1-1-2009

Characterization and Differentiation of Peripheral Blood Derived Multipotent Adult Progenitor Cells

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CHARACTERIZATION AND DIFFERENTIATION OF PERIPHERAL BLOOD DERIVED MULTIPOTENT ADULT PROGENITOR CELLS

Thesis submitted to
the Graduate College of
Marshall University

In partial fulfillment of
the requirements for the degree of
Master of Science
in Biology

by

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B.V.Sc&A.H, A.N.G.R. Agricultural University, India, 2004

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Marshall University
Summer 2009

ABSTRACT

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Stem cells are populations of undifferentiated cells that are found in most tissues and act as precursors for regeneration and maintenance. In the future, they could provide promising therapies for diseases which are to date incurable. Our lab developed a novel cell line from the peripheral blood of adult transgenic green fluorescent protein swine and designated them as Peripheral Blood Derived Multipotent Adult Progenitor Cells (PBD-MAPCs). In this study we characterized the mRNA and protein expression profiles of PBD-MAPCs before and after neural differentiation and investigated the potential of PBD-MAPCs to differentiate into myocardial or neural lineages *in vitro.* We examined the potential of various cytokines to differentiate PBD-MAPCs into cardiomyocytes. Also, as an alternative approach, we co-cultured PBD-MAPCs with neonatal cardiomyocytes or embryonic cardiomyoblasts, which produce factors to induce stem cell differentiation. These experiments did not succeed in differentiating PBD-MAPCs to a cardiac lineage. To study the expression profile of PBD-MAPCs before and after neural differentiation, we probed for the expression of stem cell marker CD133 in undifferentiated PBD-MAPCs and neural markers tyrosine hydroxylase (TH), β-tubulin III and PGP9.5 in neurally differentiated PBD-MAPCs using reverse transcription-PCR (RT-PCR) and immunoblot assays. Undifferentiated PBD-MAPCs were found to express CD133 and the neural markers TH, β-tubulin III and PGP9.5. Upon differentiation, they lost expression of CD133, TH and PGP9.5. Finally, we performed 3 dimensional cell cultures on PBD-MAPCs using various biomaterials in

neural differentiation medium. We also tested if muscle fibers added to the biomatrix provide directional support to the growing cellular processes. This 3 dimensional cell culture research is a preliminary study aimed at the development of a bridging transplant for spinal cord injuries. On differentiation, cells showed neural morphology with long cellular processes and were immunopositive for neural proteins. Cells also grew along the muscle fibers indicating that muscle fibers provide support to the growing cells. Taken together, these data suggest that PBD-MAPCs are stem cells and can be a promising stem cell population for future research in cellular therapeutics for spinal cord injury.

ACKNOWLEDGEMENTS

I would like to take this opportunity to thank each and everyone who helped me to achieve my Master's degree. I will never miss the opportunity to cherish the memory of all those who have helped me to enrich new experiences of my life. First and foremost, I would like to dedicate my work to the Goddess I believe Sri Kanaka Durga and my parents, Tata Rao Addagarla and Raja Rajeswari Devi Addagarla for what I am today. They have given me strength day after day. If it were not for them, I would not have made it this far.

I would like to express my sincere gratitude to my advisor, Dr. Elmer Price, for instilling in me the qualities of being a good researcher. His infectious enthusiasm and unlimited zeal have been major driving forces through my graduate career at Marshall University. My Master's committee members, Drs. David Mallory, Brian Antonsen and Nadja Spitzer, deserve special thanks for helping me to undo the knots in my research and critical evaluation of my research progress. Special thanks to Dr. Nadja Spitzer who supervised my work, for her patience and commitment towards making me a better researcher and for critical reading and shaping this thesis draft. I would like to thank Dr. Jaroslava Miksovska, my former advisor, for introducing me to research. Also thanks to Dr. Eric Blough for readily donating the embryonic cardiomyoblast (H9C2) cell line.

Thanks also go to all of my great teachers who have taught me in St. Aloysius High School, Dr. L.B Junior College, NTR College of Veterinary Science and Marshall University.

Thanks to all my lab members who have helped me. It was a great opportunity for us to have a nice atmosphere away from home. Thanks Jason, Jarrod, Sarah, Heather, Greg, Lindsey and Lacey. Thanks to Anne for helping me with the SpectraMax microplate reader in their lab.

Thanks to all my extended family members here in Huntington. Life was fabulous with you people and was always fun, it was as if I am in my motherland. Thanks to Sunil and his family, Anjaiah, Sriram Prasad, Anil, Ravi, Sudarsan, Satyanarayana, Madhukar, Sarath, Sreenivas, Murali, Siva and Chandu. Thanks are due to my friends in other places who have helped me reach this day, Satyanarayana, Prasad, Satish, Sudhakar, Stephen, Kiran and Hanumantha Rao.

I owe special thanks to my family members' sister Bharathi, brother-in-law Murali Krishna, my sweet little niece Rithika and my brother Lokesh for their constant love and encouragement. I thank God and my parents for giving me such a wonderful family.

I also would like to thank the Marshall University Graduate College for the "Summer thesis/dissertation research award-2007". Finally I would like to thank the Marshall University Department of Biological Sciences, for giving me this opportunity to conduct and present my Master's research.

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CHAPTER 1

INTRODUCTION

STEM CELLS

Stem cells are populations of undifferentiated cells found in most tissues that act as precursors for regeneration and maintenance. When maintained in culture, stem cells can continue to divide without differentiation, often forming non-adherent spherical aggregates. Upon placement into specific conditions, stem cells differentiate into specialized cells with defined functions. Stem cells offer great promise for repair and regeneration therapies for diseases which are caused by death of cells or loss of function in vital tissues such as liver, heart and nerves. Because these organs are vital, the practicality of procuring immunologically matching organs or tissues for transplantation is highly challenging and the need always exceeds supply. Stem cells have been of great research interest in many diseases like cardiovascular diseases (Collins and Russell, 2009), Parkinson's disease (Deierborg et al., 2008), diabetes mellitus (Guo and Hebrok, 2009), spinal cord injury (Louro and Pearse, 2008), liver diseases (Navarro-Alvarez et al., 2009), diseases of the retina (Baker and Brown, 2009) and many other pathophysiological conditions. Many different types of stem cells have been discovered and broadly they can be classified into embryonic stem cells (McDonald et al., 1999) and adult stem cells with each kind having their own advantages and disadvantages. Embryonic stem cells have a greater ability to differentiate into many different types of specialized tissues (pluripotency), and adult stem cells derived from bone marrow (Cudkowicz et al., 1964), skin (Toma et al., 2001), adipose tissue (Guilak et al., 2004) and other sources, have a relatively limited differentiation potential when compared to embryonic stem cells, and can be uni-, bi- or multipotent. Embryonic stem cells, in view of their isolation from embryonic sources, have many

ethical objections which adult stem cells do not (Strauer and Kornowski, 2003). Also due to their pluripotency, embryonic stem cells often form lethal teratomas (Hentze et al., 2009; Nussbaum et al., 2007) upon transplant which limits their use in therapy. In this study we investigated the potential of a novel type of adult stem cell in the development of therapies for myocardial infarction and spinal cord injury (SCI).

Stem cells typically express certain markers which are characteristic to them and identify them from other cell populations. These markers vary on the cell type and origin. Embryonic stem cells mainly express Oct4, Sox2, and Nanog (Boyer et al., 2005). Mesenchymal stem cells express many markers like CD9 (Cluster of Differentiation), CD29, CD41a, CD44, CD59, CD73, CD90, and CD105, while hematopoietic stem cells express markers such as CD14, CD31, CD33, CD34, CD133, and the pan-leukocyte marker CD45 (Meng et al., 2007). Stem cells typically lose expression of these markers on differentiation. However undifferentiated mouse mesenchymal stem cells were shown to express certain neural lineage specific markers like nestin, MAP2, GFAP, MBP and CNPase (Lamoury et al., 2006). Furthermore, undifferentiated human mesenchymal stem cells were reported to express neural genes (Blondheim et al., 2006). These results suggest that certain undifferentiated stem cells might be primed towards a specific fate. Also it was reported that mouse bone marrow stromal cells express a wide range of mRNA and proteins including those normally reported to be expressed in terminally differentiated neurogenic and osteogenic phenotypes. These authors report that when stem cells are subjected to osteogenic or neuronal differentiation, the neuronal or osteogenic characteristics disappeared from the stem cells indicating a selective silencing or pruning of superfluous gene clusters. They suggest that stem cells exhibit nonspecific gene expression and that, once they are directed towards a specific lineage, other lineage specific genes are silenced (Egusa et al., 2005).

PERIPHERAL BLOOD DERIVED MULTIPOTENT ADULT PROGENITOR CELLS (PBD-MAPCS)

Our lab has recently described a novel type of adult stem cell population isolated from the peripheral blood of adult transgenic green fluorescent protein (GFP) swine, designated as Peripheral Blood Derived Multipotent Adult Progenitor Cells (PBD-MAPCs) (Price et al., 2006). PBD-MAPCs are maintained in an undifferentiated form and they grow as spheroids in primordial cell media for more than 100 doublings. These cells, being from the peripheral blood, are very easy to collect, unlike stem cells from other tissues like adipose tissue and bone marrow that involve invasive and sometimes painful collection procedures. Also, as the cells are from pigs, whose overall physiology is close to human physiology (Sullivan et al., 2001); they could be used for xenotransplantation to humans in future (Ekser et al., 2008; Halperin, 2001). As these cells are obtained from adult animals they are less likely to form teratomas after transplantation and also will not attract the controversies associated with embryonic stem cells. Besides these advantages, the ease of isolating these cells from peripheral blood makes them interesting candidates for stem cell research as opposed to cells from other sources. Since the physiology of swine is similar to that of humans (Sullivan et al., 2001), we are also exploring the possibility of isolating a similar type of stem cells from humans.

STEM CELLS IN CARDIAC DISEASES

A myocardial infarction (heart attack) is caused by an acute reduction of blood to the myocardium with the direct effect of insufficient oxygen supply (Christoforou and Gearhart, 2007). Because of ischemia and associated anoxia, cardiomyocytes die and the heart tissue is replaced by a fibrous scar tissue which dramatically decreases the contractile ability of the heart tissue (Zhu et al., 2009). Stem cells are showing a huge hope in repopulating the lost myocardial

tissue and improving heart functioning. Many researchers have been working on stem cell therapies for myocardial infarctions with different kinds of stem cells. Bone marrow is one of the main cell sources that have been investigated extensively for their ability to regenerate the myocardium. In a study with bone marrow-derived hematopoietic stem cells transplanted into coronary artery ligated mice, significant recovery of heart function was observed by echocardiography and hemodynamic parameter assays when compared to sham-operated animals (Orlic et al., 2001). Autologous bone marrow-derived mononuclear cells when transendocardially injected into adult human patients with ischemic heart disease, significant improvement in symptoms and myocardial perfusion was reported after 3 months (Tse et al., 2003). On the contrary there are reports that hematopoietic stem cells (Murry et al., 2004) and skeletal muscle stem cells (Reinecke et al., 2002) do not acquire a cardiac phenotype in adult rodent hearts. Such conflicting conclusions still cast doubt on the actual applicability of cell therapy in heart diseases and thus the quest for the ideal stem cells in heart repair continues.

In vitro, many types of stem cells ranging from embryonic stem cells (Yoon et al., 2006) to adult mesenchymal stem cells (Rangappa et al., 2003) were shown to differentiate into cardiomyocytes when cultured in presence of defined growth factors like Wnt proteins, Gsk-3β inhibitor II, Oxytocin, DMSO (dimethyl sulfoxide) or 5-aza-2'-deoxycytidine. Many other studies have also reported differentiation of stem cells into cardiac lineage when co-cultured with cardiomyocytes (Li et al., 2006). We wanted to determine if PBD-MAPCs could be differentiated into cardiomyocytes *in vitro* in presence of specific cytokines and in co-culture with neonatal cardiomyocytes and embryonic cardiomyoblasts.

CHARACTERIZATION OF PBD-MAPCs

Though PBD-MAPCs were shown to differentiate into endothelial, smooth muscle, osteocyte, adipocyte and neural lineages (Price et al., 2006), we have not examined their expression profile to see if they express standard stem cell markers. We wanted to check their expression profile to prove that they not only differentiate into various lineages under specified culture conditions but also express stem cell markers. Stem cells from other sources, when cultured in defined neural differentiation media with specific cytokines will lose expression of stem cell markers and start expressing neural markers. In this study we are testing to see if PBD-MAPCs on neural differentiation express neural marker proteins and lose expression of stem cell markers.

STEM CELLS IN SPINAL CORD INJURY

SCI causes damage to white matter or myelinated fiber tracts that carry signals to and from the brain (Li et al., 1999). It also damages gray matter in the central part of the spine and the surrounding axonal tracts because of mechanical trauma and secondary ischemia (Tator and Koyanagi, 1997). It is accompanied by irreversible tissue damage and permanent loss of motor, sensory and autonomic function (Louro and Pearse, 2008). Inflammatory processes in spinal cord injury dramatically decrease the chance of regeneration in the injured area (Fawcett and Asher, 1999; Gris et al., 2007). Extensive research is being done for the development of a 3 dimensional (3D) plug of differentiated neural cells in various biologically compatible materials. This plug can be transplanted into the injured area by resecting the injured area and implanting the plug of neurally differentiated stem cells to regain the lost nervous function (Fouad et al., 2005; Kamada et al., 2005). It was reported that Schwann cells, in a poly-β-hydroxybutyrate (PHB) scaffold

plug, when implanted in spinal cord injured rats, survived and neurofilament-positive axons were observed in the biomaterial conduit which promoted axonal regeneration (Novikova et al., 2008). We wanted to determine if PBD-MAPCs could be differentiated into neural lineage cells in a 3 dimensional (3D) culture system. We also wanted to probe whether addition of muscle fibers into the biomatrix would provide a directional support for the growth of PBD-MAPCs. This particular technique of using muscle fibers was successfully used in peripheral nerve damage (Roganovic et al., 2007; Weber et al., 2000; Norris et al., 1988). It was shown that muscle basal lamina works as a temporary scaffold to guide axonal regrowth and organization (Tos et al., 2007). Our long term goal in this project is to develop a 3D plug of neurally differentiated PBD-MAPCs which can be used in the therapy of spinal cord injuries.

CHAPTER 2

PBD-MAPCs DO NOT DIFFERENTIATE INTO CARDIOMYOCYTES IN CULTURE

INTRODUCTION

The human heart has a limited regeneration potential. A heart attack (myocardial infarction) is the most serious form of heart disease and it is vital to discover novel therapies against this potentially fatal disease. Cardiomyocytes lost due to a myocardial infarction are replaced by non contractile fibrous tissue which leads to reduced functional ability and finally results in heart failure. The current treatment options for an acute myocardial infarction are very few and organ transplantation is often the only option (Zhu et al., 2009). However, this solution is limited due to a lack of donor organs and complications resulting from immune rejections. Because of these setbacks, cellular therapy has been investigated as a potential alternative. Many different types of stem cells from embryonic and adult sources are currently being investigated for their potential use in therapies for myocardial infarction. Human embryonic stem cells (Yoon et al., 2006), activated hematopoietic stem cells from adult murine hearts (Matsuura et al., 2004), an embryonic carcinoma cell line P19 (Paquin et al., 2002), skeletal myoblasts (Taylor et al., 1998), bone marrow cells (Orlic et al., 2001), mesenchymal stem cells (Toma et al., 2002; Rangappa et al., 2003; Wang et al., 2004), and adult-derived liver stem cells (Muller-Borer et al., 2004) have all been shown to differentiate into cardiomyocytes in culture.

In vitro differentiation of stem cells into cardiomyocytes occurs in response to specific cytokines including Wnt proteins (Wnt3A, recombinant human Dkk-1and recombinant mouse Frizzled 8/Fc) (Naito et al., 2006), Gsk-3β inhibitor II (Naito et al., 2006), oxytocin (Paquin et al., 2002), 5-aza-2'-deoxycytidine (Rangappa et al., 2003; Oh et al., 2003) or DMSO (Paquin et

al., 2002; Oh et al., 2003). Wnt proteins were reported to have a biphasic role in cardiomyogenesis. Activation of the Wnt pathway during the early phase enhances cardiac differentiation, whereas inhibition of Wnt signaling in the late phase enhances cardiac differentiation (Naito et al., 2006). Gsk-3β inhibitor II mimics the effects of Wnt3A. We examined the potential of these compounds to differentiate PBD-MAPCs into cardiomyocytes.

As an alternative to adding cytokines, stem cells can be co-cultured with neonatal cardiomyocytes which release factors that induce stem cell differentiation. This technique has been successful with liver stem cells (WBF344) (Muller-Borer et al., 2004), mesenchymal stem cells (Li et al., 2006), endothelial embryonic cells (Condorelli et al., 2001) and endothelial progenitor cells (EPCs) (Badorff et al., 2003). Embryonic rat cardiomyoblasts (H9C2 cells) (Murasawa et al., 2005) also induce development of a myocardial lineage from endothelial progenitor cells (EPCs) in co-culture.

When stem cells differentiate into cardiac lineage they contract spontaneously in culture because of the intrinsically contractile nature of cardiomyocytes (Planat-Benard et al., 2004). For assay of differentiated PBD-MAPCs we used mouse monoclonal antibodies for cardiac Troponin T (cTnT) (Toma et al., 2002), cardiac Troponin I (cTnI) and cardiac Myosin heavy chain (cMHC) (Min et al., 2002) which were all shown to be specific cardiac markers.

Our lab has isolated a novel type of adult stem cell from blood, Peripheral Blood-Derived Multipotent Adult Progenitor Cells, (PBD-MAPCs). Earlier findings showed that PBD-MAPCs can be differentiated into endothelial, smooth muscle, osteocyte, adipocyte and neuron like cells (Price et al., 2006). The goal of these experiments was to determine if PBD-MAPCs can differentiate into cardiomyocytes when grown in the appropriate conditions.

Specific Aims

We examined the potential of Wnt proteins, Gsk-3β inhibitor II, oxytocin, 5-aza-2'-deoxycytidine and DMSO to induce cardiomyocyte differentiation in primordial PBD-MAPCs. We also co-cultured primordial PBD-MAPCs with neonatal rat cardiomyocytes or H9C2 cells to test the cardiomyocyte differentiation potential of this unique population of adult stem cells. In order to assess differentiation, we looked for spontaneous beating behavior and expression of cardiac specific proteins, cTnT, cTnI and MHC.

Hypotheses

Hypothesis 1: Wnt proteins, Gsk-3β inhibitor II, oxytocin, DMSO or 5-aza-2'-deoxycytidine in cell culture will induce PBD-MAPCs to spontaneously beat in culture and express cardiac specific proteins.

Hypothesis 2: Neonatal rat cardiomyocytes or embryonic rat cardiomyoblast cells (H9C2 cells) when co-cultured with PBD-MAPCs induce PBD-MAPCs to spontaneously beat in culture and express cardiac specific proteins.

MATERIALS AND METHODS

Cardiac differentiation with cytokines

PBD-MAPCs were maintained in the primordial stage as previously described (Price et al., 2006). We have different clones of PBD-MAPCs, designated clone 45 and 100. For cardiac differentiation with cytokines, dissociated PBD-MAPCs were washed in PBS and resuspended in DMEM/F12 (Gibco), 10% FBS (Hyclone), Penicillin/Streptomycin (Gibco) and GlutaMAX (Invitrogen). Cells in culture medium were seeded into a 24 well tissue culture plate (Corning)

coated with gelatin (Sigma) (10uL of 2% gelatin was applied per well and left to dry) or Collagen IV (R&D Systems) (500uL of 0.4% Collagen is added per well and left to dry and washed with distilled water). Medium was changed once every 3 days. Cells were allowed to grow for 4 days and then recombinant mouse Wnt3A (R&D Systems) or Gsk3β inhibitor II (Calbiochem) were added at 100ng/ml and 0.2μM concentrations respectively. Cells were treated with Wnt3A or Gsk3β inhibitor II for 4 days, changed to regular medium for 2 days and again Wnt inhibitors, recombinant human Dkk-1 (R&D Systems) and recombinant mouse Frizzled 8/Fc Chimera (R&D Systems) were added at a concentration of 500ng/ml and 200ng/ml respectively. Cells were maintained in Wnt inhibitors for another 5 days and later probed for expression of cardiac markers.

For differentiation with oxytocin (Calbiochem) and DMSO (Fisher Scientific), cells were cultured in αMEM (Gibco) medium with 7.5% bovine serum (Gibco), 2.5% FBS, Penicillin/Streptomycin and GlutaMAX. PBD-MAPCs were initially seeded in non tissue culture plates with 10^{-7}M oxytocin (Calbiochem) or 1% DMSO and were kept rocking for 4 days. After 4 days, cells were changed to a plain medium into a tissue culture plate and were not rocked. Medium was changed once every 2 days. The culture was maintained for 14 days and later probed for cardiac specific markers using immunoblots and immunocytochemistry.

For differentiation with 5-aza-2'-deoxycytidine (Sigma) cells were cultured in DMEM/F12 with 10% FBS, Penicillin/Streptomycin and GlutaMAX. Cells were seeded on tissue culture plates coated with rat tail collagen type I (BD Biosciences). Two days after seeding, medium with 9μM of 5-aza-2'-deoxycytidine was added and cells were allowed to grow for 2 days. The medium was then replaced with plain medium and the cells cultured for additional 5 weeks and later probed for expression of cardiac markers.

Co-culture – Neonatal rat cardiomyocytes

For cardiac differentiation using co-culture technique, a 24 well tissue culture plate was coated with 2% gelatin. Neonatal rat cardiomyocytes (NRCM) (Lonza) were resuspended in DMEM/F12 with 7.5% FBS, 7.5% horse serum (Lonza), 50 mM HEPES and Penicillin/Streptomycin. NRCM were seeded into 10 wells at a density of $4x10^5$ cells per well. PBD-MAPCs were added to six wells on day 0 and the remaining 4 wells were kept as controls. Medium was changed once every 2 days. The culture was maintained for 7 days and on the 8^{th} day serum content was reduced to 5% in half the wells. Cells were maintained at 5% serum until the 19^{th} day, at which point the experiment was terminated and immunocytochemistry performed.

Co-culture – Embryonic rat cardiomyoblasts (H9C2 cells)

For cardiac differentiation using H9C2 cells, a 24 well tissue culture plate was coated with rat tail collagen type I. Embryonic rat cardiomyoblasts (H9C2 cells) (generously provided by Dr. Eric Blough, Marshall University) were seeded on culture plates in DMEM-high glucose (Gibco) with 10% FBS. Cells were allowed to settle and grow for 4 days after which PBD-MAPCs were added. Medium was changed once every 2 days for 10 days and on the 11^{th} day half of the wells were serum starved to 1% FBS. Cells were allowed to grow until the 18^{th} day, at which point the experiment was terminated and immunocytochemistry performed.

Immunoblots

Immunoblots were performed as previously described (Price et al., 2006). Briefly, after the culture period, cells in individual wells were washed with PBS and lysed using Laemmli sample buffer (2% sodium dodecyl sulfate, 6M urea, 62.5 mM Tris-Cl, pH 6.8, 160 mM

dithioerythritol, 0.005% Bromophenol Blue). Equal amounts of protein were loaded on 4-12% Bis-Tris gel (Nupage/Invitrogen) and electrophoresed at 200 mV for 1hr. The gel was transferred onto polyvinylidene fluoride (PVDF) (Millipore) membranes at 250mA for 1hr 30 min. in transfer buffer containing 0.7M glycine and 25mM Tris. Membranes were blocked for an hour in Tris-buffered saline with 0.1% Tween 20 and 5% powdered milk. The membranes were probed with a mouse monoclonal antibody specific for cardiac Troponin T (cTnT) (34/41kDa) (Santacruz Bioreagents) at a 1:200 concentration. Membranes were treated with the primary antibody overnight followed by incubation with a horseradish peroxidase conjugated goat anti-mouse secondary antibody for 1 hour (Sigma). Bands were visualized with chemiluminescence Amersham ECL Western blotting detection reagents (GE health care) and recorded on film (Kodak).

Immunocytochemistry

For immunocytochemistry, cells were washed with PBS and fixed in 4% paraformaldehyde for 30 min, after which they were washed and permeabilized in 0.1% Triton X 100 for 20 minutes. Again they were washed and incubated in 0.1% BSA for 30 min. Later they were incubated in blocking buffer containing 5% BSA, 5% goat serum and 0.1% Micro-O-Protect for 2 hours. Antibodies to cardiac Troponin I (cTnI) (Santacruz Bioreagents), cardiac Myosin heavy chain (MHC) (Affinity bioreagents) were diluted at 1:200 concentrations in a 1:10 blocking buffer in PBS and incubated in a humidified chamber overnight. After thorough washing, cells were incubated with Alexa Fluor conjugated secondary antibodies (Invitrogen) for 4 hours. Cells were washed thoroughly and incubated with 0.002% DAPI to stain the nuclei (Santa Cruz Biotechnology) for 10 minutes and then mounted in Prolong Gold (Invitrogen) and observed under an epifluorescence microscope. Micrographs were obtained with a Zeiss

AxioExplorer epifluorescent microscope using 10x and 20x air interface Neoplan objectives and the accompanying Zeiss image acquisition software. All deconvolution images were obtained with default settings. Brightness adjustments and image cropping was performed in ImageJ (NIH) and figures were assembled using Microsoft Powerpoint.

RESULTS

PBD-MAPCs in a cardiac differentiation culture do not contract spontaneously.

PBD-MAPCs were cultured in different culture conditions using the cytokines Gsk3β-inhibitor II, Oxytocin and 5-aza-2'-deoxycytidine (Fig. 2.1A-C) (n=3). Cell cultures were observed on a daily basis and assessed for spontaneous contractions, but none were observed up to 5 weeks of culture although the cells appeared healthy. Cells in culture with Wnt3A also did not contract (data not shown). When PBD-MAPCs were co-cultured with rat embryonic cardiac myoblasts (H9C2 cells), they did not contract spontaneously, as expected H9C2 cells also did not contract in culture (Meyer and Lubo, 2007). In co-culture experiments with neonatal rat cardiomyocytes, the cardiomyocytes formed foci of spontaneously contracting cells (data not shown), but none of the PBD-MAPCs exhibited such contracting foci.

Immunoblot analysis for cardiac proteins on PBD-MAPCs after cardiac differentiation with cytokines does not show cardiac specific proteins.

Fig 2.2A shows an immunoblot probed for cardiac Troponin T (cTnT) in samples after cardiac differentiation on two different extracellular matrices, gelatin and collagen (n=3). In each matrix we used cytokines Wnt 3A, Gsk3β-inhibitor II or oxytocin. Only the positive control (rat heart) expressed the expected cardiac Troponin T (cTnT) band at 34/41 kDa. The other treatment groups cultured on gelatin, Wnt3A, Gsk3β-inhibitor II, oxytocin and untreated, did not

express cTnT. Similarly the experimental groups cultured on collagen, Wnt3A, Gsk3β-inhibitor II, oxytocin or untreated, did not express cTnT. All the experimental samples, but not the rat heart control, showed an unexpected cTnT reactive doublet of about 70kDa.

Fig 2.2B shows an immunoblot probed for cardiac Troponin T (cTnT), on two different clones of PBD-MAPCs (clone 45 and clone 100) in two treatment conditions; 5-aza-2'-deoxycytidine and untreated. Again, only the positive control (rat heart) expressed a cardiac Troponin T (cTnT) band at 34/41 kDa. The PBD-MAPC samples, regardless of treatment conditions did not express cTnT. Again, all PBD-MAPC samples were immunoreactive for the 70kDa doublet observed in Fig. 2.2A. All membranes were stained with Coomassie blue and found to have equal loading of protein (data not shown).

PBD-MAPCs after cardiac differentiation with cytokines do not express cardiac proteins in immunocytochemistry.

Immunocytochemistry to detect cardiac myosin heavy chain (MHC) expression was performed on PBD-MAPCs cultured with the cytokines oxytocin (Fig. 2.3A), DMSO (Fig. 2.3B), or a combination of oxytocin, Gsk3β inhibitor II, DMSO, Dkk and Fz/F8 (Fig. 2.3C) (n=3). None of these treatments gave rise to PBD-MAPCs immunoreactive to MHC (red), although cultured neonatal rat cardiac cells processed with the same ICC protocol showed strong immunofluorescence for MHC (Fig 2.3D). The inherent green fluorescent protein (GFP) fluorescence of PBD-MAPCs allowed their visualization and nuclei were stained with DAPI.

Figure 2.1

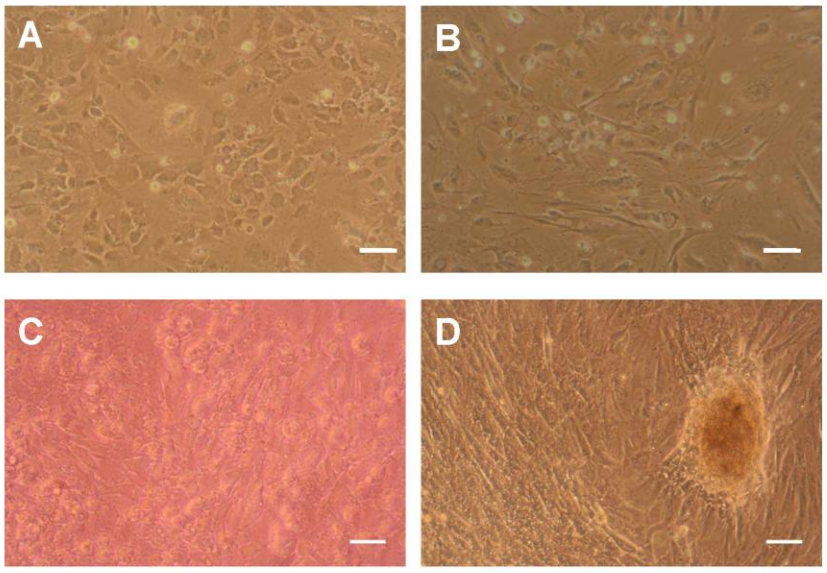

Figure 2.1: PBD-MAPCs in a cardiac differentiation culture. Bright field images of PBD-MAPCs cultured in presence of Gsk-3β inhibitor II **(A)**, Oxytocin **(B)**, 5-aza-2'-deoxycytidine **(C)** and co-cultured along with H9C2 cells **(D)**. Scale bar: 100μm

Figure 2.2

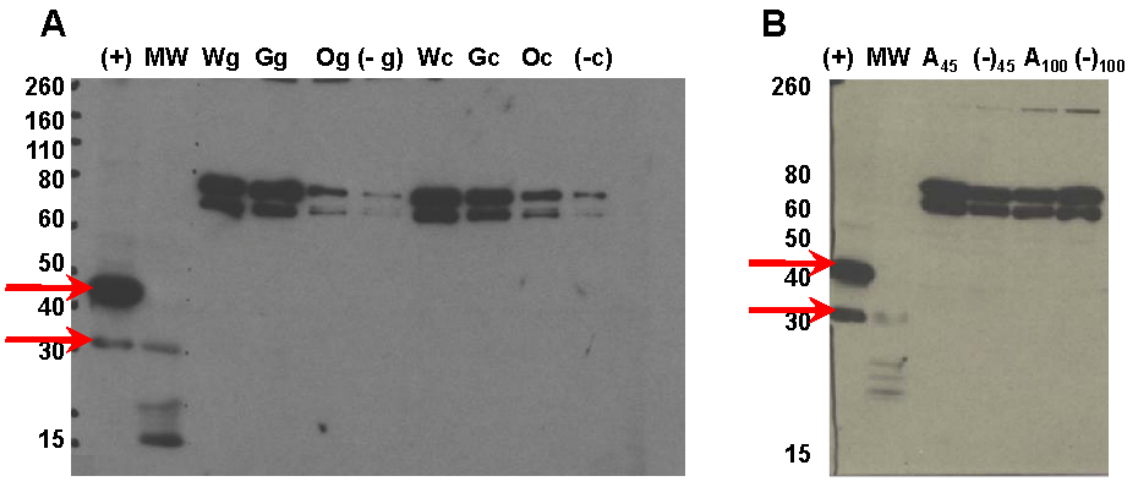

Figure 2.2: Immunoblot analysis for cardiac proteins on PBD-MAPCs after cardiac differentiation with cytokines does not show cardiac specific proteins.
A- Luminogram of blot probed for cardiac Troponin T (1:200) The positive control (+) from a mouse heart express cTnT (34/41kDa) (arrows), but the other treatments of cells grown on gelatin (g) and collagen (c) Wnt3A-gelatin (Wg), Gsk3β-gelatin (Gg), Oxytocin-gelatin (Og), untreated-gelatin (-g), Wnt3A-collagen (Wc), Gsk3β-collagen (Gc), Oxytocin-collagen (Oc), untreated-collagen (-c) do not express cTnT. All PBD-MAPC cell extracts contained a cTnT-reactive doublet at about 70kDa.
B- Luminogram of blot probed for cardiac Troponin T (1:200) The positive control (+) from a mouse heart express cTnT (34/41kDa) (arrows), but the other treatments of cells grown on collagen in presence of 5-aza-2'-deoxycytidine, clone 45 PBD-MAPCs (A_{45}), clone 100 PBD-MAPCs (A_{100}), untreated $(-)_{45}$ and $(-)_{100}$ do not express cTnT. All PBD-MAPC cell extracts contained a cTnT-reactive doublet at about 70kDa.

Figure 2.3

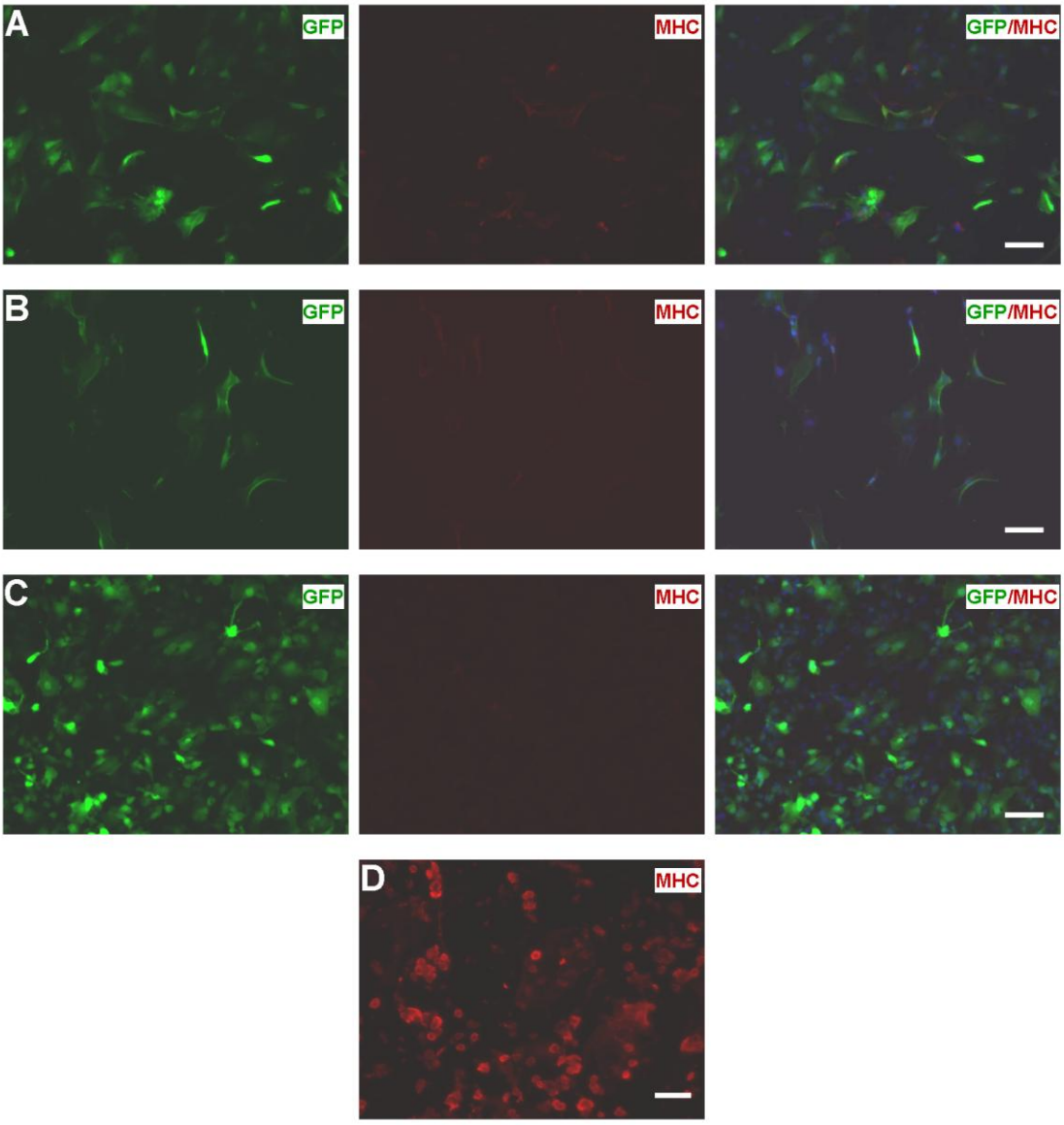

Figure 2.3: PBD-MAPCs after cardiac differentiation with cytokines do not express cardiac proteins. GFP positive (green) PBD-MAPCs treated with Oxytocin **(A)**, DMSO **(B)**, and Oxytocin, Gsk3β inhibitor II, DMSO, Dkk and Fz/F8 **(C)** do not express cardiac Myosin heavy chain (MHC) (Red). Neonatal rat cardiomyocytes express cardiac MHC **(D)**. The nuclei were stained with DAPI (blue). Scale bars in A,B,C 100μm; D 50μm

PBD-MAPCs co-cultured with neonatal rat cardiomyocytes do not express cardiac proteins in immunocytochemistry.

PBD-MAPCs were co-cultured with neonatal rat cardiomyocytes and probed for expression of cardiac proteins (n=3). Immunocytochemistry to detect myosin heavy chain (MHC) (Fig 2.4A), showed that none of the PBD-MAPCs were positive for this cardiac marker protein, only the neonatal rat cardiomyocytes were strongly immunoreactive for MHC. PBD-MAPCs and neonatal cardiac cells were in close contact with each other. Nuclei stained with DAPI were observed in PBD-MAPCs and neonatal rat cardiomyocytes. When probed for another cardiac specific marker protein, cardiac troponin I (cTnI) (Fig. 2.4B), only the neonatal cells and no PBD-MAPCs showed immunoreactivity to cTnI.

PBD-MAPCs co-cultured with embryonic rat cardiomyoblast cells (H9C2) do not express cardiac proteins.

PBD-MAPCs co-cultured with embryonic rat cardiomyoblast cells (H9C2 cells) (n=3) in two different conditions, serum starved (1% FBS) (Fig. 2.5 A) and with media containing 10% FBS (Fig. 2.5B), were probed for the expression of MHC. On immunocytochemistry, none of the PBD-MAPCs cultured in different serum concentrations were immunoreactive for MHC. Only the H9C2 cells were positive for MHC regardless of serum concentration. Nuclei were stained with DAPI and observed in both PBD-MAPCs and H9C2 cells. The H9C2 cells served as an internal control for the immunostaining procedure as they expressed high levels of the cardiac protein MHC.

Figure 2.4

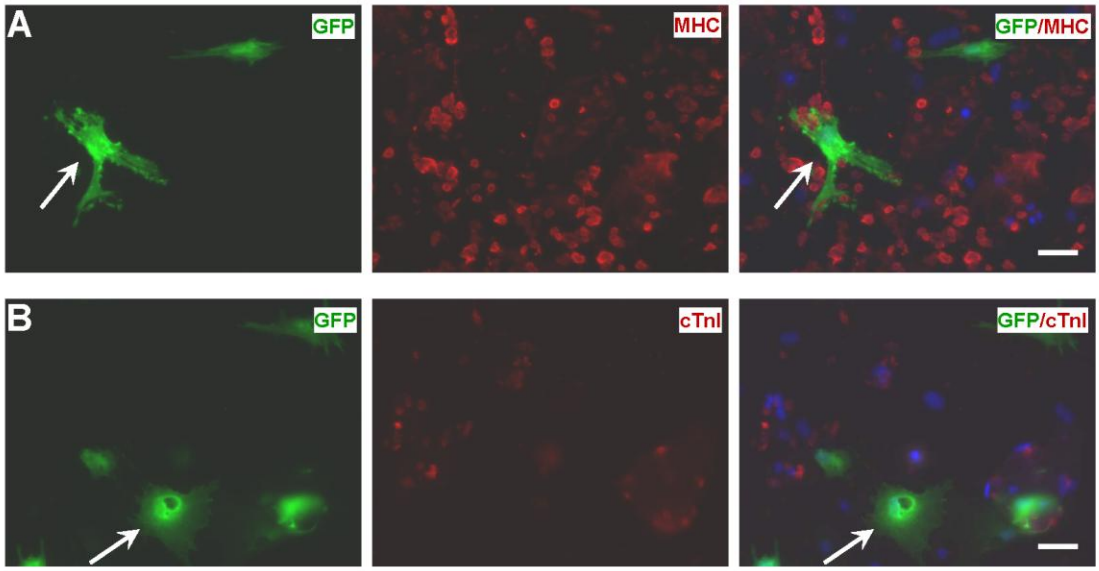

Figure 2.4: PBD-MAPCs co-cultured with neonatal rat cardiomyocytes do not express cardiac proteins. **A**-GFP positive (green) PBD-MAPCs (arrow) when co-cultured with neonatal rat cardiomyocytes do not express Myosin heavy chain (MHC) (red), only the neonatal rat cardiomyocytes express MHC
B-GFP positive (green) PBD-MAPCs (arrow) when co-cultured with neonatal rat cardiomyocytes do not express cardiac Troponin I (cTnI) (red), only the neonatal rat cardiomyocytes express cTnI. The nuclei were stained with DAPI (blue). Scale bars 50μm

Figure 2.5

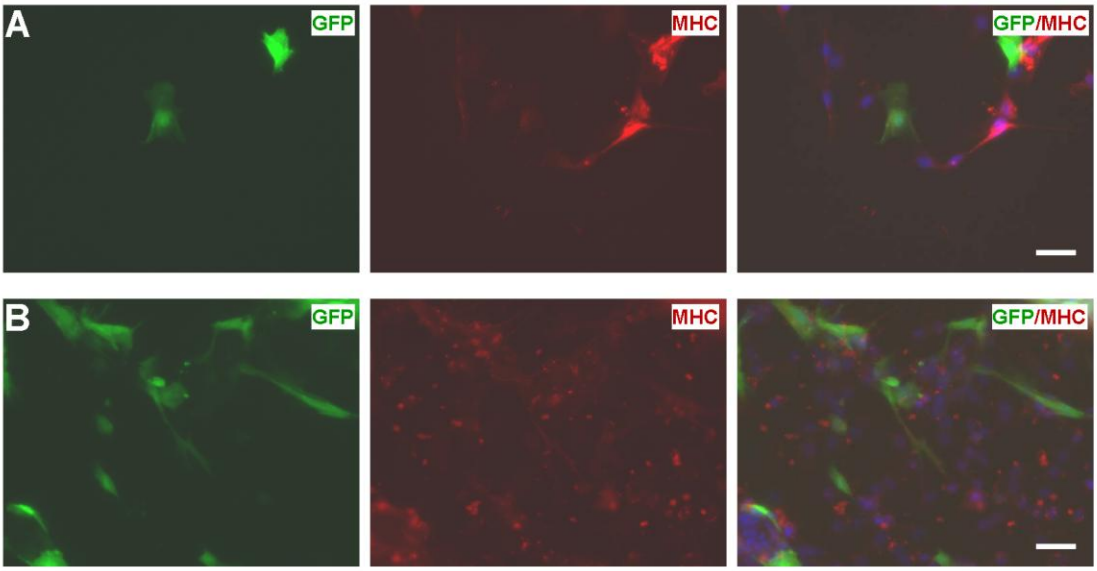

Figure 2.5: PBD-MAPCs co-cultured with H9C2 cells do not express cardiac proteins.
GFP positive (green) PBD-MAPCs when co-cultured with H9C2 cells do not express Myosin heavy chain (MHC) (red), only the H9C2 cells express MHC when cultured in 1% FBS containing medium (**A**) and in 10% FBS containing medium (**B**). The nuclei were stained with DAPI (blue). Scale bars 50 μm

DISCUSSION

Earlier findings showed that PBD-MAPCs can be differentiated into endothelial, smooth muscle, osteocyte, adipocyte and neuron like cells (Price et al., 2006) but cardiomyocyte differentiation was not attempted. In this study we used cytokines, shown to cause differentiation of stem cells into cardiomyocytes, to test the ability of PBD-MAPCs to differentiate into cardiomyocytes. Cardiomyocytes are intrinsically contractile and develop spontaneously contracting foci in culture. Whenever stem cells differentiate into cardiac lineage they also contract spontaneously in culture (Planat-Benard et al., 2004). In none of our experiments could we observe any spontaneously contracting foci of cells. Though we used various methods, none of the method employed caused apparent differentiation of PBD-MAPCs into cardiac myocytes. In this study, we establish that PBD-MAPCs are not likely to differentiate to a cardiomyocyte lineage from the primordial state in which we maintain them. Our choice of markers cTnT for immunoblots and cTnI and cardiac MHC for immunocytochemistry was due to the fact that cTnT antibody was not reactive to the positive control cardiomyocytes in immunocytochemistry and cTnI and cardiac MHC were not reactive to the heart lysate used as positive control in immunoblots. Regardless of the cytokines added, PBD-MAPCs did not express the expected 34/41 kDa cTnT band. However all PBD-MAPC cell extracts contained a cTnT-reactive doublet of about 70kDa. We could not find mention of such a doublet at that molecular weight in the literature. The doublet was not found in the positive control samples and hence could represent a cell culture induced protein expression. Immunocytochemistry on PBD-MAPCs after differentiation culture with cytokines or co-culture also did not show any cells immunoreactive to cardiac specific proteins cTnI and cardiac MHC. These results are similar to a few other published findings that mesenchymal stem cells do not differentiate into cardiomyocytes

(Martin-Rendon et al., 2008). Certain other findings also showed that co-culture did not cause differentiation or caused very limited differentiation of rodent mesenchymal stem cells (Rose et al., 2008; Gallo et al.), human endothelial progenitor cells (Gruh et al., 2006)and human bone marrow stem cells (Koninckx et al., 2009) into a cardiac lineage. In this study we did not probe to see if undifferentiated PBD-MAPCs express certain cardiac markers as was advocated by the nonspecific gene expression theory (Egusa et al., 2005) and was seen in later part of this current research in relation to neural markers. This could be a future study of interest to further validate the concepts of nonspecific gene expression.

In summary, primordial PBD-MAPCs when cultured with Wnt proteins, Gsk 3β inhibitor II, oxytocin, DMSO or 5-aza-2'-deoxycytidine under appropriate culture conditions do not differentiate into cardiomyocytes. In addition, primordial PBD-MAPCs, when co-cultured with H9C2 cells or neonatal rat cardiomyocytes, do not differentiate into cardiomyocytes. PBD-MAPCs may not be sufficiently primordial to differentiate into a myocardial lineage or perhaps these cells require additional specific factors that may play a crucial role in cardiomyogenesis. Finally, by injecting PBD-MAPCs into an ischemic heart (Van't Hof et al., 2007); we could determine if paracrine signals within the heart milieu might cause differentiation of PBD-MAPCs into cardiomyocytes. This may lead to the identification of additional signaling factors required for differentiation of PBD-MAPCs to a cardiomyocyte lineage *in vitro*. In conclusion our data does not support our hypotheses that Wnt proteins, Gsk-3β inhibitor II, oxytocin, DMSO or 5-aza-2'-deoxycytidine and co-culture with neonatal rat cardiomyocytes or H9C2 cells will induce PBD-MAPCs to spontaneously beat in culture and express cardiac specific proteins.

CHAPTER 3

COMPARING THE EXPRESSION PROFILES OF PRIMORDIAL AND NEURALIZED PBD-MAPCs

INTRODUCTION

Our lab has isolated a new kind of stem cell population from the blood of transgenic green fluorescent protein (GFP) swine and named them as Peripheral Blood Derived Multipotent Adult Progenitor Cells (PBD-MAPCs) (Price et al., 2006). *In vitro* these cells were shown to differentiate into endothelial, smooth muscle, osteocyte, adipocyte and neuron like cells (Price et al., 2006) which indicate that the newly discovered cells are a stem cell population. In general, stem cells express typical marker proteins which identify them from other cell populations. Prominin-1/CD133 (originally termed as AC133) is a plasma membrane marker found in hematopoietic stem cells. It is also found in several types of somatic stem cells, including neural stem cells (Kania et al., 2005). Though our PBD-MAPCs differentiate into many different lineages, the stem cell markers which PBD-MAPCs express were not probed to date. Since PBD-MAPCs are a novel population of blood derived stem cells and have a few advantages over traditional sources of stem cells, we wanted to examine their protein expression profiles to determine if they express the hematopoietic stem cell marker CD133.

Stem cells, when cultured in defined media with specific cytokines such as EGF, bFGF, BDNF and retinoic acid will differentiate into neuronal cells (Dasari et al., 2007; Karimi-Abdolrezaee et al., 2006; Nistor et al., 2005). Our lab has shown that PBD-MAPCs differentiate into neuron like cells in culture, in a manner similar to other described stem cells (Spitzer and Price, 2008). After differentiation, cells attain a typical neuronal morphology with long cellular processes, lose expression of primordial markers and express neuronal specific markers such as

β-tubulin III, tyrosine hydroxylase (TH) and Protein gene product 9.5 (PGP9.5). The goal of these experiments was to determine the mRNA and protein expression of undifferentiated PBD-MAPCs and PBD-MAPCs after undergoing neural differentiation.

Specific Aim

The specific aim of this project was to compare the protein expression profiles of PBD-MAPCs before and after neural differentiation. We expected that primordial PBD-MAPCs would express the stem cell marker CD133 and that after neural differentiation; they would switch their expression to neural markers such as TH, β-tubulin III and PGP9.5.

Hypotheses

Hypothesis 1: Using RT-PCR, we expect to see expression of the primordial marker CD133, and not the neuronal marker TH before subjecting PBD-MAPCs to differentiation; this expression profile is expected to reverse after neuronal differentiation.

Hypothesis 2: Using immunoblots, we expect neuralized PBD-MAPCs and not the primordial PBD-MAPCs to express the neuronal markers β-tubulin III and PGP9.5.

MATERIALS AND METHODS

Reverse transcription-polymerase chain reaction (RT-PCR)

PBD-MAPCs were maintained as previously described (Price et al., 2006). For neural differentiation, PBD-MAPCs were cultured in neuronal-specific medium as previously described (Spitzer and Price, 2008). Briefly, cells were seeded onto poly-lysine coated culture plates and

allowed to grow for 8 days in neurobasal medium with B27, N2, EGF, bFGF, FGF8b and Shh. Media was changed once every two days. For control cells we used porcine umbilical vein endothelial cells (PUVECs), which were harvested from umbilical cords of endothelial nitric oxide synthase (eNOS) over-expressing piglets (Hao et al., 2006). They were allowed to grow on tissue culture plastic in EGM-2 complete medium (Lonza) and passaged once every 5-6 days. After the culture period mRNA was isolated from cells using Trizol reagent (Invitrogen), and cDNA was synthesized with Superscript III (Invitrogen). We designed swine-specific primer sets to detect CD133, and TH to synthesize PCR products and ordered from Invitrogen. Human β-Actin primer set was ordered from Stratagene. Primers used for amplification and sequencing are shown in table 3.1. Amplified PCR products were separated by agarose or 8% polyacrylamide (Invitrogen) gel electrophoresis and DNA bands on the gel were visualized with ethidium bromide and UV light.

Sequencing

PCR products were purified with a QIAquick PCR purification kit (Qiagen). DNA concentration and purity were determined by measuring absorbance at 280 and 260nm with a Nanodrop spectrophotometer. Samples were submitted to the Marshall University DNA Core facility with the primers used for amplification and sequencing was performed there with a ABI 3130 Genetic Analyzer. Sequencing data was compared with porcine sequences in NCBI BLAST

Table 3.1

RT-PCR primers, expected product size and PCR program used for the amplification of CD133, tyrosine hydroxylase (TH) and β-actin.

			5'→ 3' primers	Expected product size	Annealing temp. Extension time, Cycles
CD133	**PCR 1**	Fw	CCIGCIA(T/C)IAA(T/C)TA(T/C)GA(A/G)ACNAA(A/G)GA		
		Rv	GC(T/C)TCNGCCATNGC(T/C)TT(A/G/T)AT	678bp	60^{o}C, 1', 40
	Nested PCR	Fw	CCTGGGGCTGTTTATTATCC		
		Rv	GACATATCACCAAGAGGGAAACG	159bp	57^{o}C, 1', 40
TH	**PCR 1**	Fw	GACCTTCGCCCAGTTCTCGC		
		Rv	AGCGTGTACGGGTCGAACTT	367bp	57^{o}C, 1', 40
	Nested PCR	Fw	AGTTTGGGCTCTGCAAACAGAACG		
		Rv	TGTCCTTGGCGTCACTGAAAACTCT	218bp	62^{o}C, 1', 40
β-actin	**PCR 1**	Fw	TGACGGGGTCACCCACACTGTGCCCATCTA		
		Rv	CTAGAAGCATTTGCGGTGGACGATGGAGGG	661bp	60^{o}C, 1'30", 40

In CD133 primers, I (Inositol) represent A/T/G/C and N represents A/T/G/C.

Immunoblots

Immunoblots were performed as described previously (Price et al., 2006). Briefly, after the culture period, cells (primordial PBD-MAPCs and newly differentiated cells) were dissolved using a Laemmli sample buffer (2% sodium dodecyl sulfate, 6M urea, 62.5 mM Tris-Cl, pH 6.8, 160 mM dithiothreitol, 0.005% Bromophenol Blue). Pig brain lysate (Abcam) was used as a positive control. Protein amounts were quantified using NanoOrange protein quantitation kit (Invitrogen). Equal amounts of protein were loaded in a 4-12% Bis-Tris gel (Nupage/Invitrogen) transferred onto polyvinylidene fluoride (PVDF) (Millipore) membranes using 0.7M glycine and 25mM Tris as a transfer buffer. Membranes were blocked for an hour in Tris-buffered saline with 0.1% Tween 20 and 5% powdered milk. The membranes were probed for mouse anti β-tubulin III monoclonal antibody (Chemicon) and rabbit anti PGP9.5 polyclonal antibody (Abcam) at a 1:500 concentration. Membranes were treated with the primary antibody overnight followed by horseradish peroxidase conjugated secondary antibody (Sigma) for 1 hour. Bands were visualized using the Amersham ECL Western blotting detection reagents (GE healthcare) using chemiluminescence and recorded on a film (Kodak).

RESULTS

Primordial PBD-MAPCs express mRNA for the stem cell marker CD133 and tyrosine hydroxylase (TH) while neuralized cells do not express TH.

RT-PCR for CD133, a hematopoietic stem cell marker (Fig. 3.1A), resulted in amplification of a fragment of the correct size (159bp) from porcine control cells, PUVECs (C+) and primordial PBD-MAPCs (P+) (n=1). In PBD-MAPCs, this expression is lost upon neural differentiation (N+). RT-PCR of tyrosine hydroxylase (TH), a dopaminergic neuron marker (Fig

3.1B) surprisingly showed that primordial PBD-MAPCs (P+) express mRNA for TH while neuralized PBD-MAPCs (N+) do not (n=1). β-actin, a housekeeping gene, was amplified to control for cDNA integrity and sample loading (n=2). Appropriately sized bands (661bp) were observed in porcine control cells (C+), primordial PBD-MAPCs (P+) and neuralized PBD-MAPCs (N+). No bands were observed in the no RT control (P-, N-) or the PCR no template control (-) samples in all experiments.

Nucleotide sequences of PCR products amplified from PBD-MAPCs are 100% identical to porcine sequences of CD133 and tyrosine hydroxylase (TH)

When the amplified RT-PCR products of CD133 (Fig. 3.2A) and TH (Fig. 3.2B) were sequenced and compared with porcine sequences in NCBI BLAST, we found them to be 100% identical (n=1). The CD133 sequence had 87% similarity to Equus caballus (horse) and 84% similarity to Bos taurus (cattle) whereas the TH sequence had 91% similarity to Homo sapiens, 96% similarity to Bos taurus (cattle) and 94% similarity to Canis familiaris (dog) sequences. This establishes that the PCR products are true porcine sequences and not from human or other species contamination and primordial PBD-MAPCs do indeed express CD133 and TH.

Immunoblot analysis for neural marker proteins in primordial and neuralized PBD-MAPCs, show expression of neural markers.

An immunoblot analysis for the cell lysate samples before and after neural differentiation showed that both primordial and neuralized PBD-MAPCs express certain neural markers. Fig 3.3A shows an immunoblot probed for β-tubulin III, a neural marker (n=2). The positive control (pig brain lysate) expressed β-tubulin III. Also both the neuralized (N) and primordial (P) cell samples showed an immunopositive β-tubulin III band at 50kDa. In addition, a second clear band

Figure 3.1

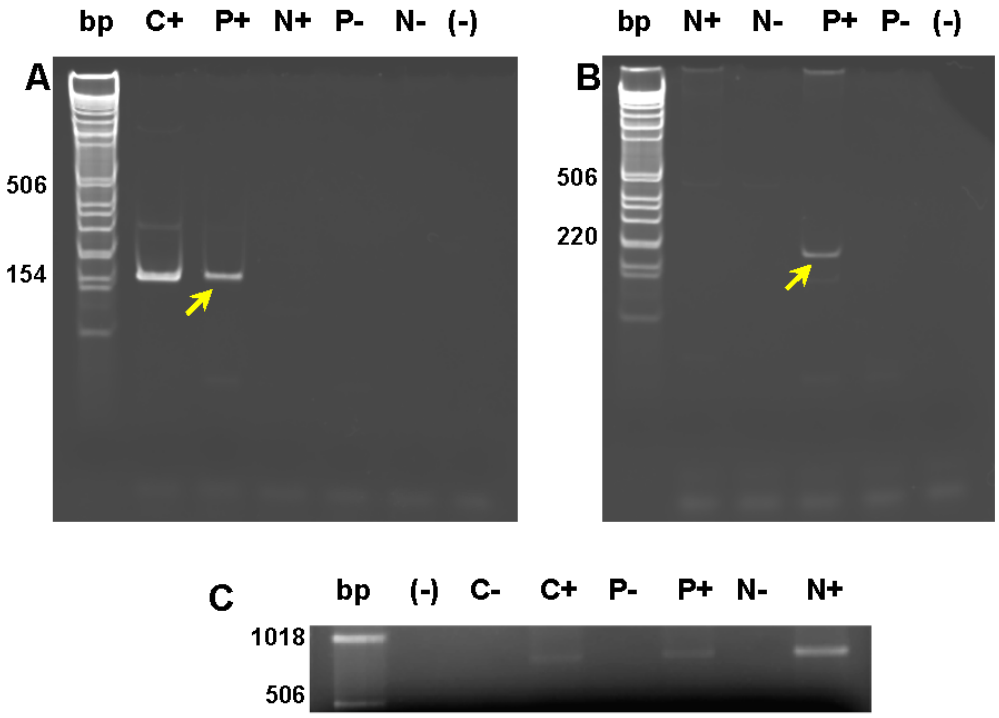

Figure 3.1: A- Primordial PBD-MAPCs (P+) express mRNA for stem cell marker CD133 (159 bp). Porcine control cells (C+) express CD133), a hematopoietic stem cell marker. Expression is also seen in primordial PBD-MAPCs (P+) (arrow) but is lost in neuralized PBD-MAPCs (N+). No bands are visible in the no RT controls (P- and N-) or the PCR negative control (-)

B- Primordial PBD-MAPCs (P+) express mRNA for neural cell marker tyrosine hydroxylase (TH) (218 bp). Primordial (P+) (arrow) but not neuralized (N+) PBD-MAPCs express TH, a marker of dopaminergic neurons. No bands are visible in the no RT controls (P- and N-) or the PCR negative control (-)

C- β-actin (661 bp), a housekeeping gene was amplified to control for cDNA integrity and sample loading. Appropriately sized bands were amplified from porcine control cells (C+), primordial PBD-MAPCs (P+) and neuralized PBD-MAPCs (N+). No bands were visible in no RT controls (C-, P- and N-) the PCR negative control (-)

Figure 3.2

Figure 3.2: Sequence of PCR products from PBD-MAPCs are 100% identical to porcine sequences of CD133 **(A)** and tyrosine hydroxylase (TH) **(B)**

Figure 3.3

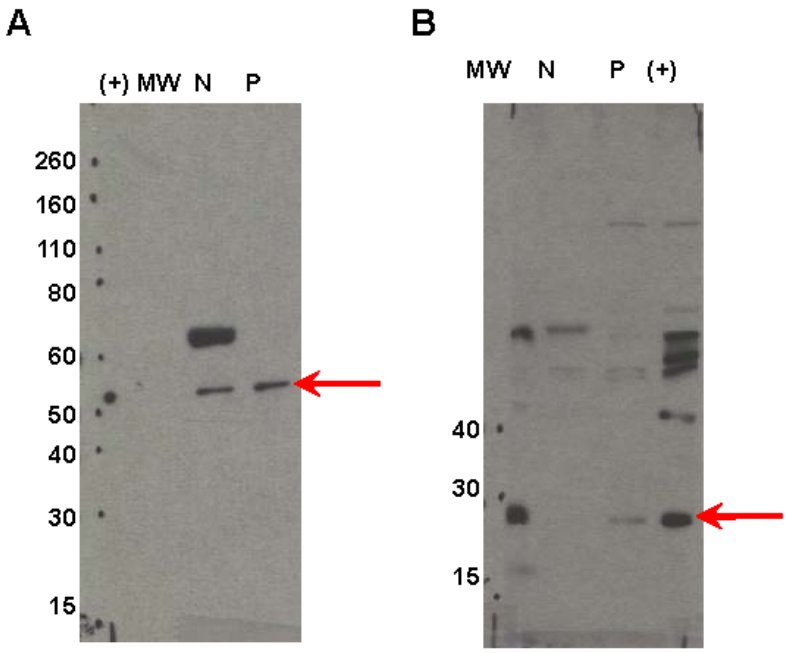

Figure 3.3: Immunoblot analysis of neural marker proteins in primordial and neuralized PBD-MAPCs show expression of neural markers.
A- Luminogram of blot probed for Mouse anti- β-tubulin (1:500). Neuralized PBD-MAPCs (N) primordial PBD-MAPCs (P) and positive control (+) express β-tubulin (50kDa), a neuronal protein (arrow).
B- Luminogram of blot probed for Rabbit anti PGP9.5 (1:500). Primordial PBD-MAPCs and positive control (+) express PGP9.5 (26.8kDa), a neuronal protein marker but neuralized PBD-MAPCs do not express PGP9.5 (arrow).

of around 60kDa was observed only in the neuralized sample but not in the positive control or the primordial PBD-MAPC samples. Fig 3.3B shows an immunoblot probed for protein gene product 9.5 (PGP9.5), a neural marker (n=2). The positive control (pig brain lysate) expressed PGP9.5. Surprisingly only the primordial (P) cells expressed PGP9.5 while the neuralized (N) cells did not. All membranes were stained with Coomassie blue stain and found to have equal loading of protein.

DISCUSSION

In this study we compared the expression profiles of primordial and neuralized PBD-MAPCs. Our choice of CD133 was made on the basis that PBD-MAPCs are blood derived and CD133 is a hematopoietic stem cell marker (Kania et al., 2005). Also due to non-availability of porcine specific antibody for CD133 we could not probe for CD133 protein expression in immunoblots. Expression of CD133 mRNA, a marker commonly found in several types of somatic stem cells, including hematopoietic and neural stem cells (Kania et al., 2005), clearly shows that PBD-MAPCs have stem cell characteristics. The fact that primordial PBD-MAPCs expressed CD133 and lost this expression after culture in neural differentiation medium confirms that they are no longer primordial and have begun their journey towards a specific lineage, in this case a neural lineage. A similar result showing loss of CD133 expression in neural stem cells from fetal brain after differentiation was shown previously (Ji et al., 2006). Also, CD133 marker expression has been reported to be lost upon terminal differentiation of endothelial progenitor cells into mature endothelial cells. (Hristov et al., 2003). CD133 was also reported to be expressed by multipotent adult progenitor cells (MAPC) from human bone marrow (Reyes et al., 2002).

Our positive control cells for RT-PCR analysis were PUVECs from piglets (porcine umbilical vein endothelial cells), which also expressed CD133. The CD133 marker is absent on mature endothelial or differentiated hematopoietic cells, however CD133 is expressed in a small population of circulating endothelial cells (Peichev et al., 2000; Salven et al., 2003). It was also reported that mature microvascular endothelial cells from human lungs express CD133 (Kahler et al., 2007) which are endothelial cells as PUVECs. Also it could be that PUVECs express CD133 because they are from a neonatal source. It was reported that late outgrowth endothelial cells derived from Wharton's jelly in human umbilical cord express CD133 and also that very few tissues such as placenta express CD133 (Wang et al., 2009). Hence this might explain PUVECs expressing CD133.

We found that primordial PBD-MAPCs expressed TH, a dopaminergic neuron marker before differentiation. Similarly, expression of neural genes by mesenchymal stem cells has been previously reported (Blondheim et al., 2006; Deng et al., 2006; Tondreau et al., 2004; Lamoury et al., 2006). Other findings report that mouse bone marrow stromal cells express a wide range of proteins including those expressed in terminally differentiated cells and suggest the theory of non specific gene expression by stem cells. In this theory it is suggested that stem cells exhibit nonspecific gene expression and when once they are directed towards a specific lineage, other lineage specific genes are silenced. Primordial cells may therefore simply express various proteins nonspecifically until they receive differentiation signals (Egusa et al., 2005). These earlier studies explain expression of TH by PBD-MAPCs. Interestingly after differentiation, neuralized PBD-MAPCs did not express TH. This might be due to insufficient signals from the culture medium or due to the TH gene silencing. Signals from the culture medium might not be sufficient to differentiate PBD-MAPCs into specialized neurons like the

dopaminergic neurons which express TH, due to this the TH genes which were being expressed by undifferentiated PBD-MAPCs might be silenced after the differentiation.

The nucleotide sequence of PCR products were observed to be identical to porcine sequences of CD133 and TH, which establishes that our PCR products are true porcine sequences and not products of human contamination.

β-tubulin III, a general neuronal marker was found to be expressed by both neuralized and primordial PBD-MAPCs. It was expected that β-tubulin III would be expressed only by differentiated cells. However, undifferentiated mesenchymal stem cells have been reported to express neural proteins, including β-tubulin III, in culture (Deng et al., 2006; Tondreau et al., 2004). This supports the theory of nonspecific gene expression in PBD-MAPCs as explained earlier. As expected, PBD-MAPCs expressed β-tubulin III on differentiation. Also primordial PBD-MAPCs expressed PGP9.5, a neural marker. This expression might be explained by the theory of nonspecific gene expression in PBD-MAPCs. However neuralized cells not expressing PGP9.5 might be due to gene silencing in differentiated PBD-MAPCs. In conclusion our data does not completely support our hypotheses. In our first hypothesis we hypothesized that PBD-MAPCs would only express CD133 and would lose its expression on differentiation. However PBD-MAPCs were seen to express both CD133 and TH. On differentiation PBD-MAPCs lost CD133 expression as expected but did not express TH. In our second hypothesis we hypothesized that only neuralized PBD-MAPCs would express neural markers, on the contrary primordial PBD-MAPCs were found to express β-tubulin III and PGP9.5. However on differentiation PBD-MAPCs expressed β-tubulin III but did not express PGP9.5.

CHAPTER 4

DIFFERENTIATION OF PBD-MAPCS INTO NEURAL LINEAGES IN A 3 DIMENSIONAL CELL CULTURE MATRIX

INTRODUCTION

Injury to the spinal cord can result in damage to the neuronal axons and causes demyelination of surviving axons, severely hampering neuronal conductance along the spinal cord (Karimi-Abdolrezaee et al., 2006; Barnabe-Heider and Frisen, 2008; Wrathall and Lytle, 2008). This greatly reduces the motor and sensory abilities of an affected person and dramatically affects his/her quality of life (Louro and Pearse, 2008). Much work is being done to regenerate the lost functional abilities due to injury, and stem cells currently offer a great promise in this direction. Many different types of stem cells from embryonic and adult sources are currently being studied, including olfactory ensheathing cells (Li et al., 1997), embryonic stem cells (McDonald et al., 1999), mesenchymal stem cells (Yang et al., 2008), Schwann cells (Xu et al., 1995) and adult rat spinal cord stem/progenitor cells (Parr et al., 2007). Stem cells have been implanted into the injury site in laboratory animals (Keirstead et al., 2005; Karimi-Abdolrezaee et al., 2006) and partial restoration of motor abilities has been reported. Recently the FDA has approved the use of stem cells in human clinical trials for spinal cord injuries. Immediately following injury, an inflammatory process begins, resulting in the formation of a glial scar that drastically reduces the possibility of regeneration or remyelination of spared fibers at the injury site (Fawcett and Asher, 1999; Gris et al., 2007). This has prompted the development of bridging transplants which consist of various biomaterials, molded into a 3 dimensional (3D) shape and are impregnated with undifferentiated stem cells, Schwann cells or cells that have been pre-differentiated into neurons (Xu et al., 1997). In this technique the injured

region is excised and replaced with the cellular plug (Novikova et al., 2008; Fouad et al., 2005; Kamada et al., 2005). In a study conducted with Schwann cells in a poly-β-hydroxybutyrate (PHB) scaffold plug implanted in spinal cord injured rats, cells survived and neurofilament-positive axons were observed in the biomaterial conduit and promoted axonal regeneration (Novikova et al., 2008).

Various biomaterials such as collagen, laminin, matrigel and fibrin have been employed in bridging transplants for spinal cord injury (Nomura et al., 2006; Cao et al., 2005). These biomaterials offer support for the growth of transplanted cells and endogenous axons. In addition to these types of biomatrices, skeletal muscle fibers have been used in nerve grafts to treat human cases of peripheral nerve damage (Roganovic et al., 2007; Weber et al., 2000; Norris et al., 1988) allowing regeneration of the lost neuronal conductance pathways and functional improvement (Brunelli et al., 1993). In this technique muscle fibers act as a guide for the growth of endogenous axons across the lesion.

Different types of stem cells, when cultured in defined media with specific cytokines such as EGF, bFGF, BDNF and Retinoic acid, will differentiate into neuronal cells (Dasari et al., 2007; Nistor et al., 2005; Karimi-Abdolrezaee et al., 2006). Upon differentiation into a neural lineage, cells show long cellular processes typical to neuronal morphology and express specific neural markers like β-tubulin III (Karimi-Abdolrezaee et al., 2006) NeuN, neurofilament-L (NF-L) (Joannides et al., 2004) which are neuronal specific markers, O4 and Myelin basic protein (MBP), both oligodendrocyte markers and glial fibrillary acidic protein (GFAP) an astrocyte marker.

We have isolated a novel type of adult stem cells from the peripheral blood of adult transgenic green fluorescent protein (GFP) swine, designated as Peripheral Blood Derived

Multipotent Adult Progenitor Cells (PBD-MAPCs). Also our lab has shown that PBD-MAPCs differentiate into neuron like cells in a 2 dimensional (2D) culture in a manner similar to other established kinds of stem cells (Spitzer and Price, 2008). In this work it was shown that neurally differentiated PBD-MAPCs in a 2D culture express β-tubulin III, a neuronal marker. At this stage we are examining if the neural differentiation model can be achieved in a 3D culture system using various biomatrices. We also embedded skeletal muscle fibers isolated from rat leg muscles in the matrices to determine if these provide directional guidance for cellular growth.

Specific aim

As a first step towards developing a bridging transplant therapy for spinal cord injury (SCI), the specific aim of this study is to determine if PBD-MAPCs can be neurally differentiated in a 3D culture system. We also tested the capacity of skeletal muscle fibers embedded in the matrices to provide directional guidance for PBD-MAPC growth.

Hypothesis

Neural differentiation medium and 3D biomatrices of collagen, laminin, matrigel or fibrin will induce primordial PBD-MAPCs into neural cells showing neural morphology with long processes and express neuronal (β-tubulin III, NF-L and NeuN) and glial cell markers (O4, MBP and GFAP). Also skeletal muscle will provide a support for the growth of cellular processes.

MATERIALS AND METHODS

Skeletal muscle isolation

Skeletal muscles were isolated from anesthetized Sprague Dawley rats and dissected into physiological rodent saline (PRS) (138mM NaCl, 2.7mM KCl, 1.8mM $CaCl_2.2H_2O$, 1.06mM

$MgCl_2.6H_2O$, 12.4mM HEPES, 5.6mM glucose, pH adjusted to 7.3). Muscle fibers are enzymatically treated with 0.2% collagenase in PRS and 10%FBS at 37^oC and 5% CO_2 for 90 minutes, with occasional shaking. Muscle fibers are thoroughly teased apart and then centrifuged and resuspended in DMEM with 10% FBS, GlutaMAX, penicillin/streptomycin and Fungizone (Gibco) in a 6 well non-tissue culture plate. Muscle fibers are incubated at 37^oC and 5% CO_2 until use.

3D cell culture

PBD-MAPCs were cultured in neuronal-specific medium (Neurobasal medium (Gibco) with Penicillin/Streptomycin 1%, NEAA 1%, GlutaMAX 1%, B27 1%, N2 1%, Matrigel without phenol red (BD Biosciences) 0.5%, EGF 60ng/ml, bFGF 10 ng/ml, BDNF 10 ng/ml, Retinoic acid 300 ng/ml). We cultured cells in 3D plugs made of collagen type I (Watanabe et al., 2007), laminin (Cultrex), fibrin (Sigma) (Ju et al., 2007) or Matrigel (LaPlaca et al., 2005) for 8 days. Media was changed every two days. For 3D biomatrices with muscle fibers, a collagen-laminin plug was used for culture and skeletal muscle fibers were added to the plug along with PBD-MAPCs.

Immunocytochemistry

After the culture period, the 3D plugs were fixed in 4% paraformaldehyde for 2 hours and embedded in 1.5% agarose containing 5% sucrose. The resulting blocks were cryoprotected in 30% sucrose in PBS at 4^oC until they were equilibrated. The agarose blocks were cryosectioned at 40uM thickness using a Leica CM1950 cryostat and mounted onto Superfrost^{TM} slides (Fisher Scientific). Sections were washed with PBS 8 times, 5 minutes each and were blocked with 10% goat serum in 0.3% PBTX (0.3% Triton X 100 in 0.1M PBS) for 60 minutes. Later they were

again washed with PBS 4 times, 5 minutes each. Sections were incubated in sufficient quantity of primary antibody (mouse anti β-tubulin III monoclonal antibody (Chemicon), mouse anti NeuN monoclonal antibody (Chemicon), Rabbit anti NF-L monoclonal antibody (Cell Signaling), (for neurons), mouse anti GFAP monoclonal antibody (Chemicon) (for astrocytes), mouse anti Oligodendrocyte marker O4 monoclonal antibody (Chemicon) and Rabbit anti Myelin basic protein MBP polyclonal antibody (Chemicon), (for oligodendrocytes) diluted at 1:500 concentrations in 1% goat serum in 0.3% PBTX and incubated in a humidified chamber at 4^{o}C overnight. After thorough washing, sections were incubated with Alexa Fluor conjugated secondary antibodies (Invitrogen) diluted at 1:500 concentrations in 1% goat serum in 0.3% PBTX and incubated in a humidified chamber at room temperature for 2 hours. Cells were washed thoroughly and incubated with 0.02% TOPRO (Invitrogen) for 10 minutes and then mounted in Prolong Gold (Invitrogen) and observed under an epifluorescence microscope. Micrographs were obtained with a Zeiss AxioExplorer epifluorescent microscope using 10x and 20x air interface Neoplan objectives and the accompanying Zeiss image acquisition software. All deconvolution images were obtained with default settings. Brightness adjustments and image cropping was performed in ImageJ (NIH) and figures were assembled using Microsoft Powerpoint.

RESULTS

PBD-MAPCs cultured in a 3D collagen matrix with neural differentiation medium express neural markers.

In this study we cultured PBD-MAPCs in four different biomatrices, collagen type I, a combination of collagen type I and laminin, Matrigel and fibrin. The fibrin plugs were completely disintegrated by day 3, terminating the experiment. All other matrices remained intact throughout the culture period. In most cases cells have long and slender processes indicative of a neuronal morphology.

Immunocytochemistry performed on sections obtained from 3D biomatrices containing PBD-MAPCs (n=3), showed that cells expressed O4, an oligodendrocyte marker (Fig. 4.1A), β-tubulin III (Fig. 4.1B), and NeuN (Fig. 4.1C) both neuronal markers. In cells expressing O4 (Fig. 4.1A), co-localization of both GFP and O4 immunofluorescence is clearly visible in the long processes of the cells (arrows). Differentiated PBD-MAPCs also expressed GFAP, an astrocyte marker (Fig. 4.1 D); MBP, an oligodendrocyte marker, (Fig. 4.1 E) and NF-L, a neuronal marker (Fig. 4.1 F). In cells expressing MBP, GFP and MBP immunofluorescence are clearly co-localized in the long cellular processes (Fig. 4.1 E, arrows).

PBD-MAPCs cultured in a 3D Matrigel matrix express neural markers when cultured in a neural differentiation medium.

PBD-MAPCs cultured within a 3D Matrigel plug developed long and slender processes which were absent in the primordial PBD-MAPCs (n=2). These cells expressed O4 (Fig. 4.2 A), an oligodendrocyte marker and GFAP, an astrocyte marker (Fig. 4.2 B). Some PBD-MAPCs

Figure 4.1

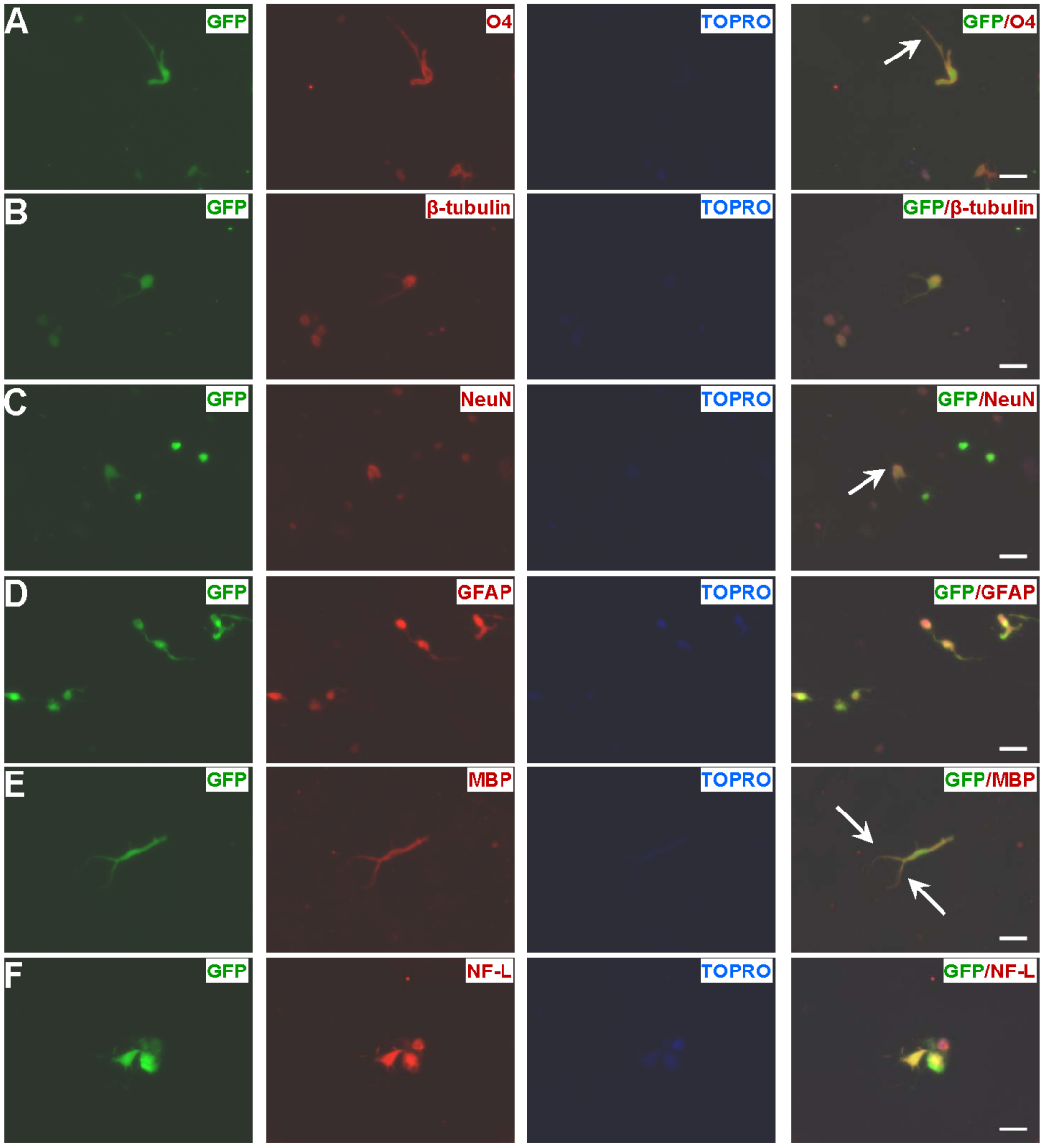

Figure 4.1: PBD-MAPCs cultured in a 3D collagen matrix express neural markers when cultured in a neural differentiation medium. GFP positive (green) PBD-MAPCs, when cultured in a 3D matrix in neural differentiation medium, express O4 an oligodendrocyte marker (red) (arrows) **(A)**, β-tubulin a mature neuron marker (red) **(B)**, NeuN a mature neuron marker (red) (arrow) **(C)**, GFAP an astrocyte marker (red) **(D)**, Myelin basic protein (MBP) an oligodendrocyte marker (red) (arrows) **(E)**, and NF-L a mature neuron marker (red) **(F)**. Merged images show co-localization of both green and red channels. The nuclei were stained with TOPRO (blue). Scale bars: 50 μm

Figure 4.2

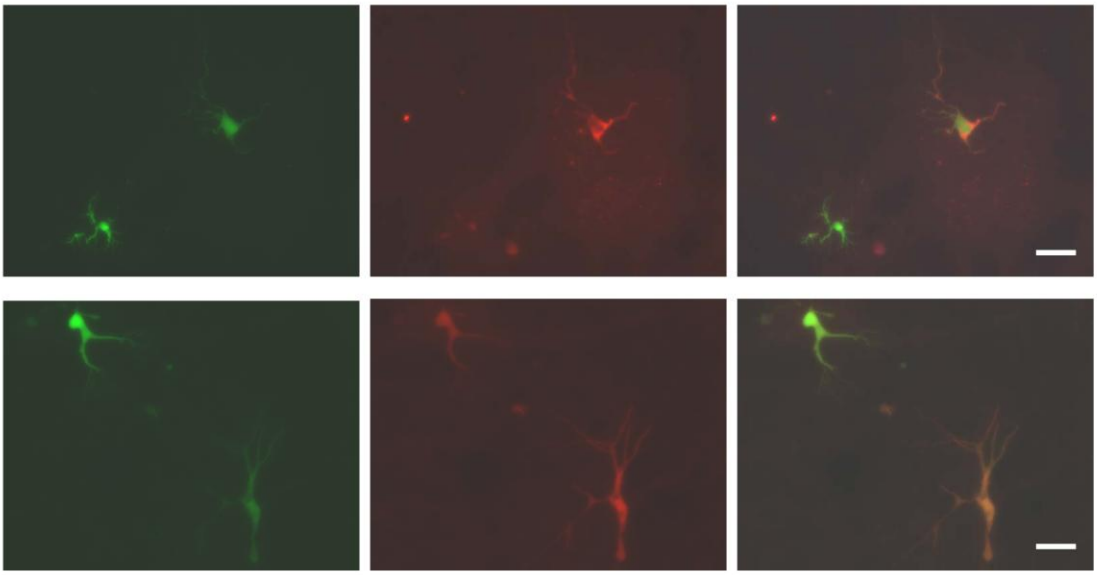

Figure 4.2: PBD-MAPCs cultured in a 3D Matrigel matrix express neural markers when cultured in a neural differentiation medium. GFP positive (green) PBD-MAPCs, when cultured in a 3D matrix in neural differentiation medium, express O4 an oligodendrocyte marker (red) **(A)**, and GFAP an astrocyte marker (red) **(B)**. Some cells express low levels of neural markers (arrowheads) compared to others (arrows). Merged images show co-localization of both green and red channels. The nuclei were stained with TOPRO (blue). Scale bars: 50 μm

Figure 4.3

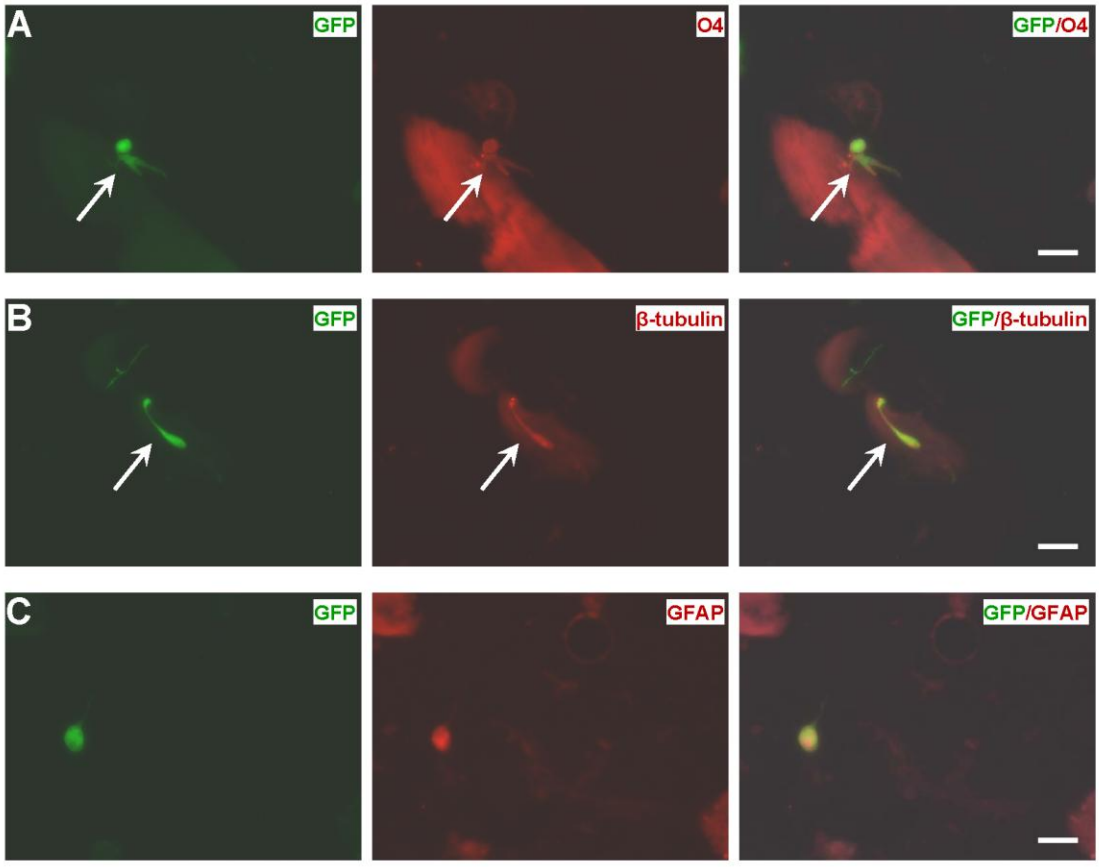

Figure 4.3:. PBD-MAPCs cultured in a 3D collagen-laminin matrix along with muscle fibers grow along muscle fibers and express neural markers when cultured in a neural differentiation medium. GFP positive (green) PBD-MAPCs (arrows), when cultured in a 3D matrix along with muscle fibers (red) grow along muscle fibers (arrows) and express O4 an oligodendrocyte marker (red) **(A)**, β-tubulin a mature neuron markers (red) **(B)** and GFAP a glial cell marker (red) **(C)**. Merged images show co-localization of both green and red channels. The nuclei were stained with TOPRO (blue). Scale bars: 50 μm

expressed low levels of neural markers (Fig. 4.2 B, arrowheads) compared to others (arrows), which were strongly immunoreactive.

PBD-MAPCs cultured with skeletal muscle fibers in a 3D collagen-laminin matrix in a neural differentiation medium grow along muscle fibers and express neural markers.

PBD-MAPCs were found to be growing along the muscle fibers when observed under a microscope (n=4). PBD-MAPCs associated with muscle fibers were immunoreactive to O4 (Fig. 4.3A), an oligodendrocyte marker, β-tubulin III, a neuronal marker (Fig. 4.3 B), and GFAP, an astrocyte marker (Fig. 4.3 C).

DISCUSSION

In this study we used 3D biomatrices to culture and neurally differentiate PBD-MAPCs. Our ultimate goal is to develop a bridging transplant consisting of a plug of differentiated cells that can be implanted at the site of a spinal cord injury. This bridge will replace the glial scar and provide a substrate and cells for regeneration of neural conduction pathways which may lead to regain the lost function.

PBD-MAPCs could not be cultured in fibrin as the fibrin plugs disintegrated. This may be because we did not include protease inhibitors which would prevent degradation of fibrin by the enzymes secreted from cells (Sieminski and Gooch, 2004; Kang et al., 2005; Willerth et al., 2006). Collagen has been reported to be a biomaterial of low immunogenicity and it supports neural cell growth (O'Connor et al., 2000). PBD-MAPCs cultured in a 3D collagen biomatrix developed long and slender processes which were absent in the primordial PBD-MAPCs clearly indicating morphological changes due to differentiation, cells also expressed neural proteins (O4,

β-tubulin III, NeuN, GFAP, MBP and NF-L) after differentiation. A similar result was reported with neural stem/precursor cells (NSPCs) from rat embryos in a collagen type I biomatrix, where NSPCs differentiated into neurons, astrocytes and oligodendrocytes (Watanabe et al., 2007). Neural differentiation was also reported for rhesus monkey embryonic stem cells with a collagen type I biomaterial (Chen et al., 2003; Michelini et al., 2006). Laminin was reported to be an important component of the extracellular matrix and is important for proper brain development (Brannvall et al., 2007). Differentiation of murine neural stem cells into neurons has also been achieved using amphiphillic nanofibers functionalized with the laminin-derived peptide IKVAV as a scaffold (Silva et al., 2004).

When PBD-MAPCs were cultured in a 3D biomatrix with muscle fibers, cells grew along the muscle fibers. The muscle fibers appeared to act as a scaffold and provided a sort of directional guidance to the growth and extension of cellular processes. Muscle grafts have been used in repair of peripheral nerve damage (Roganovic et al., 2007) and these experiments are the first steps in developing a similar approach to therapy of spinal cord injury. Skeletal muscles are an optimal addition to the 3D biomatrix bridging transplant because they provide a longitudinally oriented basal lamina and extracellular matrix components that direct and enhance regeneration of nerve fibers (Meek et al., 2004). These preliminary data indicate that PBD-MAPCs do indeed differentiate into neural lineages and grow towards and along muscle fibers in 3D biomatrix cultures. Future experiments will determine if the neuralized cells can generate and transmit action potentials, which are a key physiological function of neurons (Ban et al., 2007). Finally the data supports our hypothesis that primordial PBD-MAPCs can differentiate into neuronal cells *in vitro* in 3D biomatrices and express neuronal (β-tubulin III, NF-L and NeuN) and glial cell markers (O4, MBP and GFAP). Also, on differentiation, cells showed growth along

skeletal muscle fibers. These results indicate that a bridging transplant can be developed with PBD-MAPCs and skeletal muscle fibers embedded in biomaterials act as a scaffold and allow growth of cellular processes. Studying the possible muscle to cellular process contacts can be a potential future study to decipher the actual cell-cell interactions.

CHAPTER 5

DISCUSSION

In view of our specific aims we could show that PBD-MAPCs do not differentiate into a cardiac lineage in the present culture conditions. We were also able to show that PBD-MAPCs express the stem cell marker CD133 and are stem cells; we also demonstrated that undifferentiated PBD-MAPCs express neural markers like TH, β-tubulin III and PGP9.5. Furthermore we established that PBD-MAPCs lose expression of the stem cell marker CD133 upon differentiation. In addition, for the first time, we demonstrated neural differentiation of PBD-MAPCs in a 3D biomatrix and that PBD-MAPCs grow along muscle fibers when cultured with skeletal muscle fibers. Because of these results PBD-MAPCs are an interesting population of stem cells and show a great amount of promise for future research in spinal cord injuries. They might show cardiac differentiation with different factors and extra cellular matrices (ECM). Also, as PBD-MAPCs have been shown to differentiate into many cell types, there might still be a scope to achieve cardiac differentiation with these cells under optimal conditions which can be a promising future study.

Our attempts to differentiate PBD-MAPCs towards a cardiac lineage, regardless of the differentiation protocols used with various cytokines and co-culture, were not successful. PBD-MAPCs in culture perhaps need additional specific factors that may play a crucial role in cardiomyogenesis. Injecting PBD-MAPCs into an ischemic rat heart (Van't Hof et al., 2007), to check whether cardiac milieu induces differentiation of PBD-MAPCs towards a cardiac lineage, may help to identify additional signaling molecules required for cardiac differentiation. Future experiments could also be directed towards testing additional cytokines which have been reported to cause cardiac differentiation in stem cells such as 3,5,3’-Triiodo-L-Thyronine (T3) in

embryonal carcinoma cell line P19 (Rodriguez et al., 1994), Trichostatin A in monkey embryonic stem cells (Hosseinkhani et al., 2007). Finally, in the present study we used only gelatin and collagen as ECM in cardiac differentiation culture. It was reported in embryonic stem cells that interaction of cells with the ECM via integrins determines differentiation into mesodermal and neuroectodermal lineages. Also it was reported that loss of β1 integrin function resulted in retardation of cardiac differentiation (Czyz and Wobus, 2001). It was also reported that interactions of cells with the ECM are critical for the establishment and maintenance of stem cell self-renewal and differentiation. In a study on human embryonic stem cells (hESCs) cultured on Poly-D-Lysine (PDL), PDL/fibronectin, PDL/laminin, type I collagen and Matrigel, it was shown that laminin is a key ECM molecule and enhances neural progenitor generation, expansion and differentiation into neurons from hESCs (Ma et al., 2008). Future studies addressing the use of different ECMs would be useful in furthering our understanding of the effects of ECM and cardiac differentiation of PBD-MAPCs.

In this study involving a novel type of stem cell population, we showed that PBD-MAPCs, like many other stem cells, express CD133, a stem cell marker. Previously, it was reported that PBD-MAPCs differentiate into endothelial, smooth muscle, osteocyte, adipocyte and neuron like cells (Price et al., 2006). Hence we strongly conclude that PBD-MAPCs are stem cells and are multipotent in nature and can differentiate into various lineages when proper culture conditions are provided. Furthermore, since we found undifferentiated PBD-MAPCs to be expressing some neural markers (TH, β-tubulin III and PGP9.5) this supports the theory of nonspecific gene expression in PBD-MAPCs. Primordial cells may therefore simply express various proteins nonspecifically until they receive differentiation signals (Egusa et al., 2005).

However, expression of specific markers for other lineages like smooth muscle, osteocyte and adipocytes in undifferentiated cells was not tested. Future studies to test expression of additional stem cell markers by PBD-MAPCs may also aid in characterizing these cells. Future research could also be directed towards isolating a comparable type of peripheral blood derived multipotent adult progenitor cells from humans and to study its expression profile. This may lead to autologous stem cell therapies. In this study we also showed that PBD-MAPCs can be differentiated into a neural lineage and they lose expression of stem cell markers after undergoing neural differentiation.

Additionally in this study we have demonstrated for the first time that PBD-MAPCs, when cultured in a 3D biomatrix, differentiate into neural cells with slender processes that are typical to a neural morphology. Also we were able to show that PBD-MAPCs in a 3D biomatrix with muscle fibers grow along the fibers, supporting the idea that muscles can provide directionality to the growing cellular processes. These are preliminary data towards the development of a novel transplant therapy for spinal cord injury. Potential future experiments should be aimed at examining the ability of differentiated cells to generate and transmit action potentials, which is a key physiological function of neurons (Ban et al., 2007; Bjorklund et al., 2002; Benninger et al., 2003). When functional, active neurons can be generated from PBD-MAPCs, research should be aimed at the development of a bridging transplant for spinal cord injury. The development of a bridging transplant can eventually be tested in a rat model of spinal cord injury where the extent of regeneration of neuronal functions can be studied. In conclusion, PBD-MAPCs are an easily accessible non-controversial population of stem cells from adult porcine blood. In view of our findings that they are multipotent (Price et al., 2006) and can

differentiate into neural lineages in a 3D culture, these cells can be a promising stem cell population for further investigations in the development of cellular therapies for spinal cord injury.

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