 2.4). Results after fourteen days were similar to results after seven days with FOXA2, NODAL, and CER1 all decreasing significantly in hESCs on MEFs and SIS. hESCs on Matrigel showed NODAL and CER1 decreasing significantly and HNF4A increasing significantly (Figure 2.4). FOXA2 was also down regulated significantly more in hESCs on SIS than on MEFs after both seven and fourteen days, and NODAL was significantly different in hESCs on MEFs from hESCs on Matrigel after seven days as NODAL showed an increase in the MEF group and a decrease in the Matrigel group.

Of the 44 genes evaluated, 21 represented the mesoderm lineage making it the largest category in this study. Nine of the 21 mesoderm genes are known to be endothelial related genes while the remaining 12 are representative of various subcategories of the mesoderm lineage. For the sake of clarity, genes associated with the endothelial subcategory are discussed separately in the next section. Table 2.1 provides a partial list of the various subcategories associated with the remaining mesoderm genes. The mesoderm category is one of only two categories to show a general trend of up regulation in relative expression as a whole (Figure 2.5). After seven days in culture, only RUNX1 and GSC showed any significant change in relative expression in hESCs on MEFs for the 12 mesoderm markers. hESCs on SIS and Matrigel both showed a significant increase in relative expression for the mesodermal markers BMP4, HEY1, GATA3, and NKX2-5. hESCs on Matrigel additionally showed a significant increase in relative expression for FN1, while hESCs on SIS additionally showed a significant increase in relative expression for EPO and T (Brachyury). NKX2-5 was also up regulated significantly more in hESCs on SIS than on Matrigel. GATA4 and GSC both decreased significantly in hESCs on SIS while no significant decreases in relative expression occurred in hESCs on Matrigel.

After fourteen days in culture, hESCs on MEFs showed a significant increase in relative expression for BMP4 and GATA3, but also showed a significant decrease in Brachyury and HIF1A. hESCs on Matrigel showed a significant increase in relative expression for BMP4, HEY1, GATA3, RUNX1, BMP2R, and GATA4, which make up half of the mesodermal markers that were evaluated. There were also two markers, NKX2-5 and GSC, which decreased significantly in the Matrigel group. hESCs on SIS showed a significant increase in only three of the 12 mesodermal markers after 14 days including BMP4, HEY1, and GATA3. GATA4, HIF1A, and GSC all decreased significantly in the SIS group. The most significantly up regulated gene in this category was BMP4 which increased 53.9 fold in the Matrigel group and 22.6 fold in the SIS group after fourteen days. GATA3, which is typically a vascular or hematopoietic marker but has also been used as an endothelial marker, was second increasing 24.9 fold in hESCs on Matrigel and 11.2 fold in hESCs on SIS. GATA3 was also up regulated significantly more in hESCs on Matrigel than in hESCs on MEFs or SIS. In addition, hESCs on Matrigel also showed a significant increase in two other vascular related markers, RUNX1 and GATA4. GATA4 expression was shown to be statistically different between Matrigel and SIS groups due to GATA4 increasing significantly in the Matrigel group but decreasing significantly in the SIS group.

The endothelial subcategory of mesodermal genes is comprised of nine markers that are either endothelial specific or shown to be highly enriched in endothelial cells. This category is the only other category besides mesoderm to show a general trend of up regulation in relative expression as a whole (Figure 2.6). After seven days in culture, hESCs on MEFs, Matrigel, and SIS all showed a significant up regulation in three of the nine endothelial markers with NOS3, VEGF, and VWF increasing significantly in the MEFs group, TEK, VEGF, and KDR increasing

significantly in the Matrigel group, and CDH5, NOS3, and TEK increasing significantly in the SIS group. Only the MEF and SIS groups showed a significant reduction in relative expression of endothelial markers with KDR and FLT1 decreasing significantly in both groups. KDR was also significantly different in hESCs on MEFs and SIS from hESCs on Matrigel as KDR in the Matrigel group increased.

hESCs on MEFs and Matrigel showed little difference between day seven and day fourteen time points for endothelial markers. hESCs on MEFs showed a significant up regulation in only two of the nine endothelial markers, NOS3 and VEGF, one less than day seven samples. KDR and FLT1 remained significantly down regulated in day fourteen samples with FLT1 decreasing significantly more at day fourteen from day seven. hESCs on Matrigel also showed an up regulation in only two of the nine endothelial markers, TEK and VEGF, with only one marker, FLT1, decreasing significantly. hESCs on SIS however, showed every marker in the endothelial category as being significantly different from day zero hESCs on MEFs after fourteen days. As in day seven samples, six of the nine endothelial markers showed an increase in relative expression but now with all six, CDH5, PECAM1, CD34, NOS3, TEK, and VEGF, being up regulated significantly. The remaining three down regulated markers, KDR, FLT1, and VWF, were all down regulated significantly. The markers CDH5, PECAM1, CD34, and TEK also showed a significant up regulation from day seven to day fourteen in the SIS group. No endothelial markers showed any statistically different change in relative expression from day seven to day fourteen in the Matrigel group, despite day fourteen samples showing an up regulation over day seven samples. CDH5, a vascular endothelial adhesion protein, increased 51.4 fold in hESCs on SIS, making it the most significantly up regulated marker in this group. CDH5 was also up regulated in the Matrigel group 62.7 fold but was not significant due to large

variances between replicates. PECAM1, CD34, and NOS3 all showed similar results to CDH5 in that these markers increased slightly more in hESCs on Matrigel than on SIS, but were only significant in the SIS group, again due to large standard deviations in the Matrigel group. VEGF was the only marker that was significantly up regulated across all three treatment groups and was up regulated significantly more in hESCs on MEFs and Matrigel than in hESCs on SIS. In contrast, the VEGF receptor FLT1 was the only marker to be significantly down regulated across all three treatment groups and was also down regulated significantly more in hESCs on MEFs than on Matrigel.

The last set of genes evaluated in this study consisted of markers for general differentiation. The general trend for this set of markers was an up regulation in relative expression after seven days and then a down regulation in relative expression after fourteen days in hESCs on SIS and Matrigel, but no clear trend in expression for hESCs on MEFs (Figure 2.7). hESCs on MEFs showed a significant increase in relative expression in two of the six markers for general differentiation while hESCs on Matrigel showed a significant increase in only one of the six markers after seven days. hESCs on SIS showed a significant increase in relative expression for SMADs 1 and 3, while the activin receptor ACVR1C decreased significantly in day seven samples. By day fourteen, SMADs 1 and 2 had significantly decreased to levels half of that seen at day seven along with both activin receptors, ACVR1B and ACVR1C, giving a total of four markers that were significantly down regulated in hESCs on SIS. hESCs on Matrigel showed a similar pattern of relative expression to hESCs on SIS at day fourteen with the only differences being a significant increase in TGFb1 and no significant decrease in ACVR1C. hESCs on MEFs showed only two significant differences in relative expression at day fourteen, with SMAD1 increasing and ACVR1C decreasing.

Discussion

In this study, we demonstrate the ability of the SIS matrix to support the culture of hESCs and to directly influence the differentiation of hESCs towards an endothelial related phenotype based on the expression profiling of both endothelial and non-endothelial marker data generated from qt-PCR. The SIS matrix is a complex extracellular matrix (ECM) that allows cells to cultivate in a 3D environment similar to their own native matrix. This offers several advantages over conventional 2D culture systems in which cells are grown along the surface of culture dishes specially treated to support cell growth. The 3D nature of the ECM provides a structural framework on which cells can grow and migrate and is responsible for providing cues about the surrounding environment as well as valuable signals for maintaining cellular health and viability [34-36]. Inherent properties of the SIS matrix to naturally engraft multiple cell types *in vivo* make it an ideal candidate to study cell-ECM interactions *in vitro* [17]. SIS has been shown to support *in vitro* cell culture of multiple cell types including NIH Swiss mouse 3T3 and 3T3/J2 fibroblast, human fibroblast, human keratinocytes, human microvascular endothelial cells (HMECs) [5], human umbilical vein endothelial cells (HUVECS) [37], and bladder urothelial and smooth muscle cells [38]. It was shown that different cell types display various behaviors and morphology changes when cultured in the presence of the SIS matrix, and these behaviors can be modified when multiple cell types are cocultured together [5].

The SIS matrix has also been shown to support the culture of both fetal and adult stem cell types. Muscle derived stem cells have been shown to differentiate into contracting myotubules that are Calcium dependent and modulated by nicotinic receptors [29]. Marrow derived stem cells have been used in combination with the SIS matrix to correct bladder wall defects in rats, and *in vitro* have been stated to differentiate into myotubules after exposure to 5-

azacytidine [25]. The human embryoid body-derived stem cell line SDEC has also been shown to culture well on SIS [39]. This type of stem cell is derived by differentiating human embryonic germ cells (hEG) to embryoid bodies (EB) in the presence of endothelial growth medium, then isolating the pluripotent population of cells within the EB to produce lines of stem cells that may be predispositioned to differentiate towards specific lineages. SDECs formed a multilayered sheet when seeded onto the luminal side of the SIS matrix, and showed invasion throughout the matrix when seeded onto the abluminal side. These results are similar to our own in which we show hESCs culture well on the SIS matrix and migrate throughout the entire thickness of the matrix when seeded onto the abluminal side.

To address whether the SIS matrix could influence the differentiation of hESCs directly, we looked specifically at the expression profiles of several key pluripotent markers. The most widely accepted of these is the POU transcription factor OCT-4. OCT-4 has been shown to be an important regulator of the pluripotent state and even small changes in its expression can trigger differentiation [40]. hESCs cultured in the presence MEFs have been shown to retain the expression of OCT-4 for extended periods of time due to unknown factors secreted by the MEFs [41]. This is evident even in feeder free conditions where hESCs cultured in media conditioned by MEFs express OCT-4 stronger than hESCs cultured in non-conditioned media [42]. When hESCs are allowed to culture under general differentiating conditions, such as non-lineage directed EB formation, OCT-4 expression typically begins to diminish around day eight and is usually absent by day fourteen [43, 44]. hESCs cultured on Matrigel or other ECM type substrates such as Laminin, Fibronectin, or collagens have been shown to have diminished OCT-4 expression much sooner than hESCs on MEFs even when continually passaged [45].

We show that hESCs on both SIS and Matrigel began to differentiate as early as day seven, as key pluripotent markers such as OCT-4 were down regulated significantly in these two groups. Once more, OCT-4 expression was reduced significantly more in hESCs on SIS than on MEFs or Matrigel. These results correlate well with similar studies in which hESCs cultured on Fibronectin, a major component of the SIS matrix, shows a marked decrease in OCT-4 expression over that of hESCs cultured on MEFs, Matrigel, or Laminin, the major component of Matrigel [45]. hESCs on MEFs showed no statistical difference in OCT-4 expression between day zero and day seven, nor did they show a statistical difference in expression between day seven and day fourteen, demonstrating the role of MEFs in maintaining pluripotency during extended periods of time in culture. hESCs on SIS and Matrigel however do show a statistical difference in OCT-4 expression between days seven and fourteen, thus demonstrating the progressive differentiation of hESCs on these two matrices.

By fourteen days in culture, hESCs on all three matrices showed signs of differentiation as indicated by the significant reduction in OCT-4 expression. Other pluripotent markers such as EBAF, DNMT3B, and SOX2 have also been shown to be highly enriched in pluripotent hESCs and expression of these markers in differentiated and adult cell types is either greatly reduced or absent [31, 46]. Here we show that these markers are also significantly reduced in hESCs on SIS, and that DNMT3B and SOX2 are down regulated significantly more in hESCs on SIS and Matrigel than in hESCs on MEFs. We demonstrate that the SIS matrix is able to influence the differentiation of hESCs directly and appears to exert this influence stronger and earlier than MEFs or Matrigel.

To determine how the SIS matrix may have influenced the differentiation of hESCs differently than MEFs or Matrigel, we looked at the expression profiles for a range of various

markers across all three embryonic germ layers and compared them with expression profiles of hESCs on both MEFs and Matrigel. While the general trend in relative expression indicated that hESCs on MEFs began to differentiate by day fourteen, there did not appear to be any clear pattern of expression towards or away from any particular lineage or cell type as indicated by a lack in number of statistically different markers throughout all categories. The few markers that were statistically different in hESCs on MEFs may have been due to random differentiation typically thought to occur under *in vitro* conditions such as these [31]. In contrast, hESCs on SIS and Matrigel both appeared to be differentiating towards a mesodermal lineage after only seven days in culture as indicated by a number of significantly up regulated markers in both the mesodermal and endothelial related categories. HEY1, GATA3, and NKX2-5 were among the markers significantly expressed in both groups, along with EPO in SIS and VEGF in Matrigel, indicating the possibility of early vascular activity in both groups.

By fourteen days in culture, the direction was more definitive as hESCs on SIS and Matrigel began to show patterns of differentiation towards specific sublineages of the mesoderm fate. hESCs on Matrigel showed differentiation towards a general vascular related phenotype as indicated by the significant up regulation of several known vascular markers (Figure 2.5). These include RUNX1 for hematopoietic and blood lineages [47], GATA4 for heart development [48], GATA3 and VEGF for vasculature [49], and HEY1, which is present in developing heart, and part of the notch pathway during general cardiovascular development [50, 51]. In contrast, hESCs on SIS showed differentiation towards an endothelial related phenotype as indicated by a large majority of significantly up regulated endothelial markers (Figure 2.6). Of these, CDH5, NOS3, and TEK are all thought to be expressed almost exclusively in endothelial cells, while PECAM1, CD34, VEGF and GATA3 have all been shown to be highly enriched in endothelial

cells [44, 52-58]. Also, CDH5, PECAM1, CD34, TEK, VWF, GATA3, KDR and FLT1 have all been used by various groups for the characterization of ESC derived endothelial cells in human, monkey, and mouse and are steadily becoming the accepted standard for endothelial cell characterization [44, 59, 60]. We not only observed the combined expression of these markers in a progressive manner, but also found a reduced expression of other markers from different categories, thus strongly suggesting that hESCs on SIS are moving towards an endothelial phenotype.

The VEGF receptors KDR and FLT1 both showed a reduced expression after fourteen days in hESCs on SIS. These markers are thought to be expressed almost exclusively in vascular endothelial cells [61], thus the reduction in relative expression for these markers was somewhat unexpected. Derivation of endothelial cells from primate ESCs (RESDECs) also showed reduced or absent expression of these two markers as antibodies for both KDR and FLT1 failed to bind to RESDECs and showed very weak binding to HUVECs, although RT-PCR did reveal KDR transcript [59]. CDH5 and PECAM1 were also not detected in RESDECs but despite the absence of these markers, the presence of other defined endothelial markers such as CD34, VWF, and CD146, justified the characterization of those cells as endothelial. This argues that despite certain markers being present in control cells such as HUVECs, not all markers need be present in ESC derived cells to claim they are in fact endothelial derivations. Studies show that endothelial cells from different developmental stages or from different tissues can show different combinations of phenotypic marker expression, and therefore the reduction or absence of some endothelial markers may be acceptable when characterizing ESC derived endothelial cells [62-64]. CDH5, PECAM1, CD34, NOS3, TEK, VEGF and GATA3 have all been shown to be strong indicators of the endothelial phenotype, and results here show all have been significantly

up regulated in hESCs on SIS. These results support the contention that, despite the drop in relative expression of KDR and FLT1, there is still evidence for the movement of hESCs cultured on the SIS matrix towards an endothelial phenotype.

Not every marker in this study showed a consistent increase or decrease from day zero to day seven to day fourteen. Some markers such as NES, TEK, and SMAD1 showed an initial increase in relative expression at day seven but then a reduction in expression by day fourteen in hESCs on SIS. Others markers such as FOXA2 and GATA4 displayed the opposite trend by decreasing at day seven then increasing by day fourteen. These reversals in relative expression for various markers also occurred in both the Matrigel and MEF groups as well (Figures 2.3-2.7). It is unclear whether these reversals in expression are due to random fluctuations as a result of the culture environment, or part of the normal cyclic expression that some genes are known to display. Expression profiles of these markers were generated within three strict time windows of day zero, seven, and fourteen over the course of two weeks. Expression data between these time points is unknown making it difficult to evaluate markers that do not have a clear and consistent increase or decrease in relative expression over these time points. The large majority of markers evaluated in this study however do show a consistent pattern in expression across all time points and thus the overall trends in expression remain the same.

The underlying mechanism responsible for the differentiation pattern seen in hESCs on SIS may be due to the specific structural and biological composition of the matrix. The SIS matrix is shown to be composed primarily of collagen Types I and III, Fibronectin, and the cytokines TGFb, FGF-2, and VEGF. While TGFb is known to play important roles in general differentiation, FGF-2 and VEGF are of particular importance as they are both known to be strong angiogenic activators [65]. FGF-2 has been shown to work synergistically with VEGF to

cooperatively induce angiogenesis through up regulation of PDGF [66], and to induce VEGF expression in developing capillaries [67]. VEGF is known to direct the proliferation and differentiation of vascular endothelial progenitors into functional vascular endothelium as the first step in vasculogenesis [68]. Failure to produce VEGF, or its receptors, results in the inability to form primitive blood islands, endothelial cells, or major vascular tubules and ultimately leads to embryonic lethality [69, 70]. In the adult, it is responsible for initiating angiogenesis and the repair and maintenance of vascular networks through the recruitment and proliferation of preexisting vascular endothelial cells. This is accomplished through the KDR and FLT1 receptor pathways that lead to expression of genes responsible for the proliferation and development of vascular endothelium [68]. KDR transcript has been shown to be robustly expressed through PCR in ESCs of human [44] and through PCR and immunofluorescence in ESCs of mouse [60]. The presence of KDR on ESCs serves as an important aspect not only for early vasculogenesis, but also for the directed differentiation of endothelial cells. The presence of KDR on ESCs provides a mechanism by which the direct manipulation of the endothelial developmental pathway is possible. Various groups that have produced ESC derived endothelial cells from the ESCs of human, monkey, and mouse have all utilized this pathway by supplementing VEGF in the media to push endothelial differentiation [44, 59, 60].

Matrigel, on the other hand, is an animal derived matrix similar to mammalian basement membrane, and is isolated from the murine EHS chondrosarcoma cell line [35]. It is composed mostly of collagen Type IV and Laminin and the cytokins TGFb, FGF-2, EGF, PDGF, and IGF [71]. The presence of these cytokins in Matrigel makes it ideal for studying the influence of a basement membrane-ECM on differentiation and behavior of numerous adult cell types [35]. Matrigel is commonly used in vascular assays for its ability to influence endothelial cells to form

primitive capillary tubes and lumens. Matrigel has also been shown to induce the formation of vascular networks from mouse ESCs (mESC) without the use of additional growth factors [51]. mESCs allowed to differentiate on Matrigel were positive for primary vascular cell types including endothelium and vascular smooth muscle. These cells were also negative for markers of bone, neuron, and adipose tissue. These findings support our results in which we show Matrigel can influence the differentiation of hESCs to vascular related cell types also without the use of additional growth factors. The angiogenic effect of Matrigel on ESCs, despite the lack of VEGF, may be due primarily to PDGF, a VEGF related cytokine that has been shown to influence angiogenic processes in combination with FGF-2 similar to VEGF [72]. The presence of VEGF in the SIS matrix however, along with distinctly different architecture, is at least one difference that sets SIS apart from Matrigel, and the functional differences between these two matrices can be demonstrated through their respective effects on cellular behavior.

Initial results based on expression data generated in this study are promising for the potential production of a cell-seeded biomaterial with endothelial-like properties. It is important to establish trends in expression, and to evaluate in detail, the expression patterns of many different combinations of genes in order to determine if a concerted effort by any one of these specific combinations is contributing to cell behavior. It is important to show that, not only are hESCs on SIS differentiating towards an endothelial phenotype, but that they are also not differentiating towards cell types of other lineages such as ectoderm or endoderm. Because most genes have multiple roles during differentiation, and because the known functions of most genes are expanding everyday, it is difficult to place some genes in only one category. Nestin for example, is traditionally a strong ectoderm marker, but has also been shown to be present on developing pancreatic cells, which are endoderm. CER1, typically a key endoderm marker [73],

is also enriched in pluripotent ESCs [74]. KDR also falls into this category. It should be noted that the reduction in KDR as seen in our results is not entirely indicative that this marker is completely absent. While not generally considered a marker for pluripotency, its presence on undifferentiated ESCs brings into question the native expression levels of KDR in ESCs verses defined endothelial cells. A quantitative study comparing KDR expression in ESCs to defined endothelial cells of various origins may lead to greater understanding of endothelial development and provide information necessary for better endothelial cell engineering.

Conclusion

The SIS matrix and Matrigel are both complex extracellular matrices that differ in both their structural composition and mixture of biological molecules. These differences are undoubtedly significant in shaping the behavior of cells when cultured in their presence, and thus hESCs responded differently when cultured on these two matrices. Overall, the general trend in relative expression indicated that hESCs on MEFs remained pluripotent after seven days and began to differentiate by day fourteen but showed no clear pattern towards or away from any particular lineage or cell type. hESCs on Matrigel and SIS however, began to differentiate after only seven days in culture and by day fourteen showed a general trend of down regulation for markers representing the pluripotent, ectoderm, endoderm, and general differentiation categories while showing an up regulation for markers representing the mesoderm categories. hESCs on Matrigel appeared to be moving towards a general vascular related phenotype while hESCs on SIS appeared to be moving towards an endothelial related phenotype. Given the presence of KDR on hESCs, and VEGF in the SIS matrix, we provide an alternative method by which endothelial derivation can be achieved, and if combined with specific endothelial derivation

media, may provide an additional level of control over directed differentiation schemes currently used. Our findings should help progress tissue engineering efforts for the production of an artificial tissue with endothelial-like properties for therapeutic purposes.

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Figures for CHAPTER 2

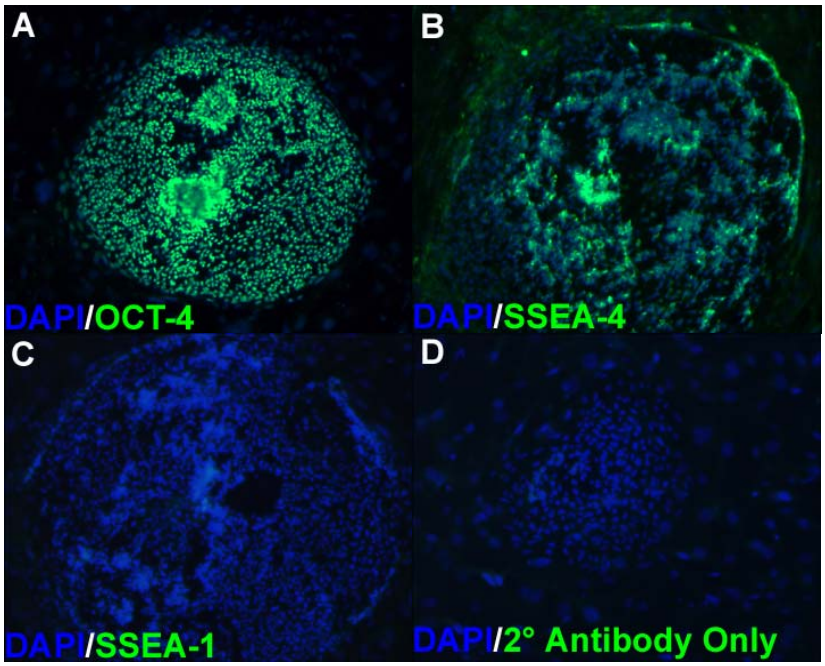

Figure 2.1 – Pluripotent Marker Expression in Day Zero Human Embryonic Stem Cells (hESC) on MEFs. hESCs were stained with immunofluorescent antibodies to verify the pluripotent state of the starting population. Cells stained positive for OCT-4 (A), and SSEA-4 (B), and negative for SSEA-1 (C). (D) Negative Control using secondary antibody only. All cells stained positive for the nuclear stain DAPI (Blue). hESCs were cultured on Mouse Embryonic Fibroblast in hES culture media. Magnification = (x100)

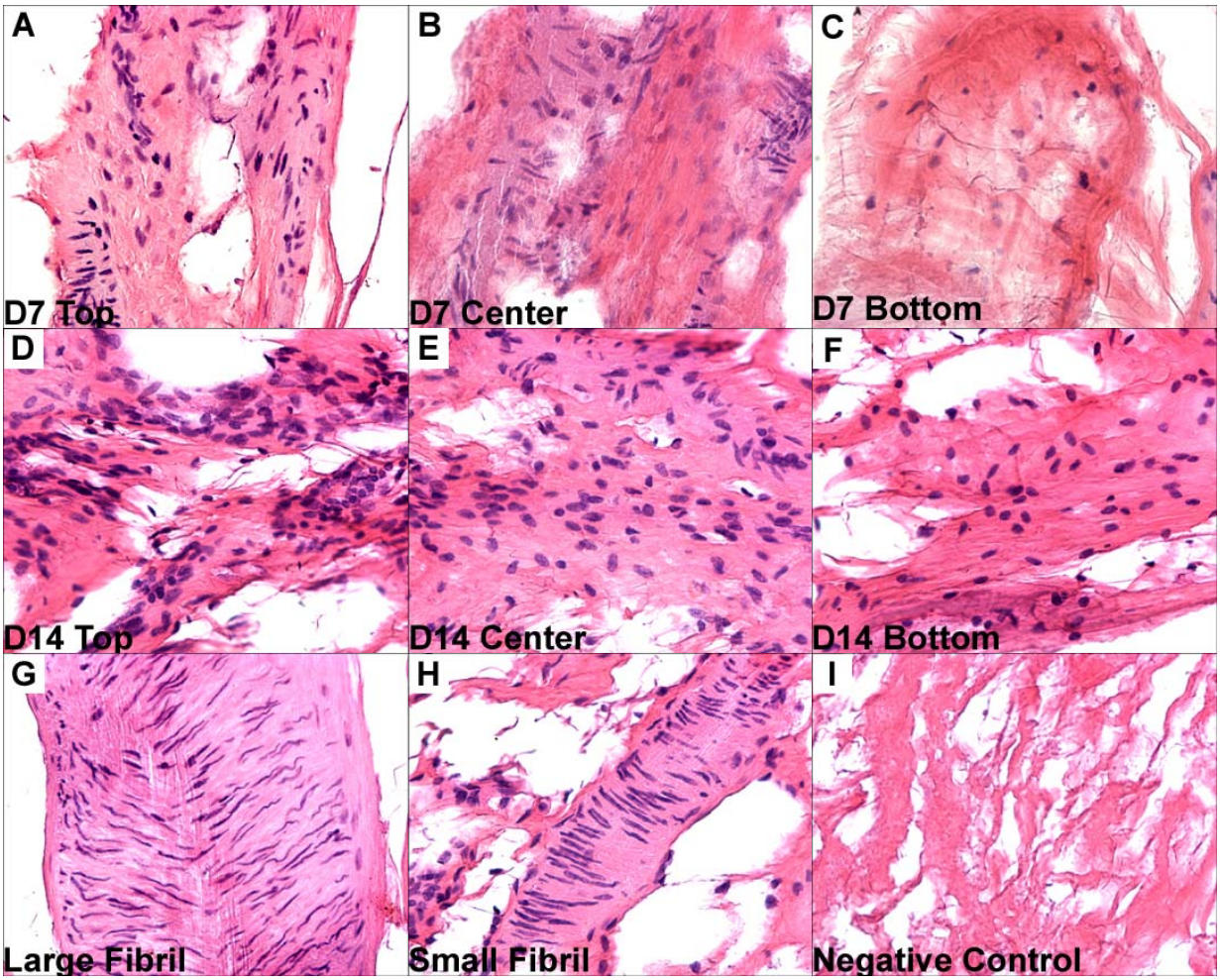

Figure 2.2 – Hematoxalyn and Eosin staining of hESCs on Small Intestinal Submucosa (SIS) at seven (A-C,G) and fourteen days (D-G,H). After seven days, cells have migrated into the matrix and have moved from the top (A) into the center of the matrix (B) with few cells moving to the bottom of the matrix (C). After fourteen days, cells are present throughout the entire matrix from top to bottom (D-F). Cells that occupy the collagen mesh take on a rounded morphology (A-F) while cells that migrate to within the major collagen fibrils take on a more flattened, elongated morphology (G-H). This second type of morphology is seen in both large (G) and small (H) fibrils. (I) shows a negative control of the SIS matrix with no cells seeded onto it. All sections are at 5um. Magnification = (x400)

Table 2.1 – List of Genes evaluated by Quantitative Real Time–Polymerase Chain Reaction

Abbreviation	*Gene Name*	*Category*	*Reference*
OCT4	POU domain, class 5, transcription factor 1	Pluripotent	[31]
DNMT3B	DNA (cytosine-5-)-methyltransferase 3 beta	Pluripotent	[31]
EBAF	Endometrial bleeding associated factor	Pluripotent	[31]
SALL2	Sal-like 2 (Drosophila)	Pluripotent	[31]
FST	Follistatin	Pluripotent	[31]
SOX2	SRY (sex determining region Y)-box 2	Pluripotent	[31]
FGF2	Fibroblast growth factor 2 (basic)	Pluripotent	[31]
FGFR4	Fibroblast growth factor receptor 4	Pluripotent	[31]
FGF4	Fibroblast growth factor 4	Pluripotent	[31]
CCNA2	Cyclin A2	Pluripotent	[31]
MSI1	Musashi homolog 1 (Drosophila)	Ecto/Neural	[75]
NES	Nestin	Ecto/Endo/Neural	[76]
NEFH	Neurofilament, heavy polypeptide	Ecto/Neural	[77]
HNF4A	Hepatocyte nuclear factor 4, alpha	Endo/Fetal Liver	[78]
FOXA2	Forkhead box A2	Endo/General	[79]
NODAL	Nodal homolog (mouse)	Endo/Meso/LR Axis	[80, 81]
CER1	Cerberus 1 homolog	Endo/Meso/Pluripote	[73, 74]
BMP4	Bone morphogenetic protein 4	Meso/General/Bone	[82]
HEY1	Hairy/Enhancer-of-split related YRPW motif1	Meso/Vascular	[51]
GATA3	GATA binding protein 3	Meso/Hema	[49]
FN1	Fibronectin 1	MesoECM	[83]
RUNX1	Runt-related transcription factor 1	Meso/Hema/Blood	[47]
EPO	Erythropoietin	Meso/Hema/Blood	[57]
NKX2-5	NK2 transcription factor related, locus 5	Meso/Vascular/Heart	[84]
T(BRAC)	T, Brachyury homolog (mouse)	Meso/Vascular/Axis	[81]
BMPR2	Bone morphogenetic protein receptor, type2	Meso/Bone	[85]
GATA4	GATA binding protein 4	Meso/Vascular/Heart	[86]
HIF1A	Hypoxia-inducible factor 1, alpha subunit	Meso	[87]
GSC	Goosecoid	Meso/Cranio Facial	[88]
CDH5	Cadherin 5, type 2, VE-cadherin (vascular)	Meso/Endothelial	[52]
PECAM1	Platelet/endothelial cell adhesion molecule	Meso/Endothelial	[44]
CD34	CD34 antigen	Meso/Endothelial	[57]
NOS3	Nitric oxide synthase 3 (endothelial)	Meso/Endothelial	[53]
TEK (TIE-2)	TEK tyrosine kinase, endothelial	Meso/Endothelial	[89]
VEGF	Vascular endothelial growth factor	Meso/Endothelial	[61]
KDR (FLK1)	Kinase insert domain receptor (VEGFR2)	Meso/Endothelial	[61]
VWF	von Willebrand factor	Meso/Endothelial	[90]
FLT1	FMS-related tyrosine kinase 1 (VEGFR1)	Meso/Endothelial	[61]
TGFB1	Transforming growth factor, beta 1	General	[91]
ACVR1C	Activin A receptor, type IC	General	[91]
ACVR1B	Activin A receptor, type IB	General	[91]
SMAD1	SMAD, mothers against DPP homolog 1	General	[91]
SMAD2	SMAD, mothers against DPP homolog 2	General	[91]
SMAD3	SMAD, mothers against DPP homolog 3	General	[91]

Abbr: Ectoderm (Ecto); Mesoderm (Meso); Endoderm (Endo); Hematopoietic (Hema)

Figure Legends for Figures (2.3-2.7)

Figure 2.3 – Relative Quantification (RQ) of Pluripotent gene expression in hESCs cultured on MEFs, Matrigel, and SIS for seven and fourteen days. Bars indicate RQ ± SD. RQ values are relative to a day zero control that has been normalized to a starting value of 1. X-axis lists pluripotent markers. (*****) indicate significant differences in expression relative to day 0. (a) or (b) indicate significant differences in relative expression between different treatment groups of the same day. (ab) indicates a group not statistically different from group (a) or group (b). No letters indicate no significant differences between treatment groups. Values are considered significant at $P < 0.05$.

Figure 2.4 – Relative Quantification (RQ) of Ectoderm and Endoderm gene expression in hESCs cultured on MEFs, Matrigel, and SIS for seven and fourteen days. Bars indicate RQ ± SD. RQ values are relative to a day zero control that has been normalized to a starting value of 1. X-axis lists ectoderm (MSI1, NES, and NEFH) and endoderm (HNF4A, FOXA2, NODAL, and CER1) markers. (*****) indicate significant differences in expression relative to day 0. (a) or (b) indicate significant differences in relative expression between different treatment groups of the same day. (ab) indicates a group not statistically different from group (a) or group (b). No letters indicate no significant differences between treatment groups. Values are considered significant at $P < 0.05$.

Figure 2.5 – Relative Quantification (RQ) of Mesoderm gene expression in hESCs cultured on MEFs, Matrigel, and SIS for seven and fourteen days. Bars indicate RQ ± SD. RQ values are relative to a day zero control that has been normalized to a starting value of 1. X-axis lists mesoderm markers. (*****) indicate significant differences in expression relative to day 0. (a) or (b) indicate significant differences in relative expression between different treatment groups of the same day. (ab) indicates a group not statistically different from group (a) or group (b). No letters indicate no significant differences between treatment groups. Values are considered significant at $P < 0.05$.

Figure 2.6 – Relative Quantification (RQ) of Endothelial gene expression in hESCs cultured on MEFs, Matrigel, and SIS for seven and fourteen days. Bars indicate RQ ± SD. RQ values are relative to a day zero control that has been normalized to a starting value of 1. X-axis lists endothelial markers. (*****) indicate significant differences in expression relative to day 0. (a) or (b) indicate significant differences in relative expression between different treatment groups of the same day. (ab) indicates a group not statistically different from group (a) or group (b). No letters indicate no significant differences between treatment groups. Values are considered significant at $P < 0.05$.

Figure 2.7 – Relative Quantification (RQ) of General Differentiation gene expression in hESCs cultured on MEFs, Matrigel, and SIS for seven and fourteen days. Bars indicate RQ ± SD. RQ values are relative to a day zero control that has been normalized to a starting value of 1. X-axis lists general differentiation markers. (*****) indicate significant differences in expression relative to day 0. (a) or (b) indicate significant differences in relative expression between different treatment groups of the same day. (ab) indicates a group not statistically different from group (a) or group (b). No letters indicate no significant differences between treatment groups. Values are considered significant at $P < 0.05$.

Relative Quantification of Pluripotent Gene Expression in Human Embryonic Stem Cells on Three Different Extracellular Matrices

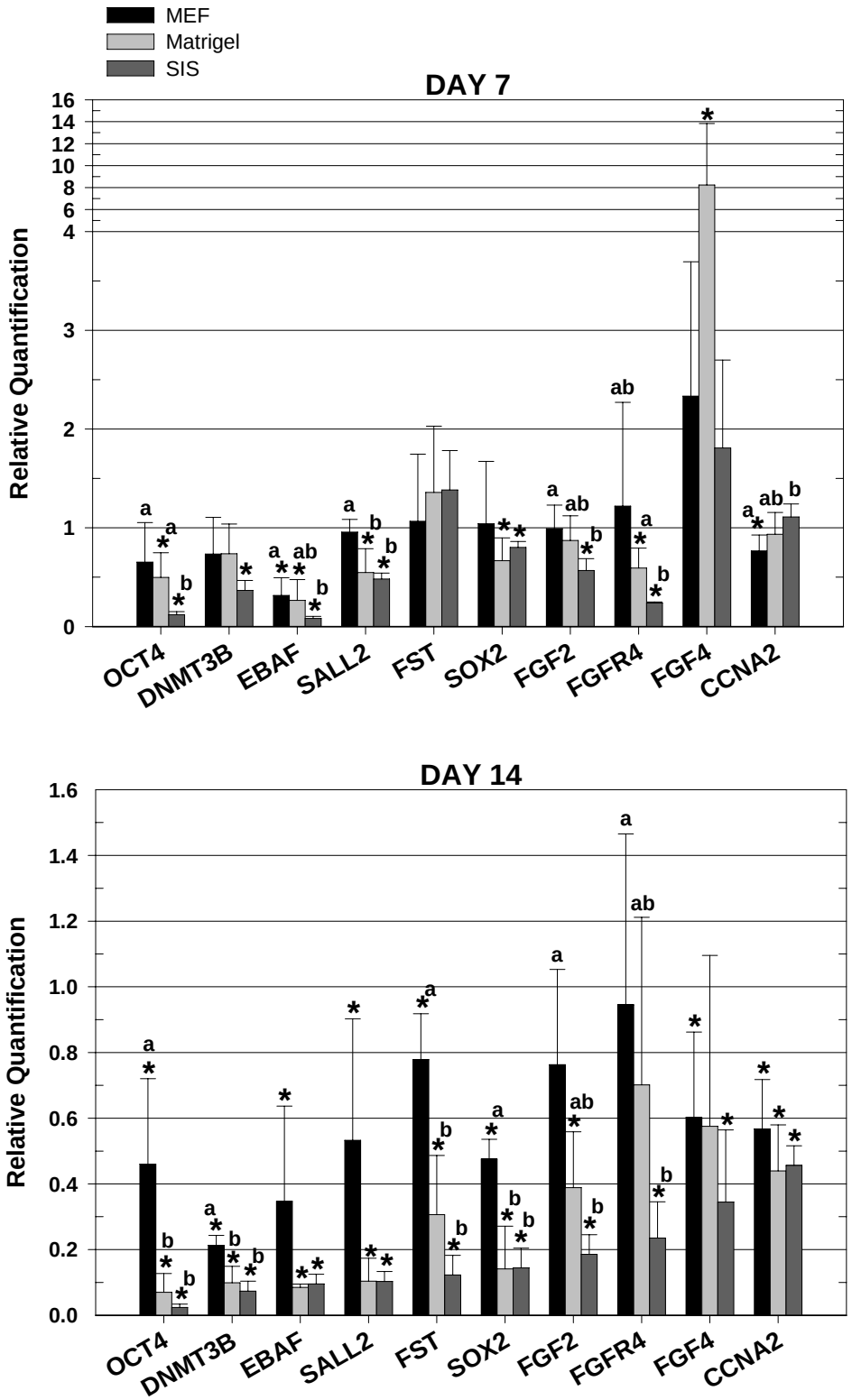

Figure 2.3

Relative Quantification of Ectoderm and Endoderm Gene Expression in Human Embryonic Stem Cells on Three Different Extracellular Matrices

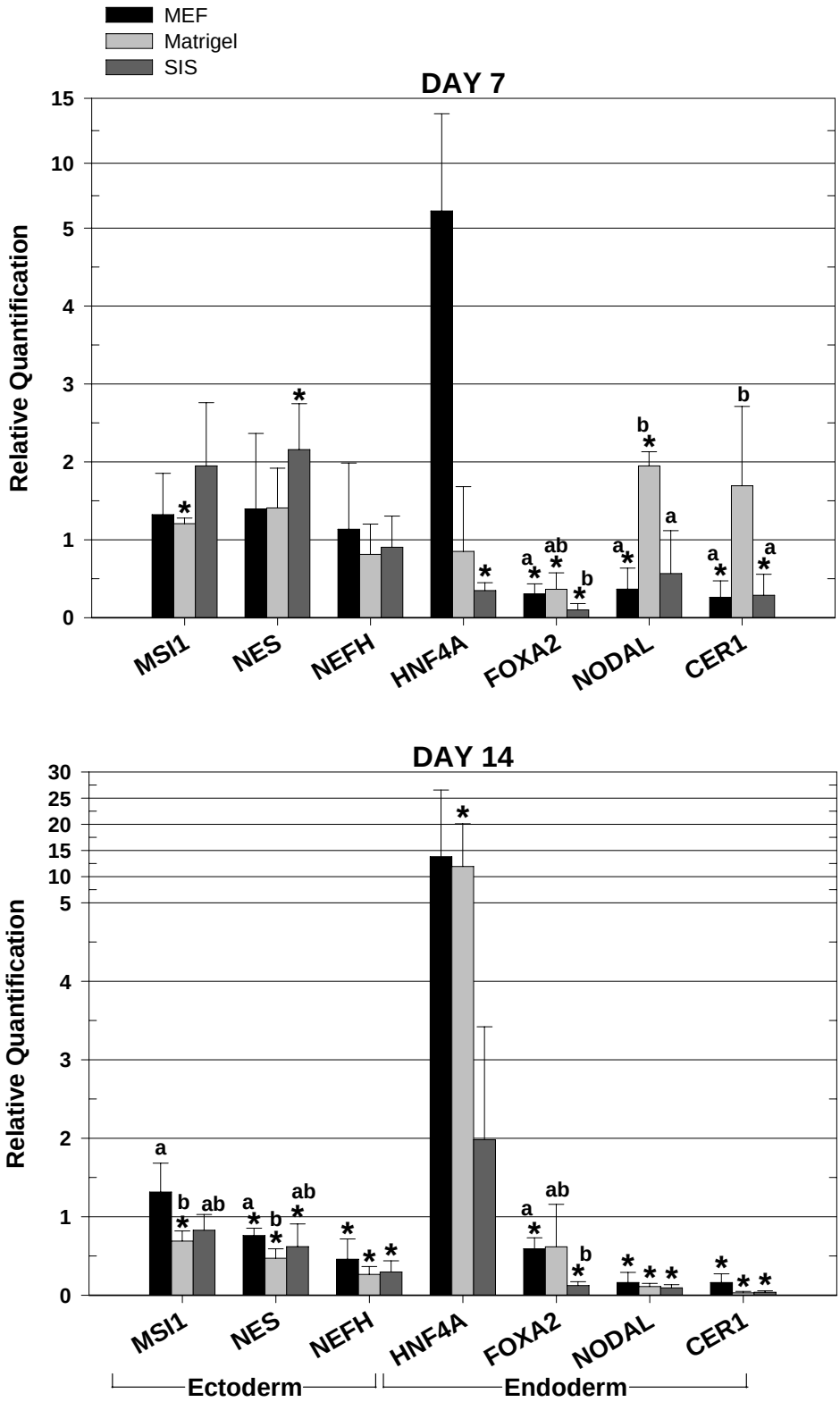

Figure 2.4

Relative Quantification of Mesoderm Gene Expression in Human Embryonic Stem Cells on Three Different Extracellular Matrices

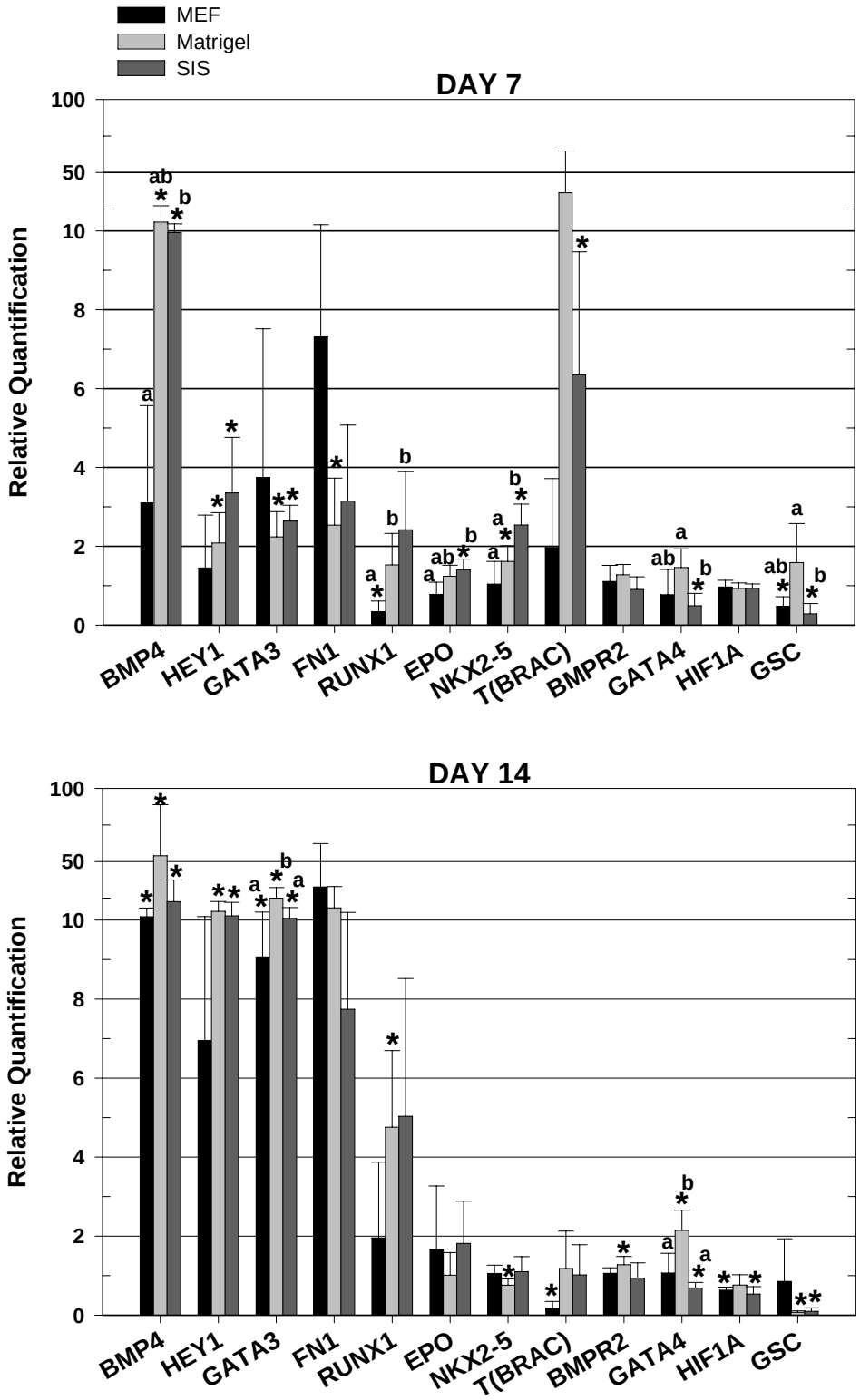

Figure 2.5

Relative Quantification of Endothelial Gene Expression in Human Embryonic Stem Cells on Three Different Extracellular Matrices

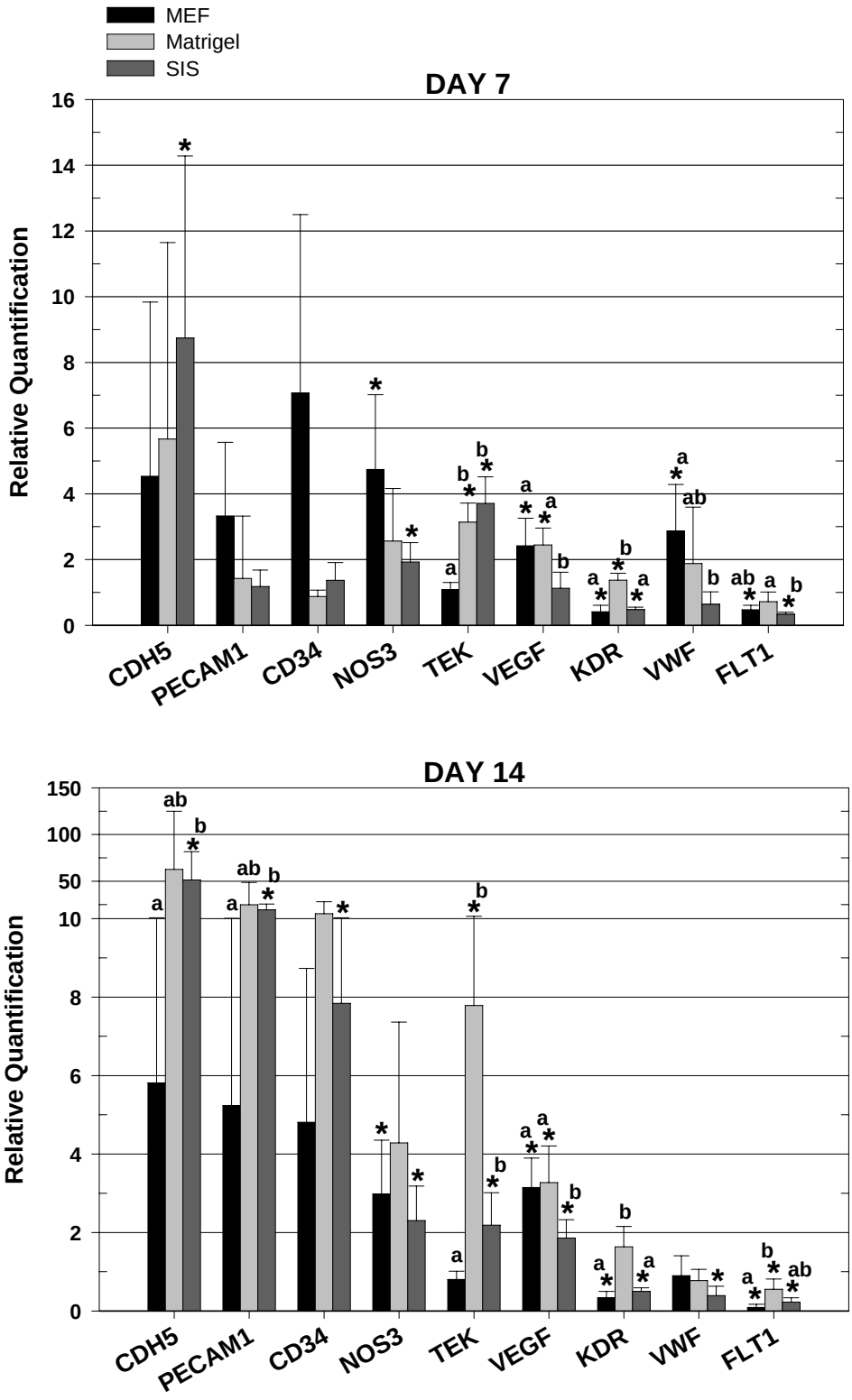

Figure 2.6

Relative Quantification of General Differentiation Gene Expression in Human Embryonic Stem Cells on Three Different Extracellular Matrices

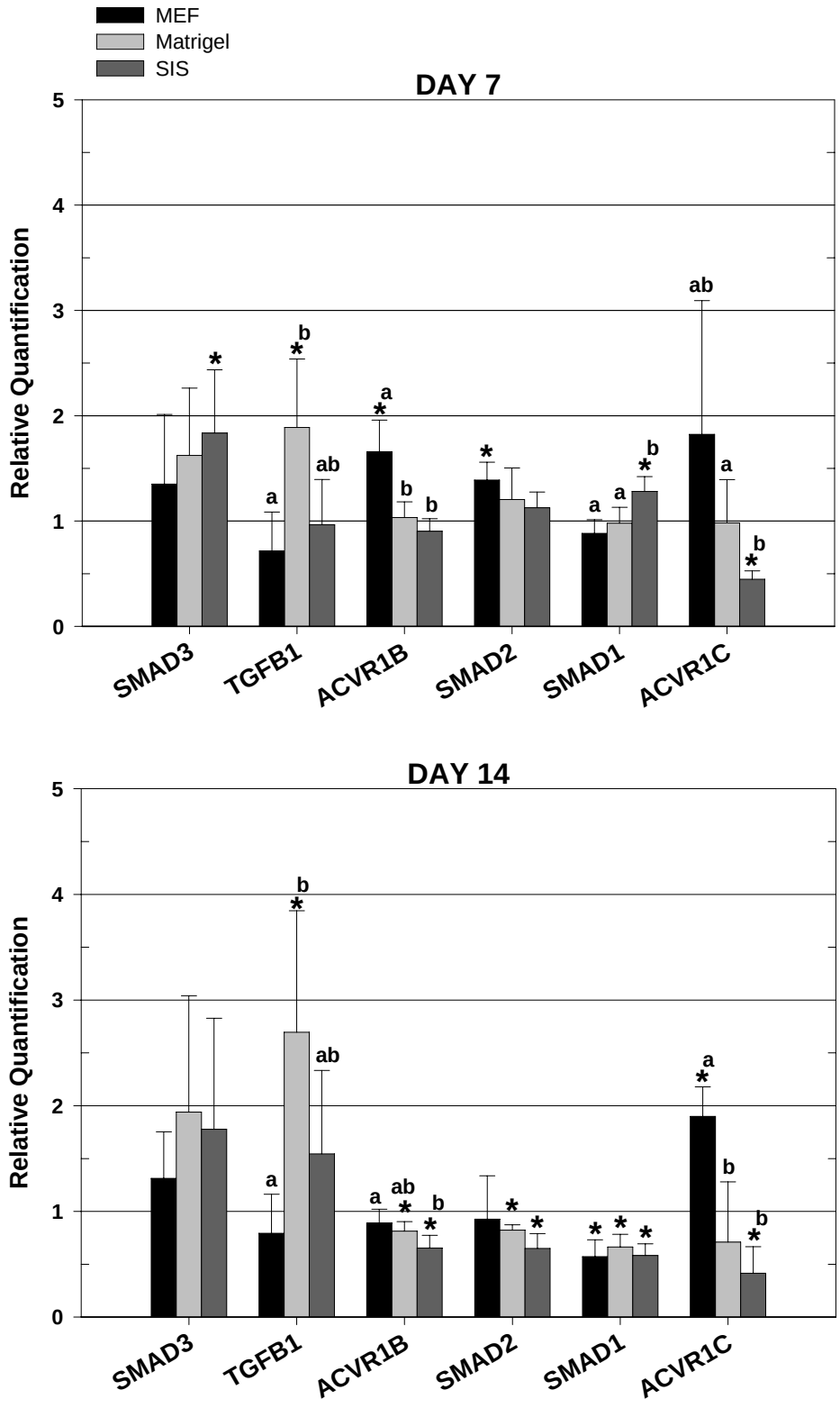

Figure 2.7

CHAPTER 3

CONCLUSIONS

The goal of this thesis was to examine the use of porcine derived Small Intestinal Submucosa (SIS) as a biomaterial to support the culture of human embryonic stem cells (hESC), and to determine, if any, the phenotypic influence of the SIS matrix over hESC differentiation. This was accomplished through the evaluation of hESCs cultured *in vitro* in the presence of the SIS matrix and the subsequent characterization of hESCs through expression profiling of various genes with quantitative RT-PCR (qt-PCR). Results suggest that the SIS matrix can support the culture of hESCs *in vitro*, and will influence the differentiation of hESCs towards a vascular endothelial related cell type. This has important implications for the engineering of endothelial-like tissues for therapeutic purposes as it provides an alternative method for hESC derived endothelial cell production with the added advantage of being engineered in a readily available cell-seeded biomatrix format that may be directly applied to tissue engineering applications. While these results are encouraging, further examination of hESCs within the SIS matrix is still needed. Further understanding of the relationship between hESC/SIS interaction, and the interaction between cell-seeded SIS and host tissues, is required before further investigation of hESC-seeded SIS as a therapeutic tool can be pursued.

The use of embryonic stem cells for cellular and tissue engineering offers an unlimited source of cellular material for potential therapeutic applications [1]. For this therapeutic potential to be reached however, more defined culture conditions must be established. The ideal conditions for hESC culture in cell based therapy aimed at humans require expansion under

feeder- and serum-free conditions [2]. The culture conditions utilized in this study consisted of hESCs cultured in serum-containing media due to the enhanced growth effects of ESCs in the presence of serum. With the quality of serum-free replacement media steadily increasing, the culture of hESCs under serum-free conditions is becoming ever more popular and well established, and thus may have been a better method for hESC culture than the one utilized here. The use of serum-free media with hESCs on SIS should be a simple first step in optimizing this method for clinical use.

One of the most critical steps will involve the repeated verification of endothelial derivation through multiple techniques. While qt-PCR serves as one of the most convincing and powerful tools for evaluating cellular and developmental events at a genetic level, the visual confirmation of gene products through antibody detection remains a tried and true method for characterization of cellular states at a morphological and phenotypic level. Positive staining for several of the major endothelial markers such as CDH5, PECAM1, and KDR, as well as negative staining for various non-endothelial markers, would have provided more convincing evidence for vascular endothelial derivation and would have supported the already strong qt-PCR data. Future investigations of hESC derived endothelial cells on the SIS matrix should undoubtedly be subjected to immunocytochemical verification.

While the expression profile of hESCs on SIS does indicate an increase in endothelial specific genes with an overall decrease in non-endothelial genes, a strict comparative study of the expression profiles between hESC derived endothelial cells via the SIS matrix and known vascular endothelial cells such as HUVECs would also have provided strong additional evidence for hESC endothelial derivation. Demonstrating that hESC derived endothelial cells match wild type endothelial cells both genetically and phenotypically is absolutely essential before use in

clinical trials. Defined endothelial cells should therefore serve as a control in all future experiments.

Future Directions

In addition to quality, the quantification and efficiency of differentiation must also be determined. It is unknown at this time the percentage of cells differentiating towards an endothelial lineage. qt-PCR data suggest the level of alternate cell types present may be low as markers representative of other lineages are decreased in hESCs on SIS, especially when compared to marker expression for hESCs on MEFs, which is typically accepted as a platform supporting random differentiation to all cell types [3]. Still, some form of screening may be necessary to determine the number of hESCs moving towards an endothelial lineage. This may prove to be challenging as it is difficult to remove intact viable cells from a 3D matrix such as SIS, although some techniques such as a collagen digest to degrade the matrix may prove useful. Cells could then be tagged with fluorescent antibodies and sorted by FACS to determine actual percentages. Fluorescent antibodies could also be used to tag endothelial cells within the SIS matrix after it has been fixed and sectioned to allow for manual counting to generate percentages.

Efficiency of derivation will be an important factor in establishing purity of the endothelial cell population. Typically, a screening step is necessary when deriving specific cell types *in vitro* due to some cells differentiating into non-target lineages. Heterogeneous cell populations may cause undesirable side effects and thus it is imperative to produce the highest levels of homogeneous cell populations possible. Current strategies for pushing ESCs towards specific lineages focus on altering media components to generate desired effects. The SIS matrix may provide an additional level of control over derivation media alone, and the use of endothelial derivation media in combination with the SIS matrix may enhance the production of

endothelial cells even further to potentially yield high quantities of homogeneous cell populations. Alternatively, cells could potentially be carried through initial differentiation events towards an endothelial cell type with endothelial derivation media, screened with FACS to select for cells with specific markers, then seeded onto the SIS matrix to allow for further endothelial development. The most interesting and potentially useful idea for enhancing endothelial cell differentiation on SIS involves the use of defined endothelial cells to condition the SIS matrix with endothelial specific matrix proteins [4]. This may allow for hESCs to receive additional endothelial signals that may not be present in the SIS matrix or commercial endothelial growth media. The use of conditioned SIS with endothelial growth media could potentially yield hESC derived endothelial cells in extremely high levels to that which are acceptable for transplant. High derivation efficiency along with multiple verification steps will be essential for making the transition from lab to clinic.

As the development of artificial tissues progress, there eventually becomes a need for evaluation under physiological conditions. *In vitro* simulations go only so far and can not take into account the vast number of variables present in living organisms. To understand the dynamic interaction between engineered transplant tissues and host organisms, physiological integration of artificial tissues within animal models is a necessary step. There have been numerous studies involving the use of cell-seeded SIS within animal models. The use of SIS matrix seeded with modified hEG cells and marrow derived stem cells to restore physiological function to bladder wall defects have now been reported [5, 6] and will prove to be a catalyst for more advanced applications. The use of hESC derived cell types in combination with bioscaffolds such as the SIS matrix will soon become an active area of research as methods to push hESCs towards specific lineages becomes more refined. The use of an animal model to

evaluate the physiological integration of hESC derived endothelial cells on the SIS matrix with host organs will be a crucial and necessary step before moving on to clinical trials.

The production of an engineered endothelial tissue will have a tremendous impact on the future of tissue engineering and transplant medicine. Endothelial cells play a key role in the proper function and maintenance of a number of organ systems [7]. They are responsible for lining the complete vascular and lymphatic system and are responsible for regulating vessel wall and circulatory function by modulating blood pressure and vascular tone. They are the primary gate keepers of the blood-tissue interface overseeing the transfer of molecules from blood to tissues and perform vital filtration functions in the kidneys and blood brain barrier. They produce and react to multiple cytokines involved in immune and inflammatory responses, and orchestrate thrombosis and fibrinolysis during injury and trauma. The endothelium is thus a necessary and crucial component of homeostasis and its dysfunction results in various disease states including atherosclerosis, hypertension, clotting disorders, and inflammatory syndromes.

Endothelial cells play central roles in angiogenesis and have the ability to transdifferentiate to intimal smooth muscle and mesenchymal cells for complete tissue remodeling in a variety of organs [7]. This makes the idea of a transplantable endothelial tissue ideal for aiding in the regeneration of extensive trauma to various tissues and organs in which the body would not normally be able to cope. Procedures such as vascular grafts, bladder and stomach wall repair and urethral reconstruction would all benefit from pre-seeded endothelial matrices. An endothelial tissue would play a crucial role in the engineering of artificial organs such as artificial blood vessels, eliminating the need for autologous transplants in procedures like coronary bypass. It could allow for internal grafts in patients of throat and mouth cancer, esophageal reconstruction, and dermal grafts for burn victims.

In conclusion, the use of hESCs with the SIS matrix has provided an alternative method towards the production of an endothelial-like tissue for regenerative medicine applications. With enhanced differentiation techniques and customized hESC lines specific to individual patients, tissue engineering technologies such as the one described here will offer great advantages over current therapies. There still remains however, much work to be done before applications such as these can be implemented into main stream medicine. With time and work, these methods will provide solutions to treating conditions for which there has previously been no other alternative.

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