STEM CELLS WITH MULTILINEAGE POTENTIAL DERIVED FROM PORCINE
SKIN

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TABLE OF CONTENTS

ACKNOWLEDGEMENTS.....	i
FIGURES.....	v
TABLES.....	vii
ABBREVIATIONS.....	viii
INTRODUCTION.....	1
I. LITERATURE REVIEW.....	2
Stem cell multipotency and its regulation.....	3
A. <i>Oct4</i>	6
B. <i>Stat3</i>	9
Adult stem cells and their therapeutic potential.....	10
Somatic Stem Cells.....	12
A. <i>Stem Cells from Skin</i>	12
a) <i>Attached isolation</i>	15
b) <i>Suspension isolation</i>	16
B. <i>Neuronal Stem Cells</i>	18
a) <i>Cells of the mammalian central nervous system</i>	18
b) <i>Neuronal stem cell isolation and characterization</i>	23
Identifying adipocytes in vitro	29
Stem cell proliferation regulation.....	31
A. <i>Stem cell division</i>	31
B. <i>Stem cell growth factors</i>	33
a) <i>Fibroblast Growth Factor</i>	34
b) <i>Epidermal Growth Factor</i>	35
Stem cells and their potential applications for livestock transgenesis	36
II. OBJECTIVE.....	42
III. SPECIFIC AIMS.....	42
IV. MATERIALS AND METHODS.....	42
<i>Cell isolation and culture</i>	43
a) <i>Complete Dissociation Method</i>	44
b) <i>Cut Method</i>	45
c) <i>Gentle Dissociation Method</i>	45
<i>Sphere Cell Number Determination</i>	47
<i>Number of spheres per cm² skin</i>	47

Cyopreservation.....	48
<i>a) Dissociated sphere cryopreservation.....</i>	<i>48</i>
<i>b) Whole sphere cryopreservation.....</i>	<i>48</i>
<i>Poly D-lysine/laminin coating of cell culture dishes</i>	49
<i>Differentiation study</i>	50
<i>Immunocytochemistry and Flow Cytometry</i>	51
<i>5-bromo-2-deoxyuridine (BrdU) incorporation assay</i>	52
<i>Oil Red O Staining</i>	53
<i>Leptin Radioimmunoassay</i>	53
<i>Total RNA isolation</i>	55
<i>Treatment of RNA with DNase I</i>	55
<i>Real Time RT-PCR</i>	56
<i>Agarose Gel Electrophoresis of Real Time Products</i>	57
<i>Recovery of DNA from agarose gel.....</i>	58
<i>Analysis of DNA by Spectrophotometry</i>	59
<i>Real time product Sequencing</i>	60
<i>Chromosome analysis (karyotype test)</i>	60
V. RESULTS.....	61
<i>Isolation and culture of PSOS cells</i>	61
<i>Cyopreservation.....</i>	63
<i>Proliferative potential of PSOS cells in vitro</i>	64
<i>Karyotype Analysis</i>	71
<i>Gene expression profile of PSOS cells</i>	71
<i>Differentiation of PSOS cells into neurons, astrocytes and adipocytes in vitro</i>	75

<i>Individual PSOS cells are multipotent in vitro</i>	90
<i>PSOS cells vs. PBOS cells.....</i>	91
VI. DISCUSSION.....	98
<i>Future Studies</i>	107
<i>Conclusion.....</i>	107
VII. REFERENCES.....	110

FIGURES

Figure 1: Expression of Oct4 during embryogenesis and ES cell differentiation.....	8
Figure 2: Differentiation of a stem cell into neuronal cell types	24
Figure 3: Micrograph of floating spheres of cells isolated from porcine fetal skin.....	62
Figure 4: Proliferation potential of PSOS cells	65
Figure 5: Comparison of various culture methods on population doubling time of PSOS cells	68
Figure 6: Growth curve of PSOS cells.....	69
Figure 7: The addition of FBS increases late passage PSOS cell proliferation.....	70
Figure 8: PSOS cells have a normal karyotype after five months culture in vitro	72
Figure 9: Immunocytochemistry depicts a subpopulation of PSOS cells expressing nestin, and a subpopulation fibronectin.....	73
Figure 10: A subpopulation of PSOS cells express nestin and fibronectin	74
Figure 11: Stat3 is expressed in a subpopulation of PSOS cells before induced differentiation	76
Figure 12: Real Time RT-PCR analysis of PSOS cells for the expression of Stat3 mRNA	77
Figure 13: Oct4 is expressed in a subpopulation of PSOS cells before induced differentiation	78
Figure 14: Real Time RT-PCR analysis of PSOS cells for the expression of Oct4 mRNA.....	79
Figure 15: Following neuronal differentiation a subpopulation of PSOS cells stain positive for GFAP and are morphologically consistent with astrocytes	81
Figure 16: Following neuronal differentiation a subpopulation of PSOS cells stain positive for β III-tubulin and are morphologically consistent with neurons	82
Figure 17: Representative acquisition graphs of flow cytometry study on the protein expression before and after induced neuronal differentiation in PSOS cells.....	83

Figure 18: Comparison of nestin mRNA expression before and after induced neuronal differentiation.....	84
Figure 19: Comparison of beta III tubulin mRNA expression before and after induced neuronal differentiation.....	85
Figure 20: Agarose gel following Real Time RT-PCR, before and after differentiation	87
Figure 21: Cells stain positive for the presence of lipid vacuoles following four weeks adipocyte differentiation.....	88
Figure 22: Real Time RT-PCR analysis of PSOS cells following four weeks induced adipocyte differentiation.....	89
Figure 23: Subpopulations of expanded PSOS cells immunostain positive for GFAP and beta III tubulin following induced neuronal differentiation	94
Figure 24: Relative percentages of differentiated cells from the clonally expanded PSOS population following neuronal differentiation	95
Figure 25: Clonally expanded PSOS cells differentiate into cells with the characteristics of adipocytes	96

TABLES

Table 1: The source and differentiation potential demonstrated of various stem cell populations.....	4
Table 2: Primers used for Real Time RT-PCR to detect the expression of various mRNA transcripts.....	57
Table 3: PSOS and PBOS marker expression prior to differentiation.....	92
Table 4: Comparison of the potential of PSOS and PBOS cells to differentiate into the neuronal and mesenchymal lineages.....	93

ABBREVIATIONS

β1	beta-1-integrin
BAT	brown adipose tissue
BrdU	bromodeoxyuridine
CNS	central nervous system
DMSO	dimethyl sulfoxide
ECM	extracellular matrix
EGF	epidermal growth factor
EGFP	enhanced green fluorescent protein
ES	embryonic stem
FBS	fetal bovine serum
FGF	fibroblast growth factor
FGF-2	fibroblast growth factor basic
FGR1	fibroblast growth factor receptor 1
GCM	glial cells missing
GFAP	glial fibrillary acidic protein
HBSS	Hank's balanced salt solution
HE	human equivalent
ICM	inner cell mass
JAK	Janus Kinase
LIF	leukemia inhibitory factor
LRC	label retaining cell
MAP	microtubule-associated protein
MBP	myelin basic protein
MSC	mesenchymal stem cell
NSC	neural stem cell
NT	nuclear transfer
PBOS	porcine brain originating sphere
PDGFαR	platelet derived growth factor α receptor
PDL/L	poly-D-lysine/laminin
PLP	proteolipid protein
PO/L	poly-ornithine/laminin
POU ^h	POU family carboxyl-terminal homeodomain
POU _s	POU family amino-terminal specific
PRR	proline-rich region
PSOS	porcine skin originated sphere
RIA	radioimmunoassay
SKP	skin-derived precursor
Stat3	signal transducer and activator of transcription 3
TA	transit amplifying
T _m	melting temperature
WAT	white adipose tissue

Introduction

In mammals, the epidermis of skin is continually refurbished every day. This rapid remodeling is maintained by stem cells that are capable of self-renewal and supplying differentiated cells in a constant manner [1]. Stem cells within the hair follicle, sebaceous gland and epidermis have been demonstrated to be involved in cutaneous renewal during postnatal life (for review [3]). Moreover, multipotent stem cells that can form neural and adipose cells have been isolated from the dermis of mouse skin [13]. These findings indicate that multiple classes of stem cells with different differentiation potentials are present in the skin, making skin a valuable source of stem cells for the study of development and differentiation. Despite many successes in the isolation and characterization of stem cells from various tissues of rodents and humans, there have been few studies on stem cells from non-rodent animals. This limits the advancement of stem cell biology and its ultimate translation to human clinical applications.

Stem cells derived from livestock animals may also offer solutions for more efficient nuclear transfer (animal cloning). The current extremely low success rate of live births from nuclear transfer is thought to be due to the reprogramming failure of the donor cell nucleus to a state equivalent to that of an early embryo [3]. It has been hypothesized that the genome of stem cells may be more easily reprogrammed to resemble the genome of the zygote, which may make stem cells more efficient donors for nuclear transfer [2]. In this report, we describe the isolation of multipotent stem cells from fetal porcine skin. Our findings represent the first report of skin-originated stem cells isolated from non-rodent animals.

I. LITERATURE REVIEW

The discovery that several stem cell populations have the ability to differentiate into other tissue types has opened new doors for improved therapeutics, and biotechnology, and has made it possible to ask biological questions that were previously out of reach. Stem cells have a variety of properties that make them unique from somatic cells. The most universally-accepted features of stem cells are prolonged self-renewal capacity, long term viability, and the ability to differentiate into cell types of multiple lineages [1].

The ability of stem cells to differentiate into multiple cell types suggests that they play an important role in tissue maintenance, and repair. Tissue maintenance in organisms involves an orderly chain of highly regulated processes involving cell proliferation, migration, differentiation, and maturation. Stem cells are the earliest cell type in this chain. The earliest stem cell in mammals is the fertilized egg, which is totipotent and is the progenitor for all of the cells that form the organism. Embryonic stem (ES) cells are derived from the inner cell mass of blastocysts, and are pluripotent. Pluripotent describes the ability of cells to generate all of the different cell types of the developing embryo (for review [2]). Later in development, stem cells are localized in tissue niches where they give rise to highly defined cell types within the organ. Therefore, together with their extensive capacity for self-renewal, stem cells also have the ability to differentiate into cells with more defined developmental potentials [3]. These cells eventually differentiate into cells with specific phenotypic characteristics and have specialized functions. Recently, a wide variety of adult tissues have been identified as

harboring stem cell populations. These include bone marrow, nervous tissue, muscle, intestine, and epidermis (Table 1). The presence of stem cells in adult tissues is required for damage repair and renewal.

Stem cell multipotency and its regulation

Initially, stem cells from different tissues were believed to be developmentally committed to a specific cell lineage, namely the lineage in which they were originally isolated. Recently, a large amount of data has been collected suggesting that these stem cells have a much broader differentiation potential than was originally thought (for review [4]). Reports have shown that a particular stem cell, while being able to differentiate into cells with characteristics of lineages in which they reside, may also give rise to cells of other lineages. For example, the hematopoietic stem cell, in addition to forming blood cells, can also form hepatic oval cells (“blood into liver”); [5]. In turn, neural stem cells, apart from forming three main cells found in the brain, also produce committed hematopoietic progenitors (“brain into blood”); [6-8]. Mesenchymal stem cells (MSCs) are able to form a variety of mesenchymal phenotypes as well as give rise to non-mesenchymal cells such as neural cells (“marrow into brain”); [9]. An early report from Bjornson *et al.* [8] showed that clonal, adult neural stem cells (NSCs) labeled with lacZ and injected into sub-lethally irradiated recipient animals repopulated the bone marrow and produced hematopoietic progeny. Also, using surface antigens specific to the donor mouse strain, they were able to show cells of donor origin in the recipient spleen, and peripheral blood by flow cytometry. This suggests that NSCs migrated into the bone marrow and differentiated into bone marrow progenitor cells capable of producing blood cells. Therefore, given the correct conditions, stem cells that normally

Table 1: The source and differentiation potential of various stem cell populations.

Stem Cell	Source	Cells Produced	References
Hematopoietic	Bone Marrow	Blood, endothelial, hepatic, and muscle cells	[5] [10] [11]
Neural	Brain Dermis	Neurons, astrocytes, oligodendrocytes, and blood cells	[6] [8] Reviewed in [12] [13]
Epithelial	Gut, epidermis	All cells in epidermal layers and epithelium crypts	[14] [15] [16]
Mesenchymal	Bone Marrow	Bone, cartilage, tendon, adipose, muscle, marrow stroma, and neural cells	Reviewed in [17] [18] [19]
Embryonic	Blastocyst	All cells	[20] [21]
Germline	Testis	Spermatogonia	[22]

make only neural cell types can produce several blood cell types. NSCs have also been demonstrated to differentiate into muscle cells, *in vitro* and *in vivo*, suggesting an even broader differentiation potential [23]. Clonally-derived NSCs were co-cultured with adult murine myoblasts for four to seven days, and the resulting cultures were tested for the presence of muscle cells. The presence of muscle cells was confirmed using the muscle specific genes MyHC and MyoD. Myogenic conversion was seen in 4-9% of the total NSCs plated. Direct contact between the NSCs and the myoblasts was required to form muscle cells. As well, single NSC-derived muscle cells and fibers composed completely of NSCs were observed, ruling out cell fusion as an explanation for the differentiation. Following induced differentiation, myotubes made up of mouse myoblasts and NSCs were also observed, suggesting the NSCs were accepted as partners during myotube formation. The ability of NSCs to differentiate into muscle cells *in vivo* was also investigated [23]. Three weeks following the injection of NSCs into damaged muscle tissue, NSC-derived muscle cells were found in the repaired tissue. These results suggest that NSCs have the potential for multilineage differentiation upon exposure to the appropriate epigenetic cues, *in vitro* and *in vivo*. Possibly, the most notable report to date is the study by Clarke *et al.* [24], which involved the injection of lacZ labeled NSCs into mouse blastocysts. They were able to show that NSCs could contribute to the formation of chimeric embryos and give rise to cells in all germ layers, but were unable to show the contribution of NSCs to the haematopoietic system. This may be due to the differing locations of NSCs utilized, since Bjornson *et al.* [8] isolated NSCs from the subventricular region, while Clarke *et al.* [24] isolated NSCs from the ependymal layer. Another study by Rietze *et al.* [25], identified a population of NSC that resides in both

the ependymal and subependymal regions. These NSCs are nestin positive, and 80% form neurospheres in culture that were able to differentiate into neurons, oligodendrocytes, and astrocytes. Under the correct culture conditions, differentiation into muscle cells was also demonstrated.

The evidence that stem cells isolated from a particular tissue, have the potential to differentiate into cell types not found in that tissue, has resulted in increased research into the genes responsible for this characteristic. One candidate gene that has been linked to pluripotency is Oct4.

A. Oct4

Cell fate with regards to differentiation is determined by transcription factors that interact with DNA, and in turn control the expression of various genes [26]. Oct4 is a transcription factor that belongs to the POU family of transcription factors [26]. The main feature of the POU family of transcription factors is the POU domain, which consists of two subdomains: a 75 amino acid amino-terminal specific (POUs) region and a 60 amino-acid carboxyl-terminal homeodomain (POUh) [27]. The two domains bind specifically to DNA through a helix-turn-helix structure and are connected by a variable linker of 15 to 56 amino-acids [27]. While the POU domain is highly conserved, sequences outside the POU region are not critical for DNA binding and vary among the POU family of transcription factors [27]. The C-terminal domain of Oct4 has been shown to be cell-type specific and is regulated through phosphorylation [28-30]. Brehm *et al.* [28] replaced the POU DNA binding domain with the binding domain of other transcription factors, such as the heterologous yeast Gal4 DNA binding domain. This replacement did not affect its transactivation function of test promoters, suggesting

that the transactivation function can be transferred to other unrelated DNA binding domains. The regulation of Oct4 expression specifically to pluripotent cells, was observed only if the C domain was linked to the POU domains of Oct4 and Oct2, but not to other binding domains, such as Pit-1 or Gal4 [28]. This suggests that the Oct4 POU-domain may function differently by serving as an interaction site for regulatory factors.

In the mouse, Oct4 expression begins at the 4-to-8 cell stage and becomes restricted to the inner cell mass (ICM) of the blastocyst (Figure 1) [31]. Following gastrulation, Oct4 expression is restricted to the germ cell lineage [32, 33]. A similar Oct4 expression pattern was also observed in human blastocysts [34, 35]. The restricted expression of Oct4 in cells with a high differentiation potential, such as the ICM and germ cells, suggests that it plays a role in maintaining cell pluripotency.

Functionally, Oct4 has been linked to the maintenance of pluripotency in ES cells. Mouse embryos homozygous for an Oct4 deletion do not form blastocysts with pluripotent ICMs, and the embryos die shortly after implantation [36]. Embryos homozygous for an Oct4 deletion produce trophoblast giant cells lacking an ICM [36]. It was shown that Oct4 is required to maintain ES cells in an undifferentiated state during *in vitro* culturing. Attempts to isolate ES cells from the ICM of homozygous Oct4 knockout embryos have failed [36]. This suggests that Oct4 expression is a requirement for the maintenance of a self-renewing, undifferentiated phenotype, in ES cells. The over and under expression of Oct4 in ES cells has also been studied [26]. A 30% decrease in Oct4 expression resulted in the differentiation of ES cells into putative endoderm, while over-expression of Oct4 resulted in differentiation of ES cells into trophectoderm *in vitro* [26].

Figure 1: Expression of Oct4 during embryogenesis and ES cell differentiation: The isolation and differentiation of ES cells *in vitro* are illustrated schematically beginning with fertilization to form a zygote. At the blastocyst stage, the inner cell mass (ICM) becomes visible and can be extracted and cultured *in vitro* to form embryonic stem (ES) cells. Cultured ES cells can be induced to differentiate into various cell types that do not express Oct4. Oct4 expression is shown in red colour, while cells not expressing Oct4 are green. There is a general correlation between Oct4 expression and totipotency.

These observations suggest that Oct4 is different from many known transcriptional regulators that appear to function in an on-off manner, and the expression of Oct4 must be tightly controlled in order for ES cells to retain a pluripotent state. Recently, it has been proposed that Oct4 is not the sole factor responsible for ES cells retaining pluripotency, and that it acts cooperatively with the Stat3 transcription factor [26].

B. Stat3

The signal transducer and activator of transcription 3 (Stat3) family, encompasses seven members, of which most are ubiquitously expressed, although individual Stat proteins can be differentially expressed in different cell types [37]. The ability of a common transcription factor to regulate a particular gene in a tissue-specific manner, is currently an active area of investigation.

The Stat3 protein is an activator of gene expression that has been linked to the maintenance of pluripotency in ES cells [38]. Stat3 is a DNA binding protein that has a conserved amino-terminus that is involved in tetramerization (for review [38]). The Stat protein is bound with activated surface receptors and becomes phosphorylated at specific sites. The phosphorylation of tyrosine position 705 (Y705) in response to cytokines is mediated by the Janus Kinase (JAK) and results in Stat3 activation [39]. The serine phosphorylation position 727 (S727) has been shown to be mediated by several different factors including ERK1, ERK2, p38, JNK, and an H-7 sensitive kinase [40]. It remains unclear whether both phosphorylation events are required for Stat3 activation, or if tyrosine phosphorylation alone results in Stat3 activation. The phosphorylated Stat3 translocates to the cell nucleus, and regulates gene expression through binding to specific

DNA elements. The DNA binding domain has specificity for the palindromic IFN- γ sequence.

Knockout of Stat3 in ES cells leads to a decrease in cell viability, and differentiation capacity [26, 41, 42]. In ES cells the activation of Stat3 is linked to the leukemia inhibitory factor (LIF). The presence of LIF, in ES cell medium, results in the activation of Stat3 and its absence results in the differentiation of ES cells. The expression of Stat3, even in the absence of LIF, is sufficient to retain ES pluripotency [42]. This suggests that while Stat3 and LIF work together, in ES cells, Stat3 is the main repressor of differentiation in this pathway.

The knockout of Stat3 leads to embryonic lethality in mice [43]. Stat3 knockout embryos rapidly degenerate just after blastocyst implantation. Due to the phenotype of embryonic lethality it has been difficult to determine the exact role Stat3 plays in embryogenesis. In order to further study the role of Stat3 beyond embryogenesis, a conditional knockout technique has been utilized. Mice that have had Stat3 ablated in the epidermis via Cre expression are viable. The phenotypic consequences of the Stat3 knockout include impaired wound healing, impaired hair growth, and an increased rate of spontaneous ulcer formation [44]. While the exact function of Stat3 remains unknown, its requirement for embryonic viability suggests an essential role in early development. The impairment to wound healing observed in mice with conditional Stat3 ablation in their skin suggests that the Stat3 protein may play a role in somatic stem cell functioning.

Adult stem cells and their therapeutic potential

The common goal of much stem cell research is the potential for therapeutic use. In this regard, mesenchymal stem cells (MSCs) are one of the few stem cell types that have been extensively studied for this purpose. Due, in part, to their relative ease of isolation from bone marrow aspirates, long term *in vitro* culturing, and their ability to be successfully transfected with exogenous genes. Depending on their microenvironment, MSCs have the ability to differentiate into different cell types.

Immune rejection of transplanted somatic cells, such as kidney, heart, and skin cells, is a major problem in the treatment of disorders. MSCs are of great interest in the field of therapeutics due, in part, to their apparent lack of surface antigens [45]. The infusion of allogeneic MSCs into organisms has resulted in no detectable immune reactions (reviewed in [46]), suggesting that it may be practical to utilize MSCs to treat patients, without the concern of immune rejection. Several strategies for treating diseases with MSCs have been utilized. Implantation of stem cells is useful in local tissue defects, while systemic dysfunction of organs and/or tissues is treated by MSC injection into the circulation. The migration ability of genetically marked human stem cells following injection into immunocompromised mice has been investigated [47]. The injected stem cells were able to home to local bone surfaces and participate functionally in repair. The ability of stem cells to migrate from the blood stream to sites of damage following infusion provides a potential means to treat many disorders. This is advantageous over direct site injection, which is only useful in certain tissue sites.

The options for treating disease are further increased by the possibility of genetically modifying stem cells to express a required therapeutic gene product. In one study, MSCs were modified to express a gene involved in bone formation, and were

transplanted to the site of fractures where the rate of osteogenesis was determined [48]. Modified MSCs were able to induce a 1.65 greater bone regeneration capacity over natural healing [48]. The potential to genetically modify stem cells and then use them to treat health disorders is an exciting possibility within the field of medicine.

The broad differentiation potential of stem cells also makes them an attractive therapeutic agent for the replacement of damaged tissues. Using stem cells to increase the rate at which damaged tissue is replaced may allow for faster recovery. A recent study examined the ability of MSCs to improve the function of damaged heart tissue [49]. Rat MSCs were induced to differentiate along the myogenic lineage and transplanted into myocardial scar tissue produced by cryoinjury. Five weeks following injection, heart function measurements were made. A reduction in scar tissue was found, and an increase in peak systolic pressure was observed in those subjects receiving the induced MSCs, suggesting that MSCs were able to integrate into the heart tissue and function properly to replace damaged tissue. This is of particular interest as heart damage is thought to be relatively permanent [49]. Stem cells have also been shown to adapt well to their surroundings and survive in a variety of tissues after systemic injection [47], suggesting that stem cells may be useful in treating a wide variety of tissues disorders.

Somatic Stem Cells

A. Stem Cells from Skin

The skin, as the largest and most accessible organ in the mammalian body, has created interest as a source of stem cells. Mammalian skin is made up of three distinct regions, the epidermis, dermis, and the subcutaneous tissue. The epidermis is the outer

most layer of skin, and is exposed to the environment. The dermis is the layer underneath the epidermis, and is composed mainly of collagen forming a fibrous connective tissue. The dermis serves the purpose of protecting the body, by storing water, maintaining temperature, and housing the sensory nerves. It has also been found to contain stem cells in mice [13]. The subcutaneous layer under the dermis layer, is mainly composed of fat cells and structures such as blood vessels, nerves, and glands. The epidermis is composed mainly of keratinocytes (95%), melanocytes, dendritic cells, and sensory receptors [50]. Epidermis is a dynamic layer that is constantly renewed, with complete renewal estimated at 7 days in mice [51, 52], and 60 days in humans [50]. It has long been known that within mammalian epidermis, there resides a population of cells that exhibit the characteristics of somatic stem cells [53]. These cells have a slow cell cycle, high proliferation potential, a protected niche, and the capacity to repair and maintain the tissue in which they reside. A widely held belief is that within the epidermis, reside stem cells that produce differentiated progeny, via transit amplifying (TA) cells. TA cells proliferate rapidly several times prior to differentiation [51]. They function to increase the number of differentiated cells formed following a single stem cell division. The slow cycling of stem cells with respect to TA cells, allows the identification of these cells through the long term retention of thymidine, or bromodeoxyuridine [53]. Cells that divide frequently quickly dilute the label, while slowly proliferating stem cells retain it longer. These label retaining cells have been isolated and shown to proliferate *in vitro* [54]. Similarly, using this approach, a population of epidermal stem cells in mice, have been located predominantly in the upper hair follicle, in an area termed the bulge [55].

Recently, a study by Liang and Bickenbach [53] utilized cell sorting based on cell size and Hoechst 33342 exclusion, to isolate a rodent epidermal stem cell population. They also isolated a purified TA population from the epidermis. While both populations expressed similar markers, only the stem cell population was able to grow clonally *in vitro*. To study the differentiation potential of these cells *in vivo*, enhanced green fluorescent protein (EGFP) labeled stem and TA cells were injected into mouse blastocysts [53]. It was found that the stem cells were incorporated into many tissues of the embryos and adult mice, including epidermis, liver, brain, and vertebra. The TA cells were unable to contribute to tissue development following blastocyst injection. This demonstrates a fundamental difference between stem cells and the more differentiated TA cells, namely the ability to contribute to tissue development *in vivo*. Liang and Bickenbach [53] were able to demonstrate that epidermal stem cells can survive blastocyst injection, proliferate during development, and contribute to all three germ layers. The contribution made by the epidermal stem cells during development persisted into adulthood suggesting a stable role.

The multilineage potential of cells from mouse skin *in vitro* has been examined recently. Toma *et al.* [13] isolated stem spheres from the dermis of mice. They discovered that these cells expressed markers of the neuronal and mesenchymal lineages, and formed neurons, astrocytes, and adipocytes upon induction of differentiation *in vitro*. In this study, nestin positive, fibronectin positive multipotent stem cells were isolated from the dermis of juvenile and adult skin. These cells were unique from mesenchymal stem cells in that they proliferated as a suspension culture as opposed to an attached culture, and did not express known mesenchymal stem cell markers such as vimentin or

cytokeratin. The skin isolated stem cells also proliferated *in vitro* for at least one year, while retaining their ability to differentiate. Therefore, multilineage stem cells reside in the dermis of mouse skin, can be easily isolated, differentiated, and have prolonged proliferation, *in vitro*.

Many different techniques are utilized to isolate populations of stem cells from different tissue types. Different isolation techniques are also utilized within a specific tissue to isolate tentatively unique stem cell populations. The following are the two major skin stem cell isolation approaches.

a) Attached Isolation

A method for the isolation of epidermal keratinocytes, which can be cultured long term, *in vitro*, has been reported in mice. Briefly, the stem cell isolation involves the enzymatic digestion of skin samples by trypsin, followed by the removal of contaminating cells through selective attachment [56]. Keratinocyte stem cells grow as an attached culture and the contaminating cells remain in suspension. Attachment is facilitated by a composite matrix of type I collagen and fibronectin, with the stem cells attaching over a 10 minute time period. Traditionally, the isolation and culture of keratinocytes has been difficult, with complex isolation protocols and the use of feeder layers to maintain an undifferentiated state [57, 58]. The protocol proposed by Hakkinen et al. [56] utilizes fibroblast conditioned medium in order to negate the necessity of culturing the stem cells on a feeder layer. The medium is also supplemented with epidermal growth factor (EGF), which is a common mitogen required by stem cells. The isolated cells have a typical keratinocyte phenotype, and maintain morphology,

proliferation, and viability *in vitro*. These cells can differentiate and produce epithelium making them potentially useful for skin grafting [56]. Keratinocyte stem cells are grown in the presence of fetal bovine serum (FBS) and maintained as an attached culture.

b) Suspension Isolation

Suspension cultures of mice skin stem cells that do not require FBS have also been examined [13]. In a similar manner to the isolation of keratinocyte stem cells, the mouse skin is carefully isolated and dissociated using trypsin digestion. Utilizing this method, contaminating cells are removed through selective attachment on uncoated culture dishes. The contaminating cells attach to the culture dish while the spherical stem cells remain in suspension. The stem cell culture medium does not contain FBS, and feeder layers are not required to maintain an undifferentiated state. The maintenance of an undifferentiated state, is achieved through the supplementation of the cell culture medium with EGF and fibroblast growth factor (FGF). These undifferentiated cells have not been differentiated into epithelium cells, as expected for skin isolated stem cells. Instead they exhibit the ability to differentiate into neuronal cell types as well as adipocytes.

While these isolation techniques follow a similar approach, the selection methods employed result in the isolation of two different stem cell populations with differing differentiation potentials from the mouse skin. The necessity of FBS for the culture of attached skin isolated stem cells makes it difficult to study the effects of a single mitogen. The ease of isolation, and more defined culture medium, of the suspension culture suggests it may be a better system for studies on stem cell biology.

Epidermal stem cells still remain poorly characterized, due in part to the absence of a specific cell surface marker to distinguish these cells from progeny and other more differentiated progenitor cells. Currently, stem cell characterization is mainly based on the verification of their differentiation potential and related gene expression profiles. Beta-1-integrin ($\beta 1$), the receptor for type IV collagen, is expressed in the thinnest portion of the interfollicular epidermis [59]. The attached nature of epidermal isolated stem cells suggests that $\beta 1$ may be a required protein. Therefore, it has been suggested $\beta 1$ is a marker for epidermal stem cells. Human epidermal cells enriched for $\beta 1$, were found to adhere more rapidly to extracellular matrix proteins, and have an increased colony forming efficiency, a characteristic of stem cells, when compared to un-enriched cells [59-61]. Cell kinetics have identified epidermal stem cells as slow cycling label retaining cells (LRC), which allows them to be separated from the more proliferatively active progeny cells that lose their label quickly [62]. Cells enriched for $\beta 1$ that quickly attach to protein matrixes, are on average made up of 50% label retaining cells (LRC) and 50% cells that do not retain DNA labels, suggesting that $\beta 1$ enrichment is made up of equal numbers of stem cells and progeny cells. This suggests that $\beta 1$ may not be an ideal epidermal stem cell marker [62].

Another approach utilized the adhesion molecule $\alpha 6$ and the proliferation-associated cell surface marker CD71 [58, 62]. The $\alpha 6$ marker is expressed in both epidermal stem cells and progeny cells, therefore the proliferation marker CD71 is utilized to allow separation of the two populations. Flow cytometry was utilized to isolate $\alpha 6^{\text{bright}}\text{CD71}^{\text{dim}}$ cells, which displayed characteristics consistent with stem cells, including high nuclear to cytoplasmic ratio, high proportion of LRC (71.95%), and the

capability of long term proliferation [58, 62]. The higher percentage of LRC in populations isolated utilizing the surface markers $\alpha 6$ and CD71, suggests they may be better markers than $\beta 1$ for attached epidermal cell isolation.

Studies to date on stem cells isolated from mammalian skin, suggest that several distinct populations are present. It also appears that mammalian skin is a source of easily obtainable cells with multilineage potential. The advantage of skin isolated stem cells is significant, as skin is the largest organ in the mammal, and could provide an alternative to ES cells or other somatic stem cells, that are more difficult to isolate. While the central nervous system (CNS) is not as accessible as skin, its limited ability to repair damage has led to extensive research into neuronal stem cells (NSC).

B. Neuronal Stem Cells

a) Cells of the mammalian central nervous system

The three main cells present in the mammalian CNS are neurons, astrocytes and oligodendrocytes. Neurons are morphologically distinct, based on the region within the CNS where they reside, the cells their axons contact, and their chemical properties [63]. The morphology of neurons is important to their biochemical signaling, and also their functioning [64]. Neurons have small cell bodies with many long processes extending outward [64, 65]. These dendritic processes have smaller processes branching off which have small spines along the entire length [64]. Their design allows neurons to make contact with thousands of other neurons. Neurons play an important role in the brain, mediating and storing vast amounts of information. They receive signals through their short branched dendrites, and send signals from their axons. Many different types of

neurons are present in the mammalian CNS, based on morphology and function. Some neuron markers such as β III tubulin, MAP2, and neurofilament, are shared by many types of neurons [66]. The unique morphology of neurons is characterized by unique cytoskeleton arrangements. Cytoskeletal structure is predominantly related to microtubules, which are a major portion of total cell protein in the brain. Microtubules function in neurite outgrowth, migration, and mitotic division (reviewed in [67]). There are several isoforms of microtubules found in different types of neurons. All microtubules share a common subunit that is a 100 kDa protein heterodimer, termed tubulin, consisting of a 50 kDa α - and 50 kDa β -polypeptide (reviewed in [67]). Protofilaments, formed by polymerization of linear units of tubulin, are polar with α and β subunits alternating along their length, forming a hollow tube. The dynamics of microtubules have been shown to differ between cell types and specific locations within a cell [68, 69].

The different isotypes of microtubules exhibit a characteristic pattern of cellular expression. All the isoforms except VI, found only in hematopoietic cells, and IVb, which is testes specific, are expressed in neuronal tissues. Class III β -tubulin is neuron specific in the mammalian CNS [67, 70]. The specific expression of β -III tubulin in neurons within the mammalian brain make it an ideal marker for the detection of neurons *in vitro*.

Glial cells in the CNS of mammals consist of astrocytes and oligodendrocytes. Astrocytes are highly fibrous, structurally complex cells that are characterized as having many processes and are located between neural elements [71]. It has been thought that astrocytes were merely supportive cells that existed to serve neurons. However, astrocytes deliver glucose and provide metabolic substrates, to the CNS and are involved

in the maintenance and tight control of ion and pH homeostasis [71]. Astrocytes also remove waste products from neuronal tissues, and play an important role in maintaining the neural network integrity, by supporting the neurotransmitters.

Recent studies suggest a more assertive role for astrocytes in the functioning and structural design of the CNS. It was shown that astrocytes transmit signals to neurons, which directly control the synaptic strength within the CNS [72, 73]. *In vitro*, neurons cultured in the presence of astrocytes, have a sevenfold increase in synapses, and synaptic efficiency, when compared to neurons cultured without astrocytes [74]. This provides evidence that astrocytes are important for the control and functioning of the mammalian CNS.

Astrocytes typically have five to eight processes, that are essentially uniformly distributed [75]. The processes surrounding an astrocyte form a 3D space, that is not intruded by other astrocyte processes. The cell body and the processes are enriched with glial fibrillary acid protein (GFAP) intermediate filaments. The finer processes are GFAP negative, and therefore the true domain of an astrocyte is not visible with GFAP staining [75]. Astrocytes are located in an organized manner within the CNS, with their cell bodies and large processes not in contact with each other. The functional significance of their organization remains unknown. It has been suggested that every synapse within the CNS is under the control of a single astrocyte [71]. Neurons are dispersed throughout the astrocytic domains, with many fine neurites being surrounded by glial processes. Astrocytic junctions are formed between the processes of a single astrocyte, and function to minimize the differences between processes, by indiscriminately sharing molecules <1000 kDa [76]. Astrocytes, unlike neurons, are

electrically non-excitable with only passive changes in membrane potential upon current application [72]. However, they do function in signaling each other and surrounding neurons by Ca^{2+} mobilization [77].

GFAP is the major intermediate filament of mature astrocytes, and its expression is commonly used as an indicator of the presence of astrocytes *in vitro* [13, 25, 78-80]. The high expression and strong conservation among invertebrates suggests it plays an important role within the CNS. The lack of spontaneous GFAP mutants in mice, suggests that GFAP null mice may be embryonic lethal. However, GFAP null mutants are viable, reproduce, and have relatively normal motor behavior [81-84]. As GFAP is the common marker used to study astrocyte morphology, it is challenging to determine morphological changes in astrocytes lacking GFAP. Ultrastructural studies suggest that the absence of GFAP does result in subtle morphological changes [83], which is supported by recent dye assays [85]. Some effects of the loss of GFAP have been found, including possible impairment of the blood-brain barrier. Geriatric null mice display signs of advanced myelin degeneration [84], impaired synaptic transmission, and enhanced long-term potential in the hippocampus [83, 86]. The astrocytes fine distal processes are responsible for supporting synapses, suggesting GFAP may play an important role in the distal processes. To date, the nature of this defect has not been well described.

The over-expression of GFAP in mice leads to serious defects, with the mice dying shortly after birth [87]. These mice had severe hypertrophy, with complex cytoplasmic aggregates of intermediate filaments [87]. It appears that the over expression of GFAP has more serious side effects, when compared to the under expression of GFAP. It is clear that GFAP is required for the proper functioning of

astrocytes. The specificity of GFAP expression in astrocytes, and the conserved homology in vertebrates, make it a commonly used marker for the identification of astrocytes *in vitro*.

Oligodendrocytes also play an important role within the mammalian CNS. They are the CNS cell that produces myelin. Myelin sheaths surround axons within the CNS, and facilitate rapid conduction of action potentials. The myelin sheath is formed as a spiraled extension of the oligodendrocyte plasma membrane. Each oligodendrocyte has several processes, which form separate myelin internodes around different axons [88]. Four types of oligodendrocytes (I-IV) have been identified in the mammalian CNS, each of which myelinate axons of different sizes [89]. Oligodendrocyte progenitors express mRNA for platelet derived growth factor α receptors (PDGF α R) [90]. These progenitors then lose PDGF α R expression and begin expressing myelin basic protein (MBP) mRNA, and label positive with the Rip antibody [91]. These committed progenitors, termed promyelinating oligodendrocytes, are distinct from premyelinating oligodendrocytes that express O4 and galactocerebroside, but do not express MBP [92]. Two myelin sheath specific markers are used to define mature oligodendrocytes, microtubule-associated protein (MAP), and myelin proteolipid protein (PLP), *in vitro* [93, 94]. Oligodendrocyte maturation results in a shift of MBP expression to the myelin sheaths alone [91].

The long term proliferation potential, and ability to differentiate into cell types of the CNS, as well as cell types of different lineages, has resulted in increased interest in neuronal stem cells [8, 24, 79, 95]. Many studies have examined the differentiation of neural progenitor cells into neuronal tissues *in vitro* (Figure 2)[24, 25, 79, 80, 96]. Several different methods have been utilized in order to differentiate stem cells into

neuronal cell types [24, 25, 79, 80, 96]. Similarly many different markers have been used in order to confirm neuronal differentiation. The resulting percentage of differentiated cells varies greatly from study to study.

b) Neuronal stem cell isolation and characterization

The ability to isolate pure neural stem cell populations from the mammalian CNS, is dependent on the identification of unique surface markers. The expression of intermediate filament proteins, has been used to define the developmental progression of CNS cells in mammals [13, 79, 80, 96-99]. Nestin is a class IV intermediate filament protein, originally found to be expressed in the early developing CNS [100]. The majority of the initial work on nestin expression was completed in rodent models; there are a few studies looking into nestin expression in the human CNS. Nestin expression has been identified in the developing human brain as early as gestation week 6 [101]. In this study, the nestin expression was found in neuroepithelial cells lining the spinal cord, and in the germinal matrix and radial glia cells of the cortex. Nestin has also been found in CNS tumors and tumor cell lines [101]. Its expression has been correlated with proliferating progenitor cells, sorted, based on their expression of nestin [102]. Nestin expression decreases following the differentiation of NSCs to mature cell types [13, 80]. The expression of nestin and markers of mature cells has also been investigated [102]. β III tubulin is a marker of mature neurons and cells expressing this marker do not co-express nestin [102]. GFAP is the major intermediate filament of mature astrocytes, and its

Figure 2: Differentiation of a stem cell into neuronal cell types: Neuronal stem cells can differentiate into neurons, astrocytes, and oligodendrocytes. Neurons have small cell bodies and few long processes while astrocytes have large cell bodies and many short processes. Oligodendrocytes have large cell bodies and finer extremity processes.

expression is commonly used as an indicator of the presence of astrocytes *in vitro* [13, 25, 78-80]. While some expression of nestin has been seen in early astrocytes expressing GFAP, the expression of nestin decreases following differentiation [102]. In other studies, nestin and GFAP were not co-expressed prior to, or, following differentiation [103]. In addition, nestin expression has been detected in cells in a transition stage, from progenitor to glial cell, in a different temporal pattern to neurons [78]. This suggests that while nestin may be expressed in cells transitioning from progenitor to mature cell types, its expression is essentially absent following differentiation within the CNS. Cells expressing nestin have been shown to proliferate and self-renew, as well as differentiate to form neurons and glia, which is a characteristic of NSCs [103]. The increase in the expression of mature CNS cell markers corresponds to a decrease in nestin expression [102]. Nestin positive cells have also been shown to be the cells forming neurospheres following isolation *in vitro* [103]. Therefore, the expression of nestin is utilized to identify cells that have the potential to differentiate into neuronal cell types. To date, nestin is the most predominant marker utilized for the labeling of neural progenitor cells [79, 80, 96, 98, 99].

While nestin is utilized frequently for the isolation of NSCs, cells initially negative for nestin expression have been shown to differentiate into neuronal cell types [97]. Nestin negative stem cells were isolated from the connective tissue of adult rat muscle. The isolated neurospheres were initially negative for nestin but began to express nestin following culture in neuronal medium. These cells were differentiated by dissociating neural spheres and plating onto dishes coated with poly-ornithine/laminin

(PO/L). Cells were induced to differentiate in medium without FGF and EGF for 4-6 days. They found 68% of the differentiated culture, stained positive for GFAP (an astrocyte marker), and 2 – 60% of the cells stained positive for β III tubulin (a neuron marker) [97]. The markers were confirmed using immunocytochemistry, and RT-PCR. The rat stem cells isolated from muscle, were morphologically consistent with stem cells isolated from the CNS, and had the potential to differentiate into neuronal cell types. Initially, the cells isolated did not express neuronal progenitor markers but were able to be induced to express these markers and differentiate *in vitro*. This suggests that stem cells not residing in the CNS may be able to be induced to display characteristics of NSCs, and have the potential for neuronal differentiation.

In another study the effect of EGF on NSC differentiation was investigated [104]. In this study stem spheres were isolated from 3 day old rat brain tissue. Neurospheres were then dissociated and plated on poly-L-ornithine coated dishes and differentiated for 1 to 21 days. Differentiation medium consisted of DMEM/F12 either in the presence or absence of EGF. Cultures were analyzed at various time points during the differentiation. Following differentiation, neurons were confirmed with MAP-2, and astrocytes with GFAP expression. The percentage of neurons following 21 days differentiation was 1%, in medium supplemented with EGF. An increase in MAP-2 expression was seen from day 1 to 21 of differentiation. An increase in astrocyte formation (GFAP positive), from 87 – 99%, was observed in the EGF supplemented medium, from day 1 to 21 respectively [104]. Differentiation in the absence of EGF resulted in an increase in neurons to 10% at 21 days differentiation, and a decrease in astrocytes from 95 – 68%, from day 1 to 21, respectively [104]. The presence of EGF resulted in a significantly lower number of

neurons following induced differentiation. The study suggests the presence of EGF results in a conflict between the growth inhibitory action of cell attachment, and the growth promoting nature of EGF resulting in apoptosis and, therefore, fewer cells differentiate into neurons. High levels of astrocytes formed following induced differentiation suggesting these cells have a higher potential to differentiate into astrocytes than neurons.

Similar stem cells were isolated from the embryonic spinal cord of rat, and were differentiated for 5 days in differentiation medium [99]. The neuronal differentiation medium consisted of DMEM/F12 supplemented with B27 without FGF or EGF. Glial differentiation medium, consisted of the above described medium supplemented with 10% fetal calf serum. Cells were dissociated and plated on poly-D-lysine/laminin (PDL/L) coated dishes. The number of β III tubulin immunoreactive cells increased to 20%, following 5 days differentiation. Following 5 days glial differentiation, 73% of cultured cells were stained positive for GFAP. These results were similar to the previously described study, with the cells showing a higher potential to differentiate into astrocytes. This study utilized separate differentiation media for the induction of astrocytes and neurons. High levels of astrocytes were formed in medium with and without serum supplementation. This suggests that NSCs have a propensity to form astrocytes under various environmental conditions.

There is increased interest in NSCs derived from the human CNS. The ability to differentiate cells *in vitro* to neuronal cell types may provide cells for tissue therapy. In order to study the differentiation potential of human neural stem cells *in vitro*, human neural precursor cells were isolated from human fetal cortical tissue [79]. The majority

(>95%) of the cells in the neurospheres were positive for nestin expression by immunocytochemistry. The undifferentiated neurospheres stained negative for TuJ1 (a neuron marker) and GFAP prior to induced differentiation. They were differentiated via plating of whole spheres onto poly-L-lysine coated coverslips. The differentiation medium consisted of DMEM supplemented with B27 (1X), without EGF or FGF, and the attached spheres were differentiated for 14 days prior to analysis. Following 14 days differentiation, approximately 30% of the culture stained positive for GFAP, and 55% of the culture stained positive for TuJ1. In a similar study, human neural precursor cells were isolated from embryonic brain tissue [80]. Prior to differentiation, 75% of the cells were positive for nestin. These cells were plated on poly-ornithine coated dishes with N2 medium supplemented with 1% fetal bovine serum (FBS). Cells were differentiated for 12 – 14 days prior to analysis. Following differentiation 15 – 50% of the culture stained positive for GFAP, and 20 – 37% of the cells stained positive for β III tubulin. The nestin expression decreased to less than 5% following induced differentiation, displaying a loss of neuronal potential.

These studies suggest that NSCs isolated from the human CNS have the ability to differentiate into neuronal cell types *in vitro*. While the studies utilized similar differentiation media, and were differentiated for the same time period prior to analysis, the results differed. In the earlier study, the majority of cells formed following differentiation were neurons, while in the later study the majority were astrocytes. This difference is interesting since both cell populations were isolated from human fetal tissue. This difference could be the result of differences in the cell populations and analysis

methods. The populations differed in the proportion of cells that expressed nestin which suggests they may have differing purities and neuronal potentials.

Limited *in vivo* studies on the potential of NSCs have been performed. In one study, murine adult NSCs were injected into the amniotic cavity of chick embryos in order to determine their differentiation potential [24]. Following the injection of dissociated neurosphere cells, no chimeric embryos were formed. However, the injection of whole neurospheres resulted in contributions to the nervous system, mesodermal derivatives, and epithelial cells. The mouse originating cells appeared morphologically consistent with surrounding cells, and expressed markers consistent with those normally found in these tissues. The ability of the stem cells to contribute to tissues in the developing mouse embryo was also studied [24]. It was found that cells derived from the neural stem cells contributed to the CNS, heart, liver, and intestine, among other tissues. The chimeric mice produced, displayed a normal phenotype, suggesting that the neural stem cells were functionally normal. This finding demonstrates a wide differentiation potential, *in vivo* of NSCs.

Identifying adipocytes in vitro

Energy homeostasis is maintained in part through the conservation of adipose tissue. In mammals, there are two distinct types of adipose tissue, white adipose tissue (WAT) and brown adipose tissue (BAT) [105]. WAT stores energy as triglyceride lipid droplets, and BAT utilizes lipids to generate heat during metabolism. Adipocytes are present in both WAT and BAT but serve different roles. In BAT, adipocytes have a high number of mitochondria which produce a proton gradient across their membrane to

generate heat. Therefore, energy can be stored in adipocytes in WAT, and consumed by adipocytes in BAT. Energy storage and utilization is only one of many adipose functions, including the production of hormones and metabolites [105].

Adipocytes are typically derived from mesenchymal precursor cells [106]. Adipocyte precursors differentiate to fibroblast-like preadipocytes, after which time they either remain dormant, or proceed to form terminally differentiated adipocytes. Mature adipocytes are commonly identified *in vitro* by staining for lipid droplets in their cytoplasm [13]. The presence of leptin following differentiation is also evidence of the presence of adipocytes. Terminally differentiated adipocytes have been shown to be the main source of leptin production and secretion [107].

Leptin is a hormone that is involved in the regulation of lipid stores in mammals. It is a 167 amino acid pre-protein that's amino terminal is cleaved during secretion and circulates as a 146 amino acid protein. The rate of leptin concentration in plasma is correlated with total fat mass [108]. It is believed that leptin is the hormone responsible for indicating the size of fat stores in the body. Leptin induces the removal of fat store via lypolysis or apoptosis [109]. The recognition that the disruption of the leptin gene is a cause of obesity, and infertility, and that adipocytes are the main secretor of leptin has led to the belief that adipocytes are a main regulator of energy metabolism and may play a role in fertility. Adipocytes secrete leptin which circulates in the body and interacts with leptin receptors. The administration of leptin to leptin knock-out mice results in a decrease in food intake and animal weight, and restored fertility [110, 111]. The actions of leptin are regulated by a family of cell-surface receptor proteins that are present on many different cell types. The hypothalamic region in mammals responsible for appetite,

reproduction, and growth, has leptin receptors and may be the primary site of leptin action [112-114].

Stem cell proliferation regulation

During development, a single fertilized egg gives rise to a functional multicellular organism, consisting of many diverse cell types. One of the fundamental questions of developmental biology is how such cellular diversity is created from a single cell. Asymmetric cell division is suggested to be one mechanism utilized by cells to take different cell fates (reviewed in [115]). Asymmetric cell division involves the production of daughter cells with different differentiation potentials. It is widely held that within tissues, reside stem cells that produce differentiated progeny via TA cells. TA cells are defined as cells that have the ability to divide three to five times prior to terminally differentiating [51]. The ability of TA cells to divide several times prior to terminal differentiation, allows the division of one stem cell to generate one stem cell, and several differentiated cells. The use of TA cells protects the original stem cell population from incurring genetic mutations, by limiting the number of times they must divide [50].

A. Stem cell division

Stem cells have been shown to undergo both symmetric and asymmetric division [116]. Asymmetric cell divisions of stem cells maintain the stem cell pool, while generating differentiated progeny. The regulation of asymmetric division in stem cells is currently under investigation. During asymmetric division cell fate determinants are asymmetrically segregated in the daughter cells. Two molecules that play an important

role in asymmetric division are Notch and Numb (suppressor of notch signaling) [117]. Notch is believed to be an important molecule in specifying cell fate. Mammalian homologues of notch have been identified and have been shown to affect stem cell fates [118, 119]. Notch may also be asymmetrically segregated in stem cells [120], and cells with high levels of Notch have been shown to continue proliferation while cells with low Notch levels differentiate. Numb is a putative Notch antagonist that interacts with Notch, and may play a role in cell fate determination [121]. Numb is a membrane associated protein that is segregated into one daughter cell, where it is required for the determination of cell fate [122]. Numb is expressed and asymmetrically segregated in stem cells [121]. Therefore, high levels of Numb results in a decrease of Notch expression, which leads to differentiation. Numb isoforms have also been linked to cell fate with those containing long proline-rich region (PRR) domains, promoting proliferation, while those lacking the domain, promoting differentiation [123]. Studies of *Drosophila* neurogenesis have found another molecule involved in asymmetric division, Prospero [117, 124]. Prospero, a nuclear transcription factor, translocates to the cell membrane prior to division and colocalizes with Numb during mitosis [122]. Both proteins are tightly correlated with the position of one of the two centrosomes [122]. Homologs of the proteins involved in asymmetric division have been identified in mammals. As well, homologs of Numb have been shown to correctly segregate into one cell in vertebrates [125]. Prospero is transiently expressed in one of the daughter cells following asymmetric cell division, perhaps until the cell is differentiated [126]. The ablation of the Prospero protein results in a defect in neuron formation or axonal growth [126]. The role of Prospero has been linked to the glial cells missing (*gcm*) gene. The loss of Gcm results in a lack of glial

cells in the resulting knockout organism [127]. The *gcm* gene acts as a molecular switch that when expressed, results in glial cells, and when ablated, results in neurons [127]. Prospero upregulates the expression of the *gcm* gene, which results in the induction of glial development [127]. When Prospero expression is upregulated, *gcm* expression is increased which results in increased glia cells [127]. The asymmetric localization of Prospero following cell division, results in the expression of *gcm*, which allows one daughter cell to differentiate into a glial cell. The daughter cell can divide again prior to differentiation, which produces a Prospero-negative daughter cell that can differentiate to form a neuron cell [127].

While several molecules have been identified that likely play a role in asymmetric cell division and cell fate determination, many questions remain to be answered. The asymmetric division of growth factors and receptors probably also play a role in stem cell proliferation and differentiation. The continued study of growth factors and their effect on proliferation may help resolve factors affecting asymmetric division.

B. Stem cell growth factors

Stem cell division and maintenance is controlled via interactions between growth factors and growth factor receptors. Though many different mitogens have been found to affect stem cell proliferation, this review describes the NSC culture system which has been extensively studied. The addition of EGF and FGF to the cell culture medium, is sufficient to maintain NSCs, *in vitro*, in a proliferatively active, undifferentiated state [80].

a) Fibroblast Growth Factor

FGFs constitute a large structurally related family of proteins found in all animals. Twenty-three members of the FGF family have been identified, of which ten are expressed in the CNS [128, 129]. Four different tyrosine kinase receptors, FGF receptors 1-4, bind FGFs [129]. The binding of FGF results in dimerization and phosphorylation of the FGR receptor, initiating an intracellular transduction cascade, with numerous signal-transducing proteins associating with the receptor and becoming tyrosine-phosphorylated [128]. Several FGF ligands and FGF receptors are expressed by neural precursor cells during development and *in vitro* [130]. FGF has been involved in a variety of biological processes, including proliferation, migration, and growth [129]. FGF basic (FGF-2) protein has been shown to be an important factor for neuronal progenitor cells *in vitro* [6]. It has also been shown to increase the total number of cell divisions, but does not effect the length of cell cycle [131]. In addition, FGF-2 has also been shown to be an important factor in maintaining the potential of neural progenitors to differentiate into neurons [128]. FGF-2 appears to act in a concentration dependant manner, with FGF-2 knock-out mice having a decreased potential to form neurons over wild-type mice [131]. Wild-type mice injected with FGF-2 during neurogenesis have increased neuron formation over non-injected mice [131]. FGF-2 is required for correct neurogenesis and is seen as early as day 9 in the rat [132]. Mice with mutant FGF-2 show abnormalities in the hippocampus and spinal cord, and in the organization of the cerebral cortex [133, 134]. The similar expression pattern between FGF-2 and the fibroblast growth factor receptor 1 (FGR1), and the high affinity binding between FGF-2 and FGR1 suggests FGR1 is the primary receptor of FGF-2 [129].

b) Epidermal Growth Factors

In addition to FGF-2, EGF has also been shown to enhance the proliferation of neural stem cells *in vitro*, via activation of the tyrosine kinase pathway [104]. In the same study, the presence of EGF in the culture medium maintained a high stem cell nestin mRNA level, and resulted in a decrease in neuron and astrocyte formation [104]. The removal of EGF and FGF from the cell culture medium resulted in stem cells differentiating into the neuronal lineage [13, 80, 97, 104]. Several EGF and EGF receptor knockout studies in mice have been completed to elucidate the function of this growth factor. Mice expressing a partially functional EGF receptor, have the phenotype of impaired hair and skin development [135, 136]. In another study, conditional EGF receptor knockout mice had the phenotype of accelerated hair loss, dermatitis, and skin ulceration [137]. It is believed that stem cells serve the purpose of tissue maintenance in mammals (for review [138]). The loss of the EGF receptor may cause a decrease in stem cell numbers which results in impaired tissue maintenance, *in vivo*. Inevitably, EGF receptor null mice died pre-implantation or early in post natal life. These studies suggest an important role of EGF and the EGF receptor for embryo development.

Early during development, neural stem cells respond only to FGF, and a loss of FGF or the FGF receptor results in a reduction of stem cell proliferation [99, 139]. Later during development stem cells require both FGF and EGF for proliferation [140, 141]. The removal of FGF-2 from stem cell culture medium results in an immediate decrease in the proliferation rate [80]. Therefore, the presence of EGF and FGF-2 is required to maintain NSCs *in vitro*.

FGF and EGF also act on precursor cells to modulate the secretion of extracellular matrix (ECM) components [95]. It has been found that FGF upregulates the expression of laminin B1 and B2 chains [142]. Laminin proteins are important for cell to cell contacts which have been shown to enhance neuronal stem cell proliferation [79]. Therefore, EGF and FGF are involved in mediating stem cell contacts as well as proliferation *in vitro*.

Stem cells and their potential applications for livestock transgenesis

Livestock transgenesis is an important research tool in the field of biological science. The introduction of disease-resistance genes, genes of interest to human therapy, as well as genes of biological interest are potentially very valuable for agriculture and biological science. Moreover, genetically modified animals can be used to address specific physiological, pathological, and developmental questions.

Until recently, the most common technique for the production of transgenic organisms was pronuclear injection. Pronuclear injection involves the injection of naked DNA into the nucleus of a recipient cell [143-145]. The nature of this technique often results in the random integration of the injected DNA, producing unpredictable results [144, 146]. The use of viral vectors has proven to be more efficient than pronuclear injection for the production of transgenic domestic animals, but also results in random integration of transgenes [143]. The limitations of these methods, such as random integration, unintended mutation of exogenous genes, and their lack of usefulness for gene knock out studies are well known [147].

An ideal system for producing transgenic livestock is “gene targeting”. This system allows researchers to precisely modify genes at specific sites. It allows exogenous DNA to be inserted into a predetermined site, resulting in controlled transgene expression, with the reduced probability of affecting the general viability of the animal [145, 148]. This requires the genome modification of cells grown in culture, involving transfection and selection, while maintaining their ability to contribute to the germ cells of the resulting animal. In mice, this is achieved with great success by employing ES cells [149]. Murine ES cells were first isolated and grown *in vitro* more than 20 years ago [150]. ES cells are isolated from the inner cell mass of pre-implantation blastocyst. ES cells remain undifferentiated *in vitro*, when grown in the presence of leukemia inhibitory factor (LIF), and on feeder layers of fibroblast cells [151]. ES cells, *in vitro*, have been shown to differentiate into cardiomyocytes, haematopoietic cells, endothelial cells, nerves, skeletal muscle, chondrocytes, adipocytes, liver cells, and pancreatic islet cells (for review [2]). In order to differentiate ES cells, LIF is removed and the cells are cultured in specific medium to induce differentiation. An attractive feature of ES cells is their ability to contribute to the germ-line of chimeras following *in vitro* culture, genetic manipulation, and injection into normal blastocysts [152]. This has allowed genetic studies utilizing gene knockout and mutation, to gain insight into gene function in a mice model.

Initially, attempts have been made to isolate ES cells from livestock. Cells are obtained from the inner cell mass (ICM) at the first visible stage of embryo differentiation, during the separation of blastomeres into the trophoblast and the ICM. The cells that contain the smallest amount of cytoplasm, and have the highest

proliferation potential are then purified as the ES cells. This method was first employed by Evans and Kaufman [149] to isolate ES cells from mice. Slightly modified methods are now used routinely to isolate mouse derived ES cells. The criteria for an ES cell line, is the ability of these cells to contribute to the germline following blastocyst injection [153]. ES-like cells have been isolated from sheep [154], pig [154, 155], rabbit [156], bovine [157], equine [158], mink [159], rat [160], and monkey [161, 162]. However, these cells have not been shown to contribute to the germline following injection into blastocysts [153]. The successful cloning of an adult mammal, has led to a change in the belief that somatic cells have a very restricted developmental potential [163]. The basic process of nuclear transfer (NT) involves fusing a single donor cell with an enucleated recipient ova. The reconstructed embryo then has the potential to develop to term. The combination of gene targeting and NT, has the potential to provide a means for selecting cells with precise genome modifications, for use in livestock transgenesis [148, 164]. Cell lines can be modified using techniques such as homologous recombination, and then be used to produce a transgenic animal. One of the main factors impeding this process, is the availability of appropriate cell lines for gene modification in domestic animals.

Currently, fetal fibroblasts have been the most commonly used donor cells for NT in the production of transgenic pigs [145, 164-166]. Fetal fibroblasts are thought to be less differentiated than other cells from adult tissue [167], and they have been shown superior to adult fibroblasts, cumulus cells, and oviduct cells at developing to blastocysts following NT [167]. This might suggest that undifferentiated cells are more amenable to reprogramming compared to cells with less developmental potential. Following NT, the parental genomes are reprogrammed in order to recapitulate normal development.

Reprogramming consists of (i) genomic methylation changes, (ii) the assembly of histones and variants into nucleosomes, and (iii) remodeling of other chromatin-associated proteins [148, 168]. The purpose of this reprogramming, is to regain the expression of genes required for early development, and the suppression of the expression of genes, that are not required or are detrimental for early development [168, 169]. It is apparent that faulty or incomplete reprogramming results in abnormal development of cloned embryos [168]. It is conceivable that the genome of undifferentiated stem cells may be more easily reprogrammed by the oocyte to resemble the genome of the early embryo, which may make stem cells more efficient donors for nuclear transfer. This hypothesis can be tested, and the efficiency of nuclear transfer may be improved in farm animals, if well characterized stem cells are made available.

It has been suggested that the poor survival of clones could also be due to genetic abnormalities, since genetic mutations have been shown to accumulate during organism aging and during cell culturing [148, 168]. This is however unlikely, due to the high frequency and cross species similarities in abnormalities seen in cloned animals, as well as the normalcy of their natural offspring [169]. Therefore, the most likely explanation for the developmental failure of NT embryos, is the failure to reprogram the epigenetic profile of the somatic donor nucleus to that of a fertilized zygote [168]. ES cells have been used successfully in mice to produce cloned animals. A comparison between somatic cells and ES cells in their ability to generate embryos following NT has been made [168]. It was found that 60 to 70% of embryos derived from the transfer of somatic nuclei into the oocyte survive to the blastocyst stage. In contrast, only 10 to 20% of transferred ES nuclei result in blastocyst formation. However, ES NT embryos that reach

the blastocyst stage develop to term at a 10 to 20 fold higher efficiency than those originating from somatic donor cells [168]. While the higher efficiency, of less differentiated cells to develop upon reaching the blastocyst stage, can be explained by less reprogramming being required, the lower blastocyst rate suggests many of the cells are not acceptable as donor cells. This may be due to the donor cell cycle not being coordinated with that of the recipient cell. Cell division is achieved through the cycle of four distinct phases: G_1 , S, G_2 , and M (for review [148]). Post mitosis, the resulting cells enter G_1 at which time the cell size increases and growth factors stimulate continuation of the cell cycle. The accumulation of G_1 factors reaches a threshold, at which time they are committed to undergo a round of DNA replication in S phase. The cells enter G_2 following DNA replication, a phase of cell growth, then proceed to the M phase at which time the DNA is segregated into two daughter cells. Eventually, cells exit the cell cycle and remain in a quiescent state termed G_0 . Several studies have looked at the effect donor cell cycle has on the success of NT. Several donor cell cycle stages promote development to term with varying efficiencies. The donor cell must be at a cell cycle stage that promotes normal ploidy in the resulting embryo. Donor cells in the G_1 or G_0 stage appear to be the most efficient for NT [170, 171]. ES cell populations typically have 60% of the cells in S phase, which results in most randomly picked cells failing to develop [168]. The transfer of a donor cell in S phase, results in abnormal ploidy following activation, thus results in a lower blastocyst rate. NT embryos that reach the blastocyst stage from ES donor cells, have a high probability of originating from a G_1 cell, and therefore a higher probability of continuing development.

Unlike ES cells and transformed cell lines, primary somatic cells have a limited proliferation capacity [145, 166]. Genetic modification and subsequent preparation for NT must be accomplished prior to the cells becoming senesce or entering crisis. The limited proliferative capacity of the primary cells in culture, is considered a significant impediment to the overall success of livestock transgenesis. It is estimated that approximately 45 population doublings are required from isolation, through genetic modification, to preparing targeted cells for NT [145]. In the porcine species, cultures of fetal fibroblasts have been reported to go through crisis at approximately 40 doublings and are associated with an aneuploid genotype upon continued growth [145]. Aneuploidy is also seen in many transformed fetal fibroblast cells which will result in the failure of NT [145]. The inability to maintain cells in a proliferative state, along with the low targeting efficiency in somatic cells, makes the identification of targeted clones very difficult. The problems above may be overcome by utilizing highly proliferative stem cells as nuclear donors.

To date few stem cell lines have been well characterized and established from domestic animals. Recent advances in the discovery of stem cells in mouse and human adult tissues [13, 53] suggest that populations of stem cells are likely also present in pigs. Stem cells can be maintained in culture for an adequate time to allow for their manipulation and selection prior to NT [148]. The fact that stem cells are undifferentiated also makes them an attractive candidate for NT [166]. The establishment of stem cells in pigs may prove an asset to porcine transgenesis as well as the study of cell differentiation, and development.

II. OBJECTIVE

To isolate and characterize stem cells from porcine skin.

III. SPECIFIC AIMS

1. To isolate floating sphere cells from porcine fetal skin.
2. To study the *in vitro* proliferation potential of these cells.
3. To study the gene expression profile of skin originating, floating sphere cells.
4. To study the multi cell-type differentiation potential of floating sphere cells *in vitro*.

IV. MATERIALS AND METHODS

Cell isolation and culture

Cells were isolated using a previously described method [13] with modifications. Briefly, fetuses were removed from the uterus of 35-50 day pregnant sows and fetal skin from the back was carefully dissected free from other tissue. The skin was then cut into small pieces (1-2 mm²) using surgical scissors in a 6 cm cell culture dish (Sarstedt). The tissue was washed from the culture dishes using 5 ml of Hank's balanced salt solution (HBSS) into a 15 ml tube (Sarstedt). The volume was then increased to a total volume of 10 ml HBSS and the samples were centrifuged at 500 x g for 6 minutes. The supernatant was then discarded and the resulting pellet washed twice by resuspending in 10 ml of HBSS and centrifuging at 500 x g for 6 minutes. The pellet was then resuspended in 3 ml of 0.2% trypsin and incubated for 35 minutes at 37°C. Following the trypsin digestion, 1 ml of 0.1% DNase was added and the samples were incubated for 1 minute at room temperature. Six ml of HBSS was then added and samples were centrifuged at 500 x g for 6 minutes. Tissue samples were washed twice with HBSS (10 ml), and three times with DMEM-F12 (1:1, 10 ml, containing antibiotics) by centrifuging at 500 x g for 6 minutes. Samples were then mechanically dissociated via vortexing for 2 minutes and pipetting in 2 ml of DMEM-F12 supplemented with B27 (Gibco) using a wide bore pipette, until the cells flowed freely through a 1000 ul pipette tip (Diamed). The resulting cell suspension was poured through a 40 uM strainer (Falcon), followed by 8 ml of DMEM supplemented with B27. The flow-through was centrifuged at 500 x g for 6 minutes, and resuspended in 10 ml of DMEM-F12, containing B-27 (Gibco), 20 ng/ml EGF and 40 ng/ml bFGF (Sigma). Cells were cultured in 100 mm tissue culture dishes (Sarstedt) in a

37°C, 5% CO₂ tissue-culture incubator. Nine independent isolations have been performed on 44 fetuses, in total. Three days following isolation, the cell culture supernatant was removed, leaving contaminating cells attached to the culture dishes, and centrifuged at 500 x g for 6 minutes. The cells were gently resuspended in 10 ml of DMEM-F12 supplemented with B-27 (Gibco), 20 ng/ml EGF and 40 ng/ml bFGF (Sigma) and plated on a new 10 cm cell culture dish. Four to six days following isolation floating spheres were passaged.

To passage floating cell spheres, designated porcine skin originated sphere (PSOS) cells, several different methods were utilized in order to determine the method providing the best viability and proliferation rate. Three different passage methods were utilized: a) Cell spheres were completely dissociated using a wide bore pipette prior to reseeding in fresh medium. b) Spheres were centrifuged and plated in 25 µL medium where they were cut into sections using a scalpel. The sphere pieces were then re-seeded in fresh medium. c) Medium containing spheres was centrifuged and the pellet was gently dissociated using a large bore pipette. This gentle dissociation resulted in different sized sphere pieces being re-plated. The cells were re-seeded in fresh stem cell medium as above. Cells were passaged every 4-6 days. Cultures were maintained using each of the three methods until passage five for comparison of the influence of subculture method on proliferation rate.

a) Complete Dissociation Method

The cell culture medium was removed, every 4-6 days, leaving cells attached to the bottom of the dish, and centrifuged at 500 x g for 6 minutes. The cell pellet was resuspended in 10 ml of PBS and centrifuged at 500 x g for 6 minutes. The cells were

then resuspended in 1 ml of cell DMEM-F12 containing B-27 (Gibco) and vigorously pipetted to ensure complete PSOS dissociation. Generally, 10-20 minutes of vigorous pipetting was required to completely dissociate the spheres. Following dissociation, 9 ml of DMEM-F12 supplemented with B-27 (Gibco) was added, and the tube was mixed gently. A drop was then added to a hemocytometer for cell counting. For cell counting, five areas were counted and averaged and this was divided by 10^{-4} and multiplied by the dilution factor. The cells were then centrifuged and resuspended in cell culture medium for replating.

b) Cut Method

The cell culture medium was removed, every 4-6 days, leaving cells attached to the bottom of the dish, and centrifuged at 500 x g for 6 minutes. The cell pellet was resuspended in 10 ml of PBS and centrifuged at 500 x g for 6 minutes. The resulting pellet was then resuspended in 20 μ L of DMEM-F12 supplemented with B-27 (Gibco). The resuspended cells were then plated in one small area of a 6 cm culture dish (Sarstedt), a number 10 scalpel was used to cut vertically and horizontally through the cells. Special care was taken to cut the majority of spheres vertically and horizontally resulting in 4 pieces from each sphere. The dish was then washed with 5 ml of DMEM-F12 supplemented with B-27 (Gibco), and poured into a 15 ml tube and an additional 5 ml of DMEM-F12 supplemented with B-27 (Gibco) added. A 250 μ L aliquot was removed for complete dissociation and counting. The remaining cell suspension was then centrifuged at 500 x g for 6 minutes and resuspended in DMEM-F12 supplemented with B-27 (Gibco), 20 ng/ml EGF and 40 ng/ml bFGF (Sigma) for reseeding.

c) Gentle Dissociation Method

Every 4-6 days the cell culture medium was removed, leaving cells attached to the bottom of the dish, and centrifuged at 500 x g for 6 minutes. The resulting cell pellet was then resuspended in 10 ml of PBS and centrifuged at 500 x g for 6 minutes. The cell pellet was then resuspended in 2 ml of DMEM-F12 supplemented with B-27 (Gibco), 20 ng/ml EGF and 40 ng/ml bFGF (Sigma). The PSOSs were then pipetted gently for approximately 2 minutes and 8 ml of DMEM-F12 supplemented with B-27 (Gibco) added. When correctly dissociated the resulting samples consisted of small PSOS pieces approximately 30 cells in size although typically a wide range of sizes were formed. A 250 μ L aliquot was removed for complete dissociation and counting. The remaining cell suspension was then centrifuged at 500 x g for 6 minutes and resuspended in DMEM-F12 supplemented with B-27 (Gibco), 20 ng/ml EGF and 40 ng/ml bFGF (Sigma) for reseeding.

In order to clonally analyze the PSOS cells, a culture of PSOS cells was completely dissociated. The dissociated PSOSs were then counted and resuspended at a concentration of 1000 cells/10 ml DMEM-F12 supplemented with B-27 (Gibco). Another dilution was then made at a concentration of 100 cells/10 ml DMEM-F12 supplemented with B-27 (Gibco), 20 ng/ml EGF and 40 ng/ml bFGF (Sigma). A 0.1 ml aliquot of this dilution was then plated in a 96 well plate. The presence of a single cell was confirmed visually using light microscopy, immediately following plating. Wells containing more than one cell or no cells were discarded from the experiment. The cells were moved to fresh medium, every 3-4 days, using a mouth pipette with a 120 μ m diameter. When the cells formed a sphere of ~100 cells, they were gently dissociated and reseeded in fresh medium. This process was repeated until enough spheres were present

to complete the required experiments. Following expansion, cells were dissociated and plated on poly-D-lysine/laminin (0.05 mg/ml poly D-lysine, 0.005 mg/ml laminin) coated dishes for differentiation. Two expanded cultures of PSOS cells were analyzed.

Sphere Cell Number Determination

In order to determine the number of cells present in a typical sphere, several spheres of different sizes were fixed in 4% paraformaldehyde for 20 minutes. The fixed spheres were then transferred into 0.5% saponin for 10 minutes for permeabilization. The cells were then washed three times with PBS and incubated for 15 minutes with Hoechst nuclear stain (1 μ M). Cells were then plated with a small volume of PBS in a 3 cm cell culture dish. Single spheres were then placed in 0.5 ml tubes in 20 μ L of PBS. The spheres were then vigorously pipetted (20-30 minutes) in order to dissociate the cells. The tubes were then centrifuged at 500 x g for 6 minutes and the supernatant was removed. The cell pellet was resuspended in 2 μ L of antifade solution and placed on a microscope slide. A coverslip was placed on the cells and the slides were visualized using a UV fluorescent microscope. The cell number was counted for three groups of different sized spheres (10 μ m, 20-30 μ m, >50 μ m). Five spheres were dissociated and counted for each size grouping.

Number of spheres per cm² skin

In order to determine the number of stem spheres isolated per cm² of fetal skin, 1 cm² of fetal skin was utilized to isolate stem spheres. The fetuses utilized were day 40 gestation. The samples were then dissociated and stem cells isolated as described above.

The skin was plated in 10 ml of stem cell medium on a 10 cm cell culture dish. This was repeated six times. Four days following the isolation, stem spheres were present. The plates were washed with PBS into a 50 ml culture tube and centrifuged at 500 x g for 6 minutes. The resulting cell pellet was then resuspended gently in 1 ml of cell culture medium. 10 μ L was then plated in a 96 well plate and the number of spheres were counted. The average of five counts was determined to calculate the total number of spheres isolated from 1 cm^2 . This number was then multiplied by 100 in order to determine the number of spheres isolated from 1 cm^2 of fetal skin.

Cryopreservation

Two methods of cryopreservation were tested for their efficiency in maintaining cell viability. Cells were either completely dissociated prior to freezing or left as whole spheres prior to cryopreservation.

a) Dissociated sphere cryopreservation

PSOS cultures were harvested by collecting the cell culture medium and centrifuging at 500 x g for 6 minutes. The resulting cell pellet was then resuspended in 1 ml of PBS and vigorously pipetted to completely dissociate the spheres. 9 ml of PBS was then added and a drop loaded on a hemocytometer for cell counting. The remaining cell suspension was centrifuged at 500 x g for 6 minutes to pellet the cells. The cells were then resuspended at a concentration of 2×10^6 cells in 500 μ L of freezing medium. Freezing medium consisted of cell culture medium supplemented with 10% dimethyl sulfoxide (DMSO).

b) Whole sphere cryopreservation

To cryopreserve the stem cells, they were first centrifuged at 500 x g for 6 minutes to pellet the cells. The cell pellet was resuspended in 10 ml of PBS and a 250 μ L aliquot removed for counting. The aliquot was then completely dissociated by vigorous pipetting and a drop loaded on a hemocytometer and counted. The remaining whole spheres are then centrifuged at 500 x g for 6 minutes and resuspended at a concentration of 2×10^6 cells per 500 μ L of freezing medium. The freezing medium consists of stem cell culture medium and 10% DMSO. The cell aliquots were immediately frozen at -80°C.

In order to determine which method preserved the PSOS cell viability the best, ten cryopreserved lines were recultured for both completely dissociated and whole sphere methods. Cells were thawed at 37°C, centrifuged to remove DMSO medium. Cells were resuspended in pre warmed (37°C) cell culture medium and immediately plated. The plated cells were then allowed 24 hours to adjust to the culture conditions prior to handling. The whole sphere cultures were subcultured as previously described. The viability of the cryopreservation methods was determined by comparing the number of spheres formed at the time of their first subculture following thawing.

Poly D-lysine/laminin coating of cell culture dishes

Poly D-lysine/laminin (PDL/L) solution was made at a concentration of 0.05 mg/ml of poly D-lysine and 0.005 mg/ml laminin in sterile H₂O. PDL/L solution was added to untreated cell culture dishes at a volume of 2 ml/10 cm dish. The plates were swirled to ensure complete coverage of the dishes. The dishes were incubated overnight at 37°C and 5% CO₂. Following incubation, the dishes were aspirated and washed twice

for 5 minutes with sterile H₂O. The liquid-free dishes were then stored at 37°C and 5% CO₂.

Differentiation study

In order to study differentiation, spheres were centrifuged; mechanically dissociated by pipetting; centrifuged, and resuspended in fresh medium DMEM-F12 containing B27 and either 1% (neuronal differentiation) or 10% (adipocyte differentiation) fetal bovine serum (FBS; Gibco). Single cells dissociated from spheres were plated onto plates coated with a solution of poly-D-lysine/laminin (0.05 mg/ml poly D-lysine, 0.005 mg/ml laminin) to facilitate cell attachment. Neuronal differentiation medium containing 1% FBS was changed 24 hours following plating to medium containing only B27. Initially 1 million PSOS cells were plated onto 6 cm cell culture dishes (Corning). Cells not attached 24 hours following plating were removed from the culture. Cells were maintained for two weeks prior to analysis in the differentiation medium, by changing half the medium every 6-7 days. Adipocyte differentiation medium was the same as stem cell medium except that the growth factors were replaced by 10% FBS. Initially, 0.5 million PSOS cells were plated per 6 cm cell culture dish (Corning). Cells were maintained in the differentiation medium by changing half the medium every 6-7 days. Cells not attached at the time of medium changing were removed. Adipocyte differentiation was conducted for four weeks prior to analysis. Three cell lines were differentiated for analysis.

Immunocytochemistry and Flow Cytometry

Culture dishes were washed with PBS twice, and fixed for 20 minutes in 4% paraformaldehyde. The cells were then washed 3 times with PBS containing 2% FBS and permeabilized with 0.5% saponin for 10 minutes. The saponin was removed and the cells were blocked with PBS containing 2% FBS for 2 hours at room temperature. Dishes were incubated with primary antibodies [nestin 1:400, β III tubulin 1:500, GFAP 1:400, Fibronectin 1:400, Oct4 1:400 human, Stat3 1:400 human] diluted in PBS containing 2% FBS for 2 hours at room temperature and washed three times with PBS containing 2% FBS. Secondary antibody (FITC 1:500, PE 1:500) was then added and incubated for 1 hour at room temperature. Dishes were washed with PBS containing 2% FBS and incubated with Hoechst (1 μ M) nuclear stain for 15 minutes at room temperature, followed by an additional three washes with PBS containing 2% FBS. One drop of antifade solution (VWR) was then added to the dish and a coverslip placed over the cells. Cells were then analyzed using a fluorescence microscope and Openlab software. Pictures were taken using a Cool Snap digital camera (RS Protometrics).

For quantification of cells producing a given marker, flow cytometry analysis of differentiated cells was performed as above following initial trypsinization and washing of cells. Analysis was performed using a FACScalibur flow cytometer (Becton Dickinson) equipped with an argon laser emission wavelength of 488 nm. FITC and PE were identified by using 530 and 585 band pass filters, respectively. Quantification analysis was completed using CellQuest software (Becton Dickinson). Ten to twenty thousand events were acquired per sample with fluorescence measured on logarithmic scales. Forward and side light scatter gates were set to exclude dead cells, debris and clumps of cells. To calculate the percentage of positive cells, markers were set on

appropriate negative controls stained with only the secondary antibody. The specificity of the assay was also confirmed using samples from fetal fibroblasts as negative controls.

Antibodies used in the assays: Antibodies for nestin (BD bioscience, monoclonal, rat), β III tubulin (Sigma, monoclonal, human), GFAP (Sigma, monoclonal, porcine), and fibronectin (Sigma, polyclonal, human) were utilized. Secondary antibodies utilized were anti-mouse IgG FITC conjugated (Sigma) for nestin, β III tubulin, and GFAP, while PE conjugated anti-rabbit IgG (Sigma) was used for the detection of fibronectin.

5-bromo-2-deoxyuridine (BrdU) incorporation assay

The BrdU incorporation assay followed a previously described protocol [172]. Spheres were cultured for 20 hours in the presence of 30 ug/ml BrdU prior to immunostaining. Cells were fixed in 70% ethanol for 30 minutes at room temperature. The cells were then incubated with diluting buffer (PBS containing 0.1% Triton X-100, 0.5% bovine serum albumin, and 100 ug/ml RNase A) for 30 minutes at 37°C. The diluting buffer was removed and the cells were incubated with 2 N HCL for 20 minutes at room temperature to denature the DNA. Dishes were washed once with HBSS followed by PBS, incubating for 3 minutes at room temperature for each wash. Anti-BrdU (Sigma; 1:500), dissolved in diluting buffer, was added and the dishes were incubated overnight at 4°C. Dishes were washed three times in diluting buffer and incubated with the secondary antibody (FITC, Sigma 1:500), for 1 hour at room temperature. The cells were then washed with diluting buffer, incubating at room temperature for 5 minutes. Cells were counterstained with propidium iodide (10 ug/ml) for 10 minutes at room temperature, washed twice with diluting buffer, and visualized. Three cultures of PSOS cells were

analyzed for BrdU uptake at early and late passage. Two late passage PSOS cultures were studied for BrdU uptake following the addition of 10% FBS. Two methods were utilized to label PSOSs with BrdU. Cells were maintained as whole spheres or dissociated immediately following 20 hours culture with BrdU. BrdU labeling was visualized using fluorescent microscopy and Openlab software.

Porcine fetal fibroblasts were utilized as controls for BrdU labeling. Fetal fibroblasts were cultured to >95% confluency and serum was withdrawn for 48 hours to stop the cell cycle in G₀. This culture was utilized as a negative control and actively dividing fetal fibroblast cells were utilized as a positive control.

Oil Red O Staining

The oil red O staining assay followed the manufacturers protocol (Sigma). Following four weeks of culture in adipocyte medium, attached cells were stained for lipid content using oil red O. Plates were washed with PBS and fixed in 4% paraformaldehyde for 2 minutes. Following fixing, cells were washed with H₂O and incubated with filtered 0.3% oil red O, diluted in 60% isopropanol, for one hour at room temperature. The oil red O stain was aspirated and the dishes washed with H₂O for 5 minutes. The dishes were then left with H₂O until analyzed. Phase contrast microscopic pictures were taken immediately following staining. Three PSOS cultures were differentiated and analyzed for adipocyte formation. Cells with adipocyte morphology and red staining were scored as positive.

Leptin Radioimmunoassay

Linco Diagnostic Services (St Charles, Missouri) performed a radioimmunoassay (RIA) on the cell culture medium to detect the presence of leptin protein. Cell culture medium was collected 28 days following the induction of adipocyte differentiation. The medium was centrifuged to remove cell debris, at 500 x g for 5 minutes, and the supernatant was collected. The supernatant was frozen at -80°C until it was shipped to Linco.

The antibody utilized in the RIA was raised against human leptin to detect the presence of leptin in a variety of species. The RIA tested for the presence of leptin protein in 100 µL of cell culture medium. The medium was incubated with 100 µL of multi-species leptin antibody and 300 µL of assay buffer overnight at 4°C. 100 µL of ¹²⁵I-Human Leptin antibody was added to each tube and the samples were incubated for 20-24 hrs at 4°C. In order to precipitate the bound leptin, a precipitating agent was added and samples were incubated at 4°C for 20 minutes. The samples were then centrifuged at 2000-3000 x g for 20 minutes forming a pellet. After removal of the supernatant, the amount of ¹²⁵I-Human leptin is quantified using a gamma counter for 1 min. The resulting data is converted to ng/ml human equivalent (HE) and represents the concentration of leptin in the cell culture medium.

The antibody utilized for the RIA is raised against human leptin but shows broad cross-reactivity with other species. The antibody has been tested by Linco with porcine leptin and was found to have 67% affinity for porcine leptin. This suggests that the results obtained with this antibody are a conservative estimate of the level of leptin in the cell culture medium.

Total RNA isolation

Cells were harvested from a 6 cm cell culture dish by scraping the plate with a rubber policeman. The cells were washed from the dish using 5 ml of PBS and pelleted by centrifuging at 500 x g for 5 minutes. Total cellular RNA was isolated using the RNeasy Mini Kit (Sigma). Cell pellets were resuspended in 350 μ L of buffer RTL with 10 μ L /ml β -mercaptoethanol by gently pipetting. The cells were homogenized by passing the lysate 5 times through a 20 gauge needle. 350 μ L of 70% ethanol was then added to the sample and it was mixed well by pipetting. The sample was then added to the RNeasy Mini column and centrifuged for 15 seconds at 10 000 rpm. 700 μ L of buffer RW1 was then pipetted into the column and it was centrifuged for 15 seconds at 10 000 rpm. The sample was then washed by passing 500 μ L of buffer through the column and it was dried by centrifuging at 10 000 rpm for 2 minutes. The column was then transferred to a new tube and the RNA was eluted by adding 30 μ L of RNase free H₂O and centrifuging for 1 minute at 10 000 rpm. The RNA was then quantified using a spectrophotometer (BioPhotometer, Eppendorf). The spectrophotometer was zeroed using sterile H₂O. Absorbance was measured at 260 nm wavelength of light and the quantity of RNA calculated using the following formula; 1 A₂₆₀ = 40 μ g/ml RNA. The absorbance at 280 nm wavelength of light was also measured and the A₂₆₀/A₂₈₀ calculated in 10 mM Tris-Hcl buffer. An A₂₆₀/A₂₈₀ ratio of 1.8-2.1 corresponds to pure RNA. The eluted RNA was then stored at -80°C until its use.

Treatment of RNA with DNase I

The total RNA isolated from cell samples was treated with DNase I prior to RT-PCR. 3 μ L of RNA was mixed with 1.8 μ L H₂O, 0.6 μ L DNase I (Invitrogen), and 0.6 μ L 10X DNase I buffer. The mixture was then vortexed and incubated at room temperature for 15 minutes. Following incubation 1 μ L of EDTA was added and the sample was then incubated at 65°C for 10 minutes in order to inactivate the DNase.

Real Time RT-PCR

Total cellular RNA was isolated from pelleted cells using the RNeasy Mini kit (Qiagen), followed by treatment with DNase I. For reverse transcription, 400 ng of RNA was used in a final volume of 30 μ L containing; 3 μ M of random hexamers, RT buffer (1X), 10 mM of dithiothreitol, 2 mM of dNTP, and 10 U MMLV. Reverse transcription was carried out at 25°C for 10 minutes, 37°C for 50 minutes, 70°C for 15 minutes, and stored at 4°C until real time PCR analysis.

Real Time PCR amplification was completed using the Smart Cycler (Cepheid). Quantitect SYBR Green PCR kit from Qiagen provided real-time quantification of the desired PCR products. The real time PCR reaction consisted of 2.5 μ L cDNA, and 0.6 pmole each of forward and reverse primers (Table 2) in a total volume of 25 μ L. The *HPRT* gene was amplified, by Real Time PCR, as a control for each RT-PCR reaction. Relative levels of mRNA were analyzed by the delta-Ct method [173]. Primer sequences were made using PRIMER DESIGNER 2.0 (Scientific Software).

Fluorescence is measured as the temperature of the Smart Cycler block is increased from in 0.2°C increments to 95°C. The SYBR green binds double stranded DNA, as the temperature is raised, single stranded DNA results, lowering fluorescence. Fluorescence is plotted against temperature as the temperature rises. The negative first

Table 2: Primers used for Real Time RT-PCR to detect the expression of various mRNA transcripts.

Transcript		Primer sequence (5'-3')	Product (bp)	Gene Bank Accession #
Nestin	Sense	ggcttctctcagcatcttgg	150	GI7050583
	Antisense	aaggctggcataggtgtgc		
β III tubulin	Sense	cagagcaagaacagcagctac tt	250	GI21595025
	Antisense	gtgaactccatctcgtccatgccctc		
GFAP	Sense	ttgacctgcgacgtggagtc	225	AF250778
	Antisense	aggtggcgatctcgatgtcc		
Leptin	Sense	caagacgattgtcaccagga	184	AF102856
	Antisense	ttggatcacattctggaagg		
Stat3	Sense	cccgtaacctgaagaccaagt	129	AF332508
	Antisense	ggttcagcagcttcaccatt		
Oct4	Sense	cacctcaggtcggagtgg	230	AJ251914
	Antisense	agcttggcaaattgttcgag		
HPRT	Sense	ccagtaaacgggcgatataa	130	U69731
	Antisense	cttgaccaaggaaagcaagg		

derivative is then calculated by the Smart Cycler software, resulting in a melting temperature (T_m) for the product. The T_m is dependent on the GC content and length of the PCR product, making a specific T_m value for a given PCR product. The presence of one peak at a specific T_m for the product indicates the presence of a single amplicon. PCR products were also run on an agarose gel to confirm the specificity of the product.

Agarose Gel Electrophoresis of Real Time PCR Products

A horizontal electrophoresis apparatus was used to run agarose gels of real time products. The gel casting trays are sealed by placing in the casting base, and a comb was placed at one end to create wells. 0.6 g of agarose (Gibco) was added to 60 ml of 1X TAE buffer and dissolved by boiling for 1 minute in a microwave oven. The solution was then cooled for 5 minutes, ethidium bromide (0.5 ug/ml) was then added, and it was slowly poured into the gel casting trays. The gel was then left to solidify at room temperature and placed in the electrophoresis apparatus containing enough TAE to completely submerge the gel.

Prior to running the real time products (10 μ L) on the agarose gel 6X loading buffer (2 μ L) was added. Samples were then loaded into the agarose gel wells and electrophoresed at 100 V until the loading dye had migrated two thirds of the total gel length. The DNA product was visualized using ultraviolet light (302 nm) and a photodocumentation station (Syngene). Pictures were analyzed using GeneSnap software.

Recovery of DNA from agarose gel

DNA was excised from the agarose gel using a sharp scalpel. The DNA in the gel slice was visualized on a UV scan bed (Chromato-vue Transilluminator), and excess agarose was removed. The DNA was extracted from the agarose gel using the Qiaex II Agarose Gel Extraction kit (Qiagen). The gel slice was weighed and 3 volumes of buffer QX1 was added to 1 volume of gel. The Qiaex II binding resin was vortexed for 1 minute, 10 μ L was added to the sample, and it was incubated at 50°C for 10 minutes with periodic vortexing to solubilize the gel. The sample was then centrifuged for 30 seconds at 14 000 rpm to pellet the bound DNA. The Qiaex pellet was then washed with 500 μ L of buffer QX1 and twice with buffer PE by resuspending the pellet and centrifuging for 30 seconds at 14 000 rpm. The resulting pellet was then air dried for 10 minutes. To elute the DNA, 20 μ L of 10 mM Tris-Cl pH 8.5 was added and the sample was vortexed. The sample was then incubated at room temperature for 5 minutes and centrifuged for 30 seconds at 14 000 rpm. The supernatant was collected and moved to a new tube.

Analysis of DNA by Spectrophotometry

The concentration of the DNA isolated from the agarose gel was determined using a spectrophotometer (BioPhotometer, Eppendorf). The absorbance of the sample was read at 260, and 280 nm wavelengths of light. The A_{260} value is used to calculate the concentration of DNA, using the following formula; 50 μ g/ml equals an A_{260} value of 1.000. The A_{260}/A_{280} ratio is used to determine the presence of impurities. An A_{260}/A_{280} ratio of ≥ 1.8 was deemed acceptable for further analysis. The spectrophotometer was zeroed using 10 mM Tris-Cl pH 8.5.

Real Time product Sequencing

Samples isolated from the agarose gel were sequenced by the Molecular Supercentre at the University of Guelph. Primers specific for the PCR product were supplied and the products were sequenced using an ABI Dye Terminator Cycle Sequencing Kit. Sequencing was completed on an ABI Prism 377 automated sequencer.

Chromosome analysis (karyotype test)

Chromosomes were analyzed with standard karyotypic analysis. Briefly, thymidine was added to the culture medium to a final concentration of 1.2 mg/ml, when cultured cells reached ~60% confluent. After overnight incubation at 5% CO₂ and 38.5 °C, culture medium was removed, and the cells were washed with PBS. Cells were then cultured in fresh medium for 6 hours and trypsinized. The harvested cells were incubated in 6 ml hypotonic solution (1:6 dilution of serum in distill H₂O) for 20 minutes at 38.5 °C, and 1ml fixative solution (3 methanol: 1 acetic acid), and centrifuged immediately at 500 g for 7 minutes. Cells were resuspended in fixative solution (4°C) and were then dropped on a cold (4°C), dry, methanol-cleaned slide, in order to make a smear. The slides were air-dried and stained with 1.5% Giemsa (EM Science) for 5 minutes. The morphology of chromosomes was examined using the image system (Openlab 2.25, Improvision Ltd.) connected to a microscope. Chromosome number was determined from a minimum of 100 separate spreads from each sample.

V. RESULTS

Isolation and culture of PSOS cells

Skin samples were dissected from day 35-45 gestation porcine fetuses, obtaining approximately 1-2 cm² of skin per fetus. Single cells were obtained via enzyme dissociation of the dissected fetal skin. The resulting single cells were plated onto a 10 cm tissue culture dish, at a density of 1 dish per fetus. The skin cells were cultured in stem cell medium for 2-4 days. Although the majority of cells adhered to the plastic plate, and some died, some small floating spheres of cells remained in suspension. These spheres were collected, gently dissociated and subcultured to a new dish with fresh stem cell medium containing growth factors. During subsequent subcultures, floating spheres could be gently dissociated into small aggregates of cells and proliferated to generate new spheres. A relatively pure population of floating sphere cells, designated PSOS (porcine skin originated sphere) cells, was obtained after a few subcultures. Figure 3 shows the different sizes of PSO spheres following 7 days of isolation. These floating cell aggregates appear morphologically consistent to previously reported neurospheres [13]. The PSOSs ranged in size from 10 μ m - >60 μ m depending on the length of time in culture.

Upon complete dissociation, the cells that make up the PSOSs are relatively homogeneous with regard to size. In order to determine the number of cells in different sized spheres, PSOSs were dissociated, permeabilized, and stained with Hoechst 33342. Cells were counted using fluorescent microscopy. The average cell number (n) of spheres approximately 10 μ m in diameter was 108 $\pm$ 5 cells per sphere (n=5). Spheres 20-30 μ m in diameter contained 252 $\pm$ 17 cells per sphere, and the >50 μ m diameter spheres

Figure 3: Micrograph of floating spheres of cells isolated from porcine fetal skin and cultured for 2 weeks (passage 3; X100). Cells isolated from fetus skin at approximately 35-45 days of gestation were cultured in vitro for 4 days before spheres began to form. Spheres were maintained in culture using gentle dissociation prior to reseedling.

contained 373 ± 19 cells per sphere (mean $\pm$ SE, n=5). The typical sphere size prior to subculture varied from 2-30 μm . If left as aggregates, and transferred to fresh medium every 3-4 days, the spheres reached a size $>120 \mu\text{m}$. When the spheres reached $>100 \mu\text{m}$ they quickly settled on the bottom of the culture dishes and attached. The PSOS cells are able to survive and proliferate from one cell to >370 cells as a floating sphere while maintaining an undifferentiated morphology.

To determine the frequency of PSOS cells within the porcine fetal skin, skin was carefully dissected from the backs of day 35-45 porcine fetuses and each measured to ensure 1 cm^2 . The 1 cm^2 piece of fetal skin was dissociated and stem cells were isolated as described above. The isolated cells were plated in 10 ml of culture medium for 4 days prior to analysis. The mean $\pm$ SE for the isolations from 1 cm^2 of skin was 584 ± 25 spheres. The number of spheres isolated from six independent experiments ranged from 381 to 991 per cm^2 skin. If one sphere results from one cell, the concentration of stem cells in porcine fetal skin can be inferred to be approximately 400-1000 cells per cm^2 .

Cyopreservation

The ability to survive freeze and thaw cultures is an important attribute of cell lines. Studies were performed to determine the potential of PSOS cells to survive cryopreservation. Two preservation methods were tested; A: PSOS cells were frozen as whole spheres, B: PSOS cells were mechanically dissociated prior to freezing. Ten individual cell lines were frozen for 2 to 5 months prior to thawing. The PSOS cells survived well with cryopreservation when left as whole spheres, while the ability of the gently dissociated spheres to reform spheres following freezing was determined to be

76%±4. PSOS cells survived cryopreservation with higher viability when frozen as whole spheres, compared to freezing following mechanical dissociation. Following complete mechanical dissociation, the percentage of viable cells was determined to be 29%±4. Mechanically dissociated thawed cultures took much longer to reform spheres, with some cell lines failing to reform spheres after extended cell culture. Some frozen dissociated cultures were morphologically inconsistent with unfrozen cultures when thawed. Cell size was increased and extended culture resulted in eventual cell death or attaching to the culture dish. The method that results in the highest cell viability and quickest return to proliferation is to freeze the PSOSs as whole spheres. To date more than twenty cell lines isolated from different fetuses and frozen at different passages have been thawed, all of which show approximately 80% viability after thawing, and were able to establish long term cultures.

Proliferative potential of PSOS cells in vitro

To study the proliferation potential of PSOS cells *in vitro*, BrdU, a homologue of thymine, incorporation assay was performed. BrdU was added to the cell culture medium, for a 20 hour period, in order to label cells that proliferated. As shown in Figure 4A, most of the cells in the outer layer of the sphere were positive for BrdU labeling (>90%), suggesting that these cells proliferate actively *in vitro*. The whole sphere assay resulted in the majority of PSOS cells staining positive. To more accurately determine the proportion of cells that proliferated during the 20 hour assay period, spheres were mechanically dissociated prior to staining for BrdU incorporation (Figure 4, B). The dissociated assay confirmed that >90% of the cells had proliferated during the 20 hour culture period.

A

B

Figure 4: Proliferation potential of PSOS cells. A) Passage 2 PSOS cells were cultured for 20 hrs in medium containing 30 ug/ml BrdU for 20 hrs before immunostaining to detect BrdU (intact sphere; 400X. B) PSOS cells cultured in the presence of BrdU for 20 hrs were completely dissociated via vigorous pipetting prior to immunostaining (100X).

We then quantitatively assessed the rate of PSOS cell proliferation, and the ability of a single PSOS cell to regenerate a new sphere, *in vitro*. Early passage PSOSs were dissociated into single cells, and plated under limiting dilution to ensure the presence of no more than one cell per well on a 96 well plate (confirmed under microscope after plating). Following 24 hours, the number of cells in each well was counted using light microscopy. In total, $28\% \pm 4$ of the wells contained one cell, while the majority of cells proliferated to become ≥ 2 cells. A small percentage of individual cells proliferated to form up to 20 cells during the 24 hour culture period ($5\% \pm 1$). This data suggests that PSOS cells are a heterogeneous population of cells with different cell cycle lengths, or, that their cell cycles progress at different speeds in response to the same culture environment. Some of the cells could divide approximately every six hours. To study if PSOS cells were capable of clonogenic growth, PSOS cells were again plated under limiting dilution to test colony formation. It was found that approximately 23% of the single PSOS cells proliferated to reform spheres. Thus, individual PSOS cells are capable of self-renewal and can regenerate whole spheres *in vitro*.

It was previously shown, that methods of subculturing impact neurosphere proliferation [79]. To study whether PSOS cells are also sensitive to culturing techniques and to identify the best method to retain PSOS cell proliferation potential and viability, *in vitro*, three protocols were tested. The gentle dissociation method involves the dissociation of PSOS cells into small aggregates via gentle pipeting. Alternatively PSOS cells were completely dissociated by vigorous pipeting to obtain a single cell population. Finally, spheres were manually cut into small pieces, using a scalpel, prior to replating. The gentle dissociation method was found to maintain both cell viability and

proliferation rate better than either complete dissociation or manual cutting (Tukey $P < 0.05$). The complete dissociation method resulted in slower reformation of spheres and a significant loss of cell viability. When PSOS cells were subcultured via gentle dissociation and cutting methods >85% of the cells remained viable, and cells proliferated to reform spheres quicker (personal observation) and a better growth curve resulted (Figure 5). Since both the gentle dissociation and the cutting method resulted in similar proliferation rates, and the gentle dissociation method is easier to use, subsequent subculture was done via the gentle dissociation method. In total, PSOS cells have been passaged over 16 times by gently dissociation of the spheres, during a five month culture period. At the end of the fifth month, the cells were still actively proliferating (Figure 6). Analysis of population growth from three independent isolations has shown that the population doubling time of PSOS cells is 55-140 hours, with longer doubling times observed in later passage cells. To study whether the decrease in proliferation is due to a smaller proportion of PSOS cells that are proliferating or a longer cell cycle length, a BrdU assay was performed on cells from both early and late passages. It was observed that the proportion of cells that synthesized DNA during the 20 hour assay decreased from 92% (passage 1, Figure 4, A, B) to 50% (passage 10, Figure 7, B), suggesting that a subpopulation of cells have stopped proliferating during late passages *in vitro*. To study whether this is due to the lack of sufficient mitogens to stimulate the full proliferation in our culture, or whether the PSOS cells have lost their proliferation potential at this stage, we cultured the late passage (passage 17, after five months culture), PSOS cells in 10% FBS for 24 hours prior to a BrdU assay. BrdU incorporation assay revealed that 90% of these PSOS cells synthesized DNA again (Figure 7, A), indicating that PSOS cells

Figure 5: Comparison of various culture methods on population doubling time of PSOS cells. PSOS cells were subcultured using three different methods for 5 passages. Dissociated; spheres were completely dissociated using vigorous pipetting prior to reseeding. Gentle; PSOSs were dissociated into cell aggregates using gentle pipetting prior to reseeding. Cut; the PSOSs were cut into aggregates using a scalpel prior to reseeding. Different alphabetical subscripts indicate a significant difference ($p < 0.05$).

Figure 6: Growth curve of PSOS cells. PSOS cells were cultured in vitro for five months in DMEM supplemented with B27, 20 ng/ml EGF, and 40 ng/ml FGF medium. The PSOS cells were gently dissociated during subculturing. The cumulative population doubling at each passage was calculated.

A

B

Figure 7: The addition of FBS increases late passage PSOS cell proliferation. A, Late passage BrdU. Late passage cells were cultured as whole spheres in the presence of 30 ug/ml BrdU for 20 hours prior to dissociating and immunostaining. The percentage of cells staining positive for BrdU incorporation was <50%. Cells that have proliferated are stained green while those that did not divide are stained red. B, BrdU following addition of 10% FBS. The addition of 10% FBS to the medium for 24 hours prior to immunostaining for the incorporation of BrdU. The percentage of late passage cells positive for BrdU increased from <50% to >90% following the addition of 10% FBS.

indeed retain self-renewal capacity after long-term culture. The PSOS cells also appear to remain healthier when cell-to-cell contacts are maintained, as a decrease in viability and proliferation is seen when PSOSs are completely dissociated.

Karyotype Analysis

We next wanted to investigate whether extended culturing of PSOS cells resulted in chromosomal abnormalities or instability. Karyotype analysis was performed using conventional staining of metaphase arrested chromosome spreads, it was found that over 97% of PSOS cells retained a normal karyotype after five months of *in vitro* culture (Figure 8). While the major of abnormalities observed in the rest of the cell population (~3%) included monosomy and trisomy, no deletions (chromosome breaks) were observed. Thus, PSOS cells are genetically stable in culture for at least five months.

Gene expression profile of PSOS cells

We next studied whether PSOS cells express markers that are indicative of an undifferentiated state. Nestin is an early marker for neural progenitors [174]. As shown in Figure 9A, the expression of nestin protein in PSOS cells was demonstrated by immunocytochemistry. A subpopulation of PSOS cells also expressed fibronectin (Figure 9,B), a protein produced by bone marrow mesenchymal stem cells [175]. Moreover, a subpopulation of PSOS cells co-expressed the markers nestin and fibronectin (6%, Figure 10). This suggests that PSOS cells express markers of both NSCs and mesenchymal stem cells.

Previous studies have suggested that the expression of several proteins correlate with the ability to differentiate into multiple lineages [26, 41, 176]. Stat3 is a

Figure 8: PSOS cells have a normal karyotype after five months culture *in vitro*: A light micrograph of a PSOS cell that had been maintained *in vitro* for 5 months (x1,000). The chromosome spread shows a normal complement of 38 chromosomes with no visible abnormalities.

A

B

Figure 9: Immunocytochemistry depicts a subpopulation of PSOS cells expressing nestin, and a subpopulation fibronectin: A, passage 2 PSOSs immunostained for nestin protein (green), cells were counterstained with Hoechst to show nucleus (x400). A subpopulation of PSOS cells stain positive for nestin. B, Immunocytochemistry shows that a subpopulation of PSOS sphere cells also express fibronectin (red; x400).

Figure 10: A subpopulation of PSOS cells express nestin and fibronectin: Flow cytometry analysis of PSOS cells double immuno-labeled with nestin and fibronectin. Early passage (P2) cells stained positive for nestin (N, A) and fibronectin (F, A) and a subset of cells demonstrated expression of both nestin and fibronectin (A).

transcription factor which plays a critical role in the maintenance of the pluripotency of murine ES cells [38]. Immunocytochemistry was used to show the expression of Stat3 in PSOS cells (Figure 11). Real-time RT-PCR revealed that *Stat3* was expressed in PSOS cells (Figure 12). Moreover, PSOS cells also expressed Oct4 (Figure 13), a hallmark of ES cells. Oct4 expression was shown to be expressed by immunocytochemistry and real-time RT-PCR (Figure 13, 14). *Oct4* is known to be primarily expressed in pluripotent cell lineages and is expressed only in germ cells of adult animals [26]. This suggests that PSOS cells may have a much wider potential for differentiation than the neuronal and mesenchymal lineages.

Differentiation of PSOS cells into neurons, astrocytes and adipocytes in vitro

As PSOS cells expressed nestin, a marker of neural progenitor cells, we next studied whether they could differentiate into neural cells. The CNS consists of three major cell types: neurons, oligodendrocytes, and astrocytes, each of which expresses characteristic antigenic markers [177, 178]. In order to determine if PSOS cells could differentiate into neurons and astrocytes, passage two PSOS cells were induced to attach to culture dishes by coating the dish with a positively charged poly-D lysine/laminin solution. Attached cells were then cultured in neural differentiation medium (stem cell medium lacking growth factors). The withdrawal of growth factors has previously been shown to promote neural differentiation [13]. Laminin was used as an attachment substrate as it has also been shown to promote neural differentiation [142]. After an initial culture for 24 hours with 1% FBS to facilitate cell attachment, the cells immediately adhered to the poly-D lysine/laminin coated dishes. Cell proliferation slowed following the removal of FBS from the medium, and the removal of FBS also

A

B

Figure 11: Stat3 is expressed in a subpopulation of PSOS cells before induced differentiation: A, A subpopulation of early passage PSOS cells stained positive for the presence of Stat3 via immunocytochemistry. B, negative control in which primary antibody was omitted in the assay.

Figure 12: Real Time RT-PCR analysis of PSOS cells for the expression of Stat3 mRNA: A representative real-time RT-PCR amplification growth curve showing *Stat3* expression in the PSOS cells (left). Melting curve of PCR product amplified demonstrating the specificity of the product (right).

A

B

Figure 13: Oct4 is expressed in a subpopulation of PSOS cells before induced differentiation: A, A subpopulation of early passage PSOS cells stained positive for the presence of Oct4 via immunocytochemistry. B, negative control in which primary antibody was omitted in the assay.

Figure 14: Real Time RT-PCR analysis of PSOS cells for the expression of Oct4 mRNA: A representative real-time RT-PCR amplification growth curve showing *Oct4* transcripts in the PSOS cells (left). Melting curve of PCR product amplified showing the specificity of the product (right).

resulted in the death and detachment of some cells. Most cells remained attached and as early as 72 hours after plating in the neural differentiation medium various new morphologies were apparent, including large flattened multipolar cells, as well as small bipolar and multipolar cells. Cells were maintained in differentiation medium for 14 days by removing half the medium and replacing with fresh medium every 3-4 days. During medium replenishment dead cells and cell debris were also removed.

Two weeks following the initial induction of differentiation, a sub-population of cells looked morphologically similar to astrocytes and stained positive for GFAP (Figure 15), a protein that has been shown to be almost exclusively expressed in astrocytes [178]. Another subpopulation of cells expressed β III tubulin, a marker of neuron cells, and showed morphology characteristic of neurons (Figure 16). These findings were confirmed and further quantitated with a flow cytometry study. As shown in Figure 17, before induced differentiation less than 0.1% of PSOS cells expressed β III tubulin and GFAP, while after induced differentiation $43\% \pm 10$ and $39\% \pm 10$ expressed the astrocytes (GFAP) and neuron markers (β III tubulin), respectively. In contrast, the proportion of nestin expressing cells decreased from $42\% \pm 9$, before differentiation, to less than 0.1% after induced differentiation. In order to study whether the response of nestin, β III tubulin, and GFAP expression to induced-differentiation was also apparent at the mRNA level, Real Time RT PCR was performed. After induced differentiation, the relative mRNA level of *nestin* was less than 0.1% of that of the undifferentiated PSOS cells (Figure 18). Conversely, the relative mRNA level of *β III tubulin* was 55 fold higher in the differentiated cells compared to that of PSOS cells before induced differentiation (Figure 19). Similar trends in response to differentiation were observed when *GFAP*

Figure 15: Following neuronal differentiation a subpopulation of PSOS cells stain positive for GFAP and are morphologically consistent with astrocytes: A, Morphology of neuronal differentiation following 2 weeks differentiation. Cells are present with the characteristics of neurons, small cell body and few long processes, and astrocytes, large cell body with many processes. B, A subpopulation of PSOS cells express GFAP and they are morphologically similar to astrocytes following 2 weeks neuronal differentiation (x400). Cells staining positive have large cell bodies and many fibrous processes.

Figure 16: Following neuronal differentiation a subpopulation of PSOS cells stain positive for β III-tubulin and are morphologically consistent with neurons: A, White light micrograph of cells morphologically consistent with neurons. Cells were present with small cell bodies and long processes. B, After induced differentiation, immunocytochemistry indicates a subpopulation of PSOS cells expressing β III-tubulin, and they are morphologically consistent with neurons (x600).

Figure 17: Representative acquisition graphs of flow cytometry study on the protein expression before and after induced neuronal differentiation in PSOS cells: Upper panels depict the expression levels of nestin, βIII tubulin, and GFAP in PSOS cells prior to induced differentiation. Lower panels show the expression levels of nestin, βIII tubulin, and GFAP two weeks after induced differentiation. Prior to differentiation the PSOS cells expressed nestin but did not express βIII tubulin or GFAP. Following induced neuronal differentiation the nestin expression was absent but βIII tubulin, and GFAP expression was up-regulated. Ten to twenty thousand events were acquired per sample with fluorescence measured on logarithmic scales, histograms show the gated expression positive events. Data are representative of three independent experiments.

Figure 18: Comparison of nestin mRNA expression before and after induced neuronal differentiation: PSOS cell mRNA abundance of *nestin* before and after differentiation measured by real time RT-PCR. All samples were normalized using *HPRT* expression as an internal control in each real-time PCR reaction. Relative levels of *nestin* mRNA were analyzed by the delta-Ct method.

Figure 19: Comparison of βIII tubulin mRNA expression before and after induced neuronal differentiation: PSOS cell mRNA abundance of βIII tubulin before and after differentiation measured by real time RT-PCR. All samples were normalized using *HPRT* expression as an internal control in each real-time PCR reaction. Relative levels of βIII tubulin mRNA were analyzed by the delta-Ct method.

mRNA level was determined; although the exact fold increased could not be calculated as *GFAP* mRNA was not detectable in PSOS cells before induced differentiation (Figure 20). Our findings suggest that PSOS cells can be induced to differentiate into neuron and astrocytes-like cell *in vitro*. The specificity and size of HPRT, Nestin, β III tubulin, GFAP, Oct4 and Stat3 Real Time RT-PCR products is depicted in Figure 20.

The expression of fibronectin suggested that PSOS cells may also have the ability to differentiate into mesenchymal cell types. Therefore, we next determined if PSOS cells could be induced to differentiate into cells of the mesenchymal lineage. PSOS cells were cultured in poly-D lysine/laminin coated dishes in adipocyte differentiation medium containing 10% FBS. Cells cultured in this medium proliferated rapidly to form a confluent lawn. A number of cells began to form visible vacuoles in their cytoplasm, following two weeks. The vacuoles increased in size and became more pronounced by the fourth week of culture. We examined two characteristics of adipocytes; the presence of lipid droplets in the cytoplasm and the ability to express leptin, a hormone that is mainly produced by adipocytes [179]. Four weeks after induced differentiation, oil red O staining revealed that approximately 2-3% of the cells contained cytoplasmic lipid droplets (Figure 21). Real time RT-PCR shown that while absent in PSOS cells before differentiation, *leptin* is expressed by cells induced to form adipocytes (Figure 22).

The production and secretion of leptin protein by the differentiated PSOS cells was investigated. At day 28 of induced differentiation, culture medium was collected and an RIA was performed to determine leptin protein content. It was shown that leptin was present in the culture medium at 0.3 ng/ml, while the differentiation medium tested negative for leptin. Therefore, PSOS cells after differentiation contain lipid inclusions

Figure 20: Agarose gel electrophoresis of Real Time RT-PCR products. Real Time PCR products, were electrophoresed on a 1.0% agarose gel, and visualized under UV light. 100bp DNA ladders are included to indicate the size of products (HPRT 130bp, Nestin, 150bp, GFAP 225bp, β III tubulin 250bp, Oct4 230bp, Stat3 129bp). The intensity of bands, following PCR, was not used to determine relative product levels. B – before induced differentiation. A – after induced neuronal differentiation.

Figure 21: Cells stain positive for the presence of lipid vacuoles following four weeks adipocyte differentiation: Four weeks after induced differentiation, a subpopulation of cells were positive for oil red O staining (x100). Cells staining positive have visible vacuoles present in their cytoplasm. The vacuoles stain red with oil red O demonstrating the presence of lipids. Control groups of porcine fetal fibroblasts were included to confirm the specificity of the assay.

Figure 22: Real Time RT-PCR analysis of PSOS cells following four weeks induced adipocyte differentiation: A representative real-time RT-PCR amplification growth curve showing *Leptin* transcripts in cells from the after (red) but not before (blue) differentiation group (left). Melting curve of PCR product amplified showing the specificity of the product (right). Data shown are representative of two experiments.

that stain positive for oil red O, express the *leptin* transcript, and secrete the leptin hormone. This suggests that PSOS cells are able to differentiate into cells with adipocyte characteristics *in vitro*.

Individual PSOS cells are multipotent in vitro

The fact that PSOS sphere cells as a whole population could differentiate into multiple cell types could be due to two possibilities: 1) PSOS cells are stem cells whose individual cells have multipotent differentiation potential; or 2) PSOS cells are a mixed population of different precursor cells, whose fates have partially been determined, and each subpopulation could become a more specific cell type upon induced differentiation.

To distinguish between these two possibilities, clonal analysis was performed. PSOS cells were dissociated into single cells, and plated under limiting dilution to ensure ≤ 1 one cell per well (confirmed under microscope after plating). A proportion of single cells grew to form spheres, resulting in a single cell-originated population after a few subcultures. Similar to what was observed in our whole cell population experiment, a proportion (33%) of these cells immunostained positive for nestin. Next, the clonal population was differentiated, following attachment on poly-D lysine/laminin coated plates, in neuronal differentiation medium. Similar to what was observed in the whole population differentiation study upon attachment, the cells began to proliferate and flatten out. Following removal of FBS at 24 hours, the cells ceased to proliferate and began to extend axons. The neuronal differentiation cultures were maintained for two weeks prior to analysis. After differentiation, these differentiated cells were negative for nestin expression, while proportions of the cells stained positive for β III tubulin and GFAP. The morphological characteristics of the cells staining positive for β III tubulin or GFAP

were consistent with the morphology of neurons and astrocytes respectively (Figure 23). On average, 15% of the differentiated clonal population expressed β III tubulin, and 24% expressed GFAP (Figure 24).

In order to determine if a clonal population had the ability to differentiate into cells from the mesenchymal lineage, a portion of the clonal population was plated on poly-D lysine/laminin coated plates and differentiated in adipocyte medium for four weeks. Cells from these clonal populations formed cells with visible vacuoles, characteristic of adipocytes *in vitro* (Figure 25,A). The clonal population also expressed leptin as determined via Real Time RT-PCR (Figure 25, B,C). This suggests that single PSOS cells have the potential to differentiate into multiple cell types.

PSOS cells vs. PBOS cells

We also isolated porcine brain originating sphere (PBOS) cells, in order to compare marker expression and differentiation potential. PBOS cells also expressed nestin which was confirmed with immunocytochemistry and flow cytometry (Table 3). The PBOS cells did not express fibronectin (Table 3). We also tested the ability of PSOB cells to differentiate into neuronal cell types. PBOS cells were differentiated for two weeks in neuronal differentiation medium prior to analysis. Immunocytochemistry following two weeks differentiation demonstrated a decrease in nestin expression and an increase in GFAP and β III tubulin expression (Table 4), similar to that observed for the PSOS cells. In order to quantify changes in marker expression following differentiation, a flow cytometry study was completed. The proportion of PBOS cells expressing nestin expression decreased to <0.1%, following two weeks induced differentiation, from 62% $\pm$ 16, prior to differentiation. β III tubulin and GFAP expression increased from

Table 3: PSOS and PBOS marker expression prior to differentiation. A comparison of the marker expression in PSOS cells and PBOS marker expression using flow cytometry. Data shown is percent expressing.

	PSOS	PBOS
Nestin	56.39	82.54
βIII tubulin	0.23	13.03
GFAP	0.08	3.86
Fibronectin	6.42	0.03

Table 4: Comparison of the potential of PSOS and PBOS cells to differentiate into the neuronal and mesenchymal lineages. Data shown is percent expressing.

	PSOS Before	PBOS Before	PSOS After	PBOS After
Nestin	33.06	60	2.43	0
βIII Tubulin	1.45	3.42	63.89	60
GFAP	2.17	0	30	38
Fibronectin	5	0	1.35	0
Oil Red O	0	0	2-3%	0
Leptin (mRNA)	No	No	Yes	No
Leptin (Protein)	NA	NA	Yes	No

Figure 23: Subpopulations of expanded PSOS cells immunostain positive for GFAP and βIII tubulin following two weeks induced neuronal differentiation: A, PSOS cells were disassociated into single cells and plated as ≤ 1 cell/well, a proportion of the single cells grew to form spheres, and were expanded into a single cell originated population. When induced to differentiation, some of these single cell originated cells immunostained positive for GFAP (a; $\times 1,000$), and were morphologically consistent with astrocytes. B, When induced to differentiation, some of these single cell originated cells immunostained positive for βIII tubulin (a; $\times 1,000$), and were morphologically consistent with neurons.

Figure 24: Relative percentages of differentiated cells from the clonally expanded PSOS population following two weeks induced neuronal differentiation: Following clonal expansion proportions of the cells stained positive for β III tubulin (neurons), and GFAP (astrocytes) while a proportion was not stained, as determined by immunocytochemistry.

A

B

C

Figure 25: Clonally expanded PSOS cells differentiate into cells with the characteristics of adipocytes: A, PSOS cells were disassociated into single cells and plated as ≤ 1 cell/well, a proportion of the single cells grew to form spheres, and were expanded into a single cell originated population. After induced differentiation in adipocyte differentiation medium, a subpopulation was morphologically consistent with adipocytes (c; x400). B, A representative real-time RT-PCR amplification growth curve showing *Leptin* transcripts in the clonal population from the after (blue) differentiation group (left). Melting curve of PCR product amplified showing the specificity of the product (right). HPRT was included in the real time reaction as an internal control. Data shown are representative of two experiments. C, Clonal cell differentiation. Real-time PCR amplification of RNA before differentiation lane 1 and after differentiation lane 2.

<01% to 47%±13, and 31%±7, respectively. This suggests that PBOS cells have the potential to differentiate into neuronal cell types.

We were also interested to see whether PSOS cells and PBOS cells had similar potential to differentiate into cells from the mesenchymal lineage. The lack of fibronectin expression in the PBOS cells suggested they may not have the potential to differentiate into cells of this lineage (Table 4). PBOS cells were differentiated in differentiation medium containing 10% FBS for four weeks. Following differentiation, PBOS cells did not stain positive for oil red O or form visible lipid droplets (Table 4). Real time RT-PCR was used to test for expression of *leptin* in the differentiated PBOS cells. No *leptin* expression was detected in the PBOS cells following induced adipocyte differentiation. We also tested culture medium at day 28 of differentiation for the presence of secreted leptin using an RIA. Leptin protein was not detected in PBOS differentiated medium. This suggests that unlike PSOS cells, PBOS cells do not have the potential to differentiate into adipocytes *in vitro*.

The differences in the ability of PSOS and PBOSs to differentiate into cells of the mesenchymal lineage, corresponds with the differences in marker expression (Table 4). PSOS and PBOS cells both expressed nestin, a neuronal marker, and had the ability to differentiate into neuronal cell types. The PSOS cells also expressed fibronectin, a mesenchymal marker, and had the ability to differentiate into adipocytes, while PBOS cells did not express fibronectin and were unable to differentiate into adipocytes (Table 4).

VI. DISCUSSION

The data presented here indicates that a large number of PSOS stem cells with differentiation potential of at least two different lineages can be isolated from porcine fetal skin. The number of spheres generated from 1 cm² of skin, ranged from 400 – 1000, from six separate isolations. In a previous study investigating similar cells, in mouse dermis, an average of 1000 spheres were recovered from 1 cm² of skin [13]. The variation in the number of spheres formed from the dissociation of 1 cm² of skin appeared to be greater in our study. In the mouse, spheres of 5 – 30 cells formed 10 days following isolation [13], while in our study, spheres were found as early as 3-4 days following isolation and typically contained 20 – 50 cells. It is possible that the spheres isolated in this report originated from more than one stem cell, which resulted in the earlier appearance of spheres with higher cell numbers. This is unlikely however, as the cells were plated at a similar density as the mouse skin originating cells [13]. We have also demonstrated that a small fraction of the stem cells have the capacity to divide to a total of 20 cells in a 24 hour period. Therefore, it is feasible for spheres to be present three days following isolation with cell numbers as high as 50 cells. This data suggests that overall percentage of stem cells in the skin is relatively low. A recent study investigating the frequency of epidermal stem cells determined 1 in 35,000 total epidermal cells displayed stem cell characteristics [180]. The number of cells in 1 cm² of human skin has been estimated at 4X10⁶ cells [57]. We isolated approximately 400 to 1000 stem cells from 1 cm² of porcine skin, assuming a total cell number close to 4X10⁶, our frequency was approximately 1 stem cell in 4000 – 10 000 total cells. This suggests that the stem cell population we have isolated is present at a higher frequency than classic

epidermal stem cells. The ability to isolate a large number of stem cells from an accessible tissue source such as the skin is an advantage.

We have also provided further evidence that maintaining cell-to-cell contact following subculturing results in a higher proliferation rate, as well as cumulative population doubling [79]. We found the complete dissociation of spheres resulted in the death of some cells and a slow return of the appearance of spheres. Dissociated cells typically took 48 hours to resume proliferation, and initially proliferated very slowly. Maintaining the cells in small aggregates resulted in a low frequency of cell death, and a much faster return of spheres. The aggregates of cells become spherical within 24 hours following reseeding and appear to resume replication. It is likely that the extended pipetting that is required to completely dissociate the spheres results in increased mechanical injuries, thereby affecting overall viability. Cells remaining viable following subculturing by the standard complete dissociation method may still have cellular trauma to their membranes, possibly causing damage to cell surface receptors. This has been suggested to result in a lack of response to growth factors, which may explain the slow proliferation rate observed with the complete dissociation method [79].

PSOS cells express nestin and fibronectin, marker proteins for progenitor cells of both neural and mesenchymal lineages. The expression of marker genes of differentiated neural cells and adipocytes was either not detectable or minimal before induced differentiation, suggesting the undifferentiated state of PSOS cells. The finding that PSOS cells expressed Oct4 was indeed surprising because it has been well documented that Oct4 is almost exclusively expressed in ES cells and germ cells [36, 181-183]. Oct4 is a transcription factor that has been recently suggested to play a critical role in

pluripotency (for review see [184]). In mice, loss of Oct4 function results in the formation of embryos without pluripotent inner cell mass [184]. In addition, Stat3 activation is fundamental for ES cell self-renewal [182, 185]. Overexpression of a dominant negative Stat3 resulted in the differentiation of ES cells [182, 186]. Interestingly, Stat3 is also highly expressed in PSOS cells. It has been recently suggested that both Stat3 and Oct4 play a critical role in maintaining cellular pluripotency by their cooperative function in the regulation of gene expression ([26]; also reviewed by [158, 187]). Our finding that individual PSOS cells can differentiate into neuron-, astrocyte- and adipocyte-like cells suggests the multipotency of PSOS cells. The co-expression of these two pluripotent related genes in this cell type suggests that PSOS cells may be far more “plastic” than has been demonstrated in the current study.

Compared to previously reported skin-derived precursors (SKPs) from mice, PSOS cells are more biased towards neural cell differentiation. While in the current study, only 3-6% and 9-11% of mouse SKPs generated neurons and glial cells, respectively [13], 43% and 38% of PSOS cells differentiated into cells expressing neuron and astrocyte markers, respectively. Both SKPs and PSOS cells were isolated via selective growth of floating sphere cells, and differentiated in the same neural differentiation medium. Thus, their difference in neural differentiation potential may reflect species diversity. Interestingly, although only 42% of PSOS cells were positive for the expression of nestin, a neural precursor marker, before induced differentiation, on average 81% of these cells become either a neuron-like or astrocyte-like cell following two weeks of differentiation. This may suggest that a portion of the nestin negative PSOS cells took the path of neural differentiation when they were placed in a

microenvironment that was favorable for neural differentiation. Alternatively, a portion of the non-neural cells may have died of apoptosis in the growth factor free medium, making the percentage of neural cells appear to be higher than 42% at the end of the differentiation period. In fact, we did observe a percentage of dead cells after serum and growth factor withdrawal at the initial stage of neural differentiation. During the initial 24 hour attachment period, a percentage of PSOS cells failed to attach and were subsequently removed from the culture system. It is thus possible that the cells attaching to the culture dishes were predominantly nestin positive while the cells remaining in suspension and dying are nestin negative.

Rodent isolated SKPs were also differentiated into adipocyte cells using a similar method [13]. PSOS cells and rodent skin isolated stem cells displayed a similar potential for adipocyte differentiation with both producing approximately 3% adipocytes *in vitro*. In the current study, we provided further evidence of adipocyte formation, by demonstrating the expression and secretion of leptin by differentiated PSOS cells. This is important as the morphological analysis is unable to provide evidence of adipocyte function. The ability of cells in the culture following induced differentiation to express and secrete leptin supports the presence of functional adipocytes. The level of oil red O staining may also be a conservative estimate of the number of adipocytes following differentiation, as many cells had small lipid vacuoles that were not scored as positive due to insufficient dye uptake. Given more time, it is likely that more cells would stain positive for oil red O. The level of leptin detected in the culture medium (0.3 ng/ml) suggests more than the 3% of cells labeled with oil red O were producing and secreting leptin [188]. The adipocyte culture following differentiation contains many cells that do

not stain positive with oil red O and have a large flat cell morphology. These cultures do not express β III tubulin or GFAP and are not morphologically consistent with neurons or astrocytes. While the majority of cells remain undefined, following induced differentiation further investigation will identify more cell types that formed in the adipocyte differentiation culture.

The study on rodent SKPs demonstrated that the precursor cells are originated from dermis[13]. Interestingly, in this study, nestin positive cells were also detected in the dermis of porcine skin via immunohistochemistry (preliminary data). This suggests that the PSOS cells may be the porcine counterpart of the mouse SKPs. In order to conclusively localize the PSOS cells within the skin a reliable marker must first be identified which is expressed in all PSOS cells. Only a subpopulation of PSOS cells expressed nestin and a marker expressed by all the PSOS cells will be required to accurately locate the cells. The fetal skin from which the PSOS cells originate is composed of the epidermis, dermis, and some subcutaneous tissue, and the PSOS cells may originate from any of these tissues. Previously, populations of stem cells that have epithelium potential have been isolated from the epidermis [53, 56, 62], however these cells have not been shown to differentiate into neuronal lineages. It is thus possible that stem cells with different potential bias and *in vitro* growth characteristics are located in different layers of the skin.

In order to compare the gene expression and differentiation potential of PSOS cells to NSCs, porcine brain originating sphere (PBOS) cells were isolated. Morphologically PSOS and PBOS cells were very similar. The PBOS cells also expressed the stem cell markers Oct4, Stat3, and nestin. Interestingly, fibronectin, a

protein that is produced by bone marrow mesenchymal stem cells, was detected in PSOS cells but not PBOS cells. Of the PSOS cells staining positive for fibronectin, some of these cells also stained positive for nestin (Figure 10), suggesting that PSOS cells have a multilineage potential. To study whether expression of these markers is indeed correlated to their lineage potential, we studied the ability of PBOS and PSOS cells to differentiate into mesenchymal cell types. PSOS and PBOS cells were differentiated in medium previously described to generate mesodermal cell types [13]. Following four weeks of differentiation, oil red O and Real Time RT-PCR identified cells containing lipid droplets and leptin mRNA expression in the PSOS group. PBOS cultures did not express leptin and stained negative for oil red O. The culture medium following induced adipocyte differentiation of PSOS and PBOS cells was also tested for the presence of the leptin hormone. Leptin protein was present in PSOS culture medium following induced differentiation, but remained absent in the PBOS cell culture medium. These findings suggest that the PSOS cells are a separate population of cells from PBOS cells and are not a global fetal population. In addition, a higher percent of PBOS cells express nestin than PSOS cells, suggesting that PBOS cells may have enhanced potential to differentiate into neuronal cell types. As expected, following two weeks neuronal differentiation, the number of cells with neuron and astrocyte characteristics were higher in the PBOS group (Table 4). PBOS cells had a higher potential to differentiate into neurons, and lower potential to differentiate into astrocytes than PSOS cells.

Stem cells are thought to undergo asymmetric division to yield one daughter stem cell and one daughter cell destined to differentiate. It has long been suggested that stem cells cycle slowly, while the descendent daughter cells, can be amplified for a period of

time before terminally differentiating [189, 190]. In addition, the possibility that a population of stem cells can be self-maintaining when some divisions yield two stem cell daughters and others yield two differentiating daughters was also proposed [190]. Our proliferation study suggests that individual PSOSs are composed of a heterogeneous population of cells with different cell cycle rates; 15% did not divide during 24 hours of culture, while approximately 8% divided up to four times. Assuming that a sphere originates from a single cell, our data provides additional evidence of stem cell asymmetric division *in vitro*. Our on going study on the comparison of the differentiation potential of clonal PSOS cells from fast and slow proliferating groups should provide direct evidences to support or reject the above proposed models.

The observation that some individual PSOS cells divided into 20 cells (>4 division) within a 24 hours culture is noteworthy. This represents a four fold higher proliferation rate compared to that of somatic cells from the same stage of fetal development (24h/division, personal observation). This may be one of the self-protection mechanisms in mammals, where tissue can be quickly regenerated after an injury. With the current culture system, PSOS cells show a decreased population doubling rate after long term culture. Our BrdU incorporation assay also indicated that after three months of *in vitro* culture, only 50% of the PSOS cells underwent DNA synthesis during the 20 hour assay period. It is unlikely that the rest of the cells had lost their proliferation potential, since the addition of 10% fetal bovine serum (contains various types of growth factors) into the culture medium could reactivate proliferation of the late passage PSOS cells (>90%). Maintenance of stem cell self-renewal potential *in vitro* is currently a major challenge in stem cell biology research in general. The medium utilized to culture

PSOS cells in the current study was adapted from a previous study in culture of rodent neural stem cells, which contains only EGF and bFGF as growth factors [191]. Further optimization of culture medium, with the inclusion of some skin specific growth factors, may help to improve the self-renewal capacity of PSOS cells *in vitro*.

The PSOS cells isolated from porcine fetal skin appear to be a heterogeneous population. The ability of the stem cells to differentiate into various cell types could be due to one of two possibilities: 1) PSOS cells are stem cells whose individual cells have multipotent differentiation potential; or 2) PSOS cells are a mixed population of different precursor cells, whose fates have been partially determined. In order to clarify this, we clonally expanded our cell population suggesting that the demonstrated differentiation was a characteristic of individual cells. It is essential to clonally expand a putative stem cell population prior to differentiation in order to confidently label them as stem cells. Clonally expanded PSOS cells retained the potential to differentiate into neuronal cell types, as well as cells with adipocyte characteristics suggesting that at least a population of these cells have multilineage potential.

We were also able to show that PSOS cells have the ability to differentiate into neuronal cell types, as well as adipocyte cells. This multilineage potential, taken with the fact that PSOS cells express Oct4 and Stat3, suggests that they may have a wider potential for differentiation. The undifferentiated phenotype of PSOS cells suggests they may be good candidate donor cells for nuclear transfer. It has been suggested that undifferentiated cells may be more amenable to reprogramming, or require less reprogramming following NT than differentiated cells [148, 168]. In fact, this notion has been tested in a rodent model in which a significantly higher percentage of blastocysts

cloned from ES cell developed to term and survived to adulthood compared to those cloned from other somatic cell types [192-194]. With the availability of PSOS cells, this hypothesis can be tested in non-rodent animals such as the pig. Although the isolation of porcine ES-like cells has been reported [146], the overall efficiency of producing ES cells from blastocysts is low and only 1.3% of the ES like-cells maintained ES morphology at past passage 4 [146]. PSOS cells are able to maintain an undifferentiated morphology for at least 6 months, *in vitro*. Transformed ES cells have also been used with blastocyst injection to generate chimeric piglets [195], however, the transgene was not shown to be faithfully transmitted to the germline cells in the chimeras [143, 195]. Unlike ES cells, PSOS cells are easy to isolate, available in large number (400-1000 spheres/cm² of skin) and proliferate well without a feeder layer. In addition, PSOS cells maintain a normal karyotype even after five months of culture suggesting that they are genetically stable *in vitro*. This is critical for nuclear transfer to be successful. Thus, in addition to providing an additional valuable model for stem cell biology research, PSOS cells may offer novel means for the advancement of non-rodent animal cloning.

The culture system of PSOS cells also makes them a good cell model for stem cell biology studies. The culture medium lacks FBS and consists only of B27 supplement and the growth factors EGF and FGF. FBS consists of a wide variety of mitogens and factors which makes it difficult to study the effects of specific factors on stem cells. The addition of a particular factor may result in interactions with factors already present in FBS making it difficult to determine what causes the observed results. The fact that PSOS cells can grow in serum-free medium allows the precise modification of the culture system to determine the effects of different factors on stem cells. This is an advantage as

many other stem cell populations require the addition of FBS, including porcine mesenchymal stem cells [196], adipose isolated stem cells [1], and epidermal stem cells [53, 56].

Future Studies

Recently, there is increased interest in using stem cells, as an autologous therapy, for the treatment of disease. Stem cells isolated from the skin, could feasibly be isolated from patients and modified prior to replacement. A recent study has demonstrated the existence of stem cells in human skin [13], which provides the prospect of completely removing any ethical concerns as well as immune problems associated with ES cells. While some stem cells, such as mesenchymal stem cells (MSCs), have shown the ability to escape immune detection, it would still be beneficial to use a patients own stem cells to decrease the risk of the transmission of detrimental genes from cell donors. At this time it is not possible to screen the complete human genome for propensity to all diseases, therefore the use of donor cells in a therapeutic situation would present the risk of adverse health effects to the patient. As mentioned, it may be feasible to culture stem cells isolated from skin from an individual with relative ease. This provides the foundation for autologous transplantation. In order to safely treat patients with their own stem cells, more studies involving their capacity to differentiate and function *in vivo* must be completed.

Given the multi cell-type potential of PSOS cells and their ease of handling *in vitro*, they are potentially a powerful tool in tissue engineering. The ability of PSOS cells to proliferate at a high rate makes them an excellent target for gene targeting. Animal

and human MSCs have been successfully transfected with exogenous genes using several different vectors, without influencing their stem cell properties [48, 197-199]. This can be used for two purposes; 1) the tracking of genetically labeled stem cells *in vivo*; and 2) the introduction of new genes into tissues. MSCs have been transfected with genes for growth factors and cytokines, and have been shown to express these proteins *in vitro* through multiple rounds of cell division [197]. The ability of human MSCs to be transfected and express reporter genes such as EGFP and DsRed has also been carefully studied [200]. Lentiviral reporter vectors have also been used to transfect mouse MSCs. The study showed that MSCs were able to be efficiently transfected and express reporter genes while maintaining their ability to differentiate.

The ability to efficiently transfect stem cells provides many potential uses in the field of biotechnology. Genetically modified stem cells used for nuclear transfer may allow for the production of transgenic animals. Our ongoing study into the potential of PSOS cells as cell donors in nuclear transfer and their ability to be genetically modified, *in vitro*, will help determine their potential for transgenesis.

Conclusion

In summary, PSOS cells grow in cell culture as suspended aggregates of cells and proliferate *in vitro* for an extended period of time. Morphologically PSOS cells appear similar to NSCs and express nestin, a marker expressed in NSCs. PSOS cells are unique from NSCs in that they express fibronectin. They also express Oct4 and Stat3, which are genes linked to the maintenance of pluripotency in ES cells.

PSOS cells formed cells with the characteristics of neurons and astrocytes following the removal of growth factors for two weeks *in vitro*. The cells with neuronal characteristics expressed β III tubulin and were morphologically consistent with neurons, while astrocytes-like cells expressed GFAP and had large flattened cell bodies with many processes, which is morphologically consistent with astrocytes. The observed increase in β III tubulin and GFAP expression coincided with decreased nestin expression, which is typical of NSCs following *in vitro* differentiation.

The expression of fibronectin, a marker expressed by mesenchymal stem cells, suggested PSOS cells may have mesodermal potential. PSOS cells were induced to differentiate into the mesodermal lineage for four weeks via the addition of 10% FBS to the culture medium. Following differentiation, a subpopulation of the cells had characteristics consistent with adipocytes. Morphologically, the differentiated cells had visible cytoplasmic lipid inclusions which stained positive for oil red O staining, which preferentially binds lipids and is a characteristic of adipocytes. We also observed expression of *leptin* mRNA, a hormone expressed by adipocytes, following induced differentiation. Interestingly, leptin hormone was also detected in the culture medium, following differentiation, providing evidence that the adipocyte cells were functional.

In conclusion we have demonstrated that a population of stem cells is present in porcine fetus skin and can be readily isolated in large numbers. They express markers consistent with pluripotent cell types. These cells can be maintained *in vitro* and induced to differentiate into cells of multiple lineages.

VII. REFERENCES

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