Establishment of Methods for Isolation of Pnmt+ Cardiac Progenitor Cells

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ESTABLISHMENT OF METHODS FOR ISOLATION OF CARDIAC PROGENITOR CELLS

by

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ABSTRACT

Cardiovascular disease is the leading cause of death in the United States. Millions of patients suffer each year from endothelial dysfunction and/or debilitating myocardial damage resulting in decreased quality of life and increased risk of death or disablement. Current pharmacological approaches are only partly effective at treating cardiovascular disease, and hence, better strategies are needed to provide significant improvements in treatment options. Cardiac stem/progenitor cells have the potential to regenerate myocardial tissue and repair damaged heart muscle. There are many different types of cardiac progenitor cells, and each may have certain unique properties and characteristics that would likely be useful for particular clinical applications. A current challenge in the field is to identify, isolate, and test specific cardiac stem/progenitor cell populations for their ability to repair/regenerate myocardial tissue. Our laboratory has discovered a new type of cardiac progenitor cell that expresses the enzyme, Phenylethanolamine-n-methyltransferase (Pnmt). My initial studies focused on identification of Pnmt+ cells based on knock-in of a nuclear-localized Enhanced Green Fluorescent Protein (nEGFP) reporter gene into exon 1 of the Pnmt gene in a stable recombinant Pnmt-nEGFP mouse embryonic stem cell (mESC) line. These cells were differentiated into cardiomyocytes, and I identified nEGFP+ cells using fluorescence, immunofluorescence, and phase-contrast microscopy techniques. Our results showed that only about 0.025% (1 per 4000) of the cardiac-differentiating stem cells expressed the nEGFP+ marker. Because of the relative rarity of these cells, optimization of isolation methods proved initially challenging. To overcome this technical barrier, I used a surrogate cell culture system to establish the methods of isolation based on expression of either a fluorescent cell marker (EGFP), or a unique cell surface receptor represented by an inactivated (truncated) version of the human low-affinity nerve growth factor receptor (LNGFR). Plasmid DNA containing these reporter genes was transiently transfected into a permissive cell line (RS1), and reporter gene expression was used to identify

and isolate transfected from non-transfected cells using either Fluorescence-Activated Cell Sorting (FACS) or Magnetic-Activated Cell Sorting (MACS) methods. The main objective of the study was to establish the isolation techniques based on the expression of reporter genes (EGFP and LNGFR) in RS1 cells. Following transfection, EGFP+ cells were successfully isolated via FACS as verified by flow cytometric and microscopic analyses, which showed that approximately 96% of the isolated cells were indeed EGFP+. Despite the relative purity of the isolated cell population, however, their viability in culture following FACS was substantially compromised (50% attrition). In contrast, MACS enabled efficient isolation of LNGFR+ cells, and the vast majority of these (90%) retained viability in culture following MACS. The LNGFR expression was verified using RT-PCR. Further, MACS methods enabled isolation of marked cells in about 5-7 mins, whereas it took 2-4 hours to using FACS to perform similar isolations from the same amount of starting material (10^6 cells). In addition, MACS is a more economical method in that it does not require the use of an expensive laser-based instrument to perform the sorting. These results suggest that MACS was a more efficient, gentle, and feasible technique than FACS for isolation of reporter-tagged mammalian cells. Consequently, future studies aimed at isolation of Pnmt+ cardiac progenitor cells will thus primarily focus on MACS methods.

I dedicate this work to my brother who has been the support and guidance in my life. He has made me who I am today. I express my gratitude towards my parents who have been my strength throughout my life.

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CHAPTER 1: INTRODUCTION

Cardiovascular disease is the leading cause of death in the world. Cardiac tissue is damaged due to several cardiac diseases such as myocardial infarction, ischemia, heart failure etc. The inability of the cardiac tissue to regenerate itself has encouraged various researchers to study the processes leading to cardiac regeneration since organ transplantations have their limitations with availability of the organ and or rejection.

Stem cell therapy seems to be showing a promising potential in the area of cardiac regeneration. Stem cells have the ability to get differentiated into cardiomyocytes and hence they can be transplanted into the damaged cardiac tissue [1, 3, 9, 2, 4]. Several tissue-specific markers have been used which help in identification, isolation and characterization of differentiated stem cells such as neurons, muscle cells, endothelial cells [9, 11, 12, 13, 14, 15, 5]. Some of the key markers used as cardiac markers are Wnt11, wnt7a, Id2, Nkx2.5, Tbx5 [15, 16, 17, 6, 10, 8, 7]. One such unique cardiac marker used in differentiating stem cells into cardiomyocytes is the enzyme Phenylethanolamine n-methyl transferase (Pnmt). Pnmt is one of the enzymes in the biosynthetic pathway of the adrenergic hormones which helps in conversion of noradrenaline to adrenaline. It serves as an important cardiac marker as the adrenergic system is found to play a key role in the development of early embryonic heart [18]. A considerable number of cardiac cells are found to express this enzyme in the developing and adult heart [23].

Adrenaline is an important hormone and neurotransmitter which plays a pivotal role in the fight or flight system of the body. It also performs various physiological functions. The adrenergic hormones were identified in the early embryonic development of chick heart. Huang et al identified a specific population of cells in the fetal mammalian heart called the Intrinsic Cardiac Adrenergic Cells (ICA cells) capable of producing adrenergic hormones in vitro [20]. They showed that these

adrenergic cells were present in the heart even before the sympathetic innnervation of the heart.

One might expect Pnmt to be primarily expressed in adrenal gland primarily during embryonic development as this is the major site of expression in the adult. However, this was not the case. Ebert et al showed that Pnmt is expressed in the embryonic rat heart during embryonic day E11.0 and E18 [18]. E11 is the time when the embryonic heart develops in the rat whereas during E18 adrenal gland is in the process of development. This shows that Pnmt is expressed in the heart before the onset of adrenal gland development. This leads us to a hypothesis that these Pnmt+ cells are useful in cardiogenesis of the heart during early embryonic development.

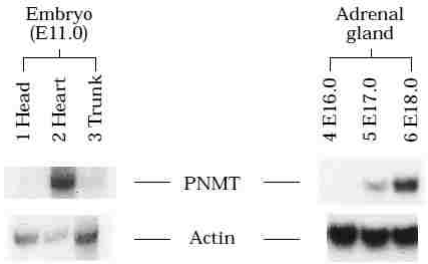

Figure 1.1: PNMT mRNA analysis of embryonic tissue and adrenal gland samples [18].

RNase protection assays were performed using total RNA samples from the head, heart, and trunk regions of E11.0 rat embryos (lanes 1-3, respectively). In addition, total RNA adrenal glands at E16.0, E17.0, and E18.0 was analysed (lanes 4-6, respectively). The amount of β- actin mRNA present in each sample is also shown. Figure reprinted from [18].

The expression of Pnmt is found in the developing heart specifically in the SA node of the embryonic heart. This expression was seen during embryonic day 11.5. Figure 1.2 shows Pnmt+ cells identified using the Pnmt-Cre/R26R mouse model [21]. These cells contribute to the conduction and pacemaking system of the heart in adults. Further analysis of these cells showed that these adrenergic-derived cells were found to be concentrated on the left region of the heart specifically to the left atrium as well as near the base and the midline of the left ventricle [23, 24, 19]. These cells were positive for sarcomeric -actinin which is a marker for myocytes.

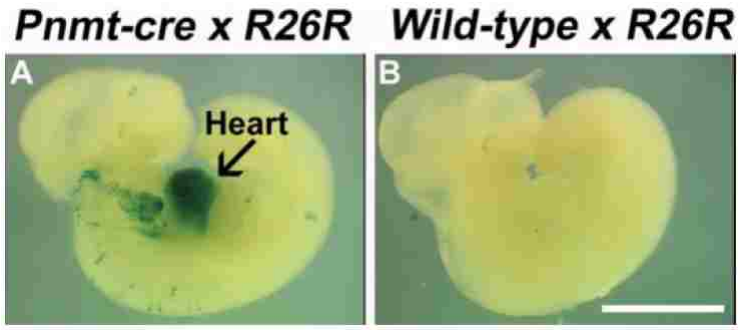

Figure 1.2: Whole-mount XGAL staining of embryonic day E10.5 Pnmt-Cre/R26R embryo.

A: LacZ expression seen in heart, brainstem region, 2nd branchial arch and sporadically along the dorsal surface of the embryo **B**: Wild Type mice crossed with R26R mice. Figure reprinted from [21].

The above data suggests that the stem cells expressing Pnmt could serve as promising candidates for regenerative applications of the heart. Mouse embryonic stem cell lines which are adrenergic-derived, expressing Pnmt as a marker along with additional markers is a novel way for efficient identification, isolation, and characterization of stem cell generated cardiomyocytes that could be used in regenerative therapies for damaged cardiac tissue.

In this study, we discuss two different mouse embryonic stem cell (mESC) lines which shall be used for generating effective cardiac progenitor cells. The two cells lines are Pnmt-nEGFP (nuclear enhanced green fluorescent protein)and Pnmt-Δ LNGFR (low affinity nerve growth factor receptor). My initial objective was to differentiate these mESCs into Pnmt$^+$ cardiac progenitor cells, isolate and study the characteristics of these cardiomyocytes.

My thesis focuses mainly on establishing the techniques for isolation of these Pnmt+ cardiac progenitor cells. We hypothesize that:

Fluorescence-Activated Cell Sorting (FACS) and Magentic-ACtivated Cell Sorting (MACS) are effective techniques for isolation of Pnmt$^+$ cardiac progenitor cells.

We have the following aims to test the above stated hypothesis:

1. To isolate enhanced green fluorescent (EGFP$^+$) cells using fluorescent activated cell sorting (FACS) technique.

In this step we will try to differentiate the Pnmt-nEGFP mESCs into beating cardiomyocytes and verify Pnmt expression in them and analyze as well as identify the number of nEGFP$^+$ cells. To establish the FACS technique we will used the adrenergic-derived RS1 cells as the surrogate cells and transfect with the EGFP plasmid. These EGFP$^+$ cells were then verified using laser-scanning confocal microscope and will be isolated using FACS technique.

2. To identify and isolate low-affinity nerve growth factor receptor ($LNGFR^+$) cells using magnetic activated cells sorting (MACS) techniques.

For this particular aim we utilize the PnmtΔ LNGFR cell line. The first step is to characterize these mESCs by genomic screening to verify proper insertion in the mouse genome. The second step is to establish the MACS technique of isolation where we shall be utilizing the RS1 cells and transfect it with pMACS LNGFR. These $LNGFR^+$ cells will be isolated using the MACS technique and LNGFR expression will be verified by RT-PCR analysis.

CHAPTER 2: ISOLATION OF nEGFP+ CELLS USING FACS

2.1 Introduction

The purpose of this chapter was to isolate nuclear enhanced green fluorescent protein (nEGFP+) cells using fluorescent activated cell sorting technique. The Pnmt-nEGFP mouse model was made by inserting the GFP gene in exon 1 of the Pnmt gene locus [25]. Figure 2.1 is the vector diagram for the Pnmt-nEGFP mouse model. The Pnmt gene encodes for Pnmt enzyme which helps in conversion of nor- adrenaline to adrenaline in the catecholamine synthetic pathway. The GFP gene was tagged with an NLS nuclear localization sequence so as to improve the sensitivity and detection of GFP expression in Pnmt+ cells [25]. The nEGFP gene was targeted under the regulatory sequences of Pnmt gene so as to co-localize the nEGFP expression with that of the Pnmt expression [25]. Figure 2.2 shows nEGFP expression in the adult adrenal glands isolated from the mouse model. The Pnmt-nEGFP mouse model was characterized by verifying the co-localization of the Pnmt expression in the chromaffin cells of the adult adrenal medullary region in the Pnmt$^{+/nEGFP}$ mice [25]. The nuclear localized expression of the EGFP protein was confirmed by co-staining the adrenal sections with Pnmt IF staining as seen in figure 2.3 [25]. EGFP expression was also seen in the atrio-ventricular sections of the adult heart in this mouse model to confirm that this mouse model successfully tracks the adrenergic or Pnmt expressing cells [25]. Our main aim was to isolate Pnmt+ cardiomyocyte progenitor cells from the mouse embryonic stem cells, but due to some technical difficulties it was hard to differentiate the mESCs into beating cardiomyocytes. Hence, we focused on getting the methods of isolation worked out so as to facilitate the isolation of differentiated cardiomyocytes.

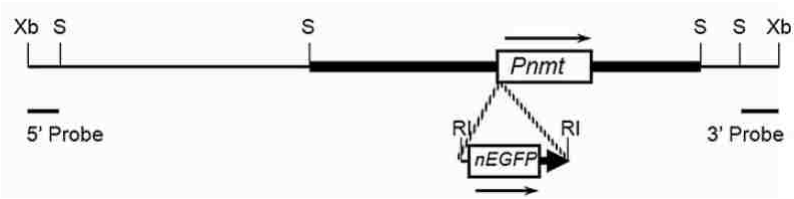

Figure 2.1: Pnmt GFP alleles. Reprinted from [25]

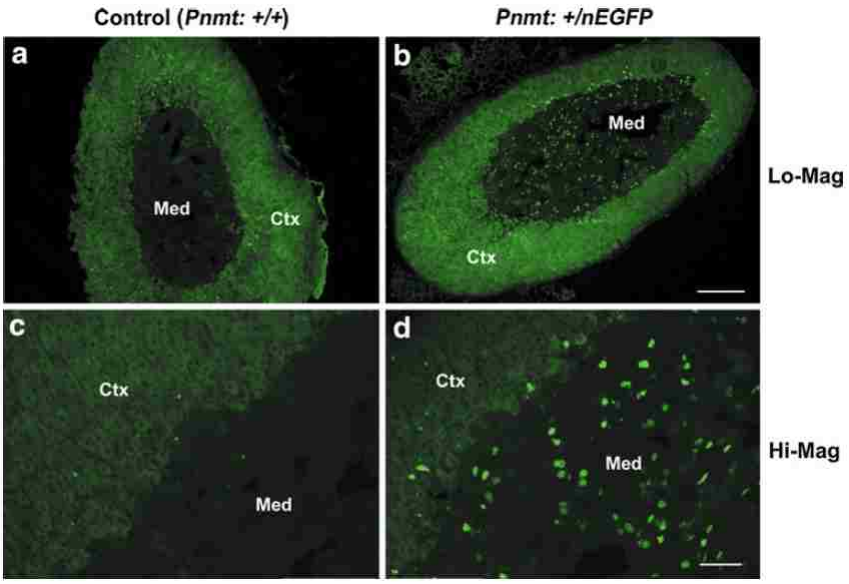

Figure 2.2: nEGFP expression in mouse adrenal glands

a,b Low magnification views of fluorescent imaging of adrenal gland sections from wild type control and Pnmt$^{+/nEGFP}$ mice, respectively. Scale bar, 100μm c,d higher magnification of same image. Scale bar, 50μm Ctx cortex, Med medulla. Reprinted from [25].

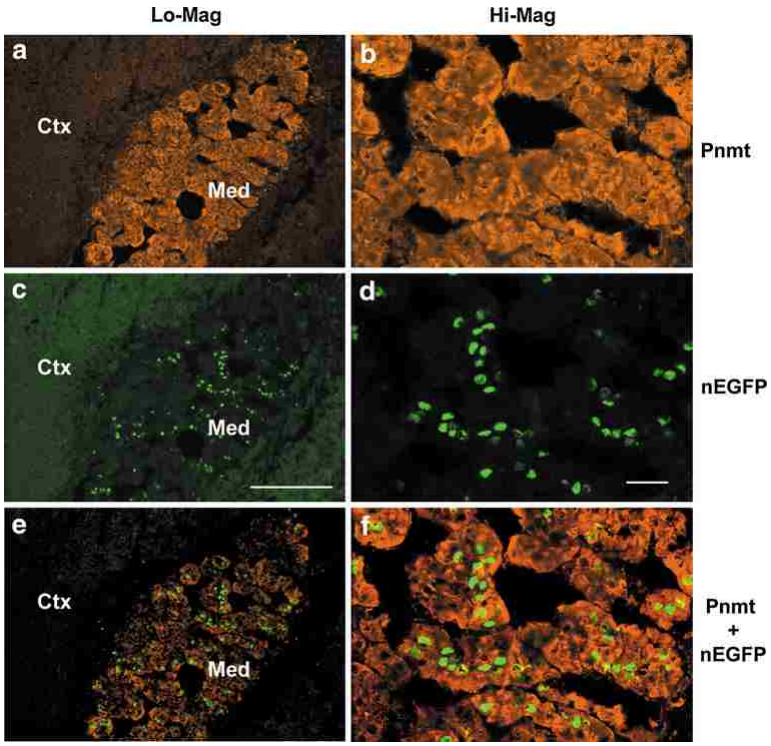

Figure 2.3: Identification of nEGFP and endogenous Pnmt in adrenal chromaffin cells.

a,b Pnmt IF histochemical staining in adult mouse adrenal gland sections as visualized for red fluorescence c,d EGFP expression in the same adrenal sections but visualized for green fluorescence (GFP filter) e,f Overlay of Pnmt and nEGFP staining for each section a,c, e Lo-mag low-magnification: scale bar, 200 μm. b, d, f Hi-Mag high-magnification: scale bar, 30 μm. Reprinted from [25].

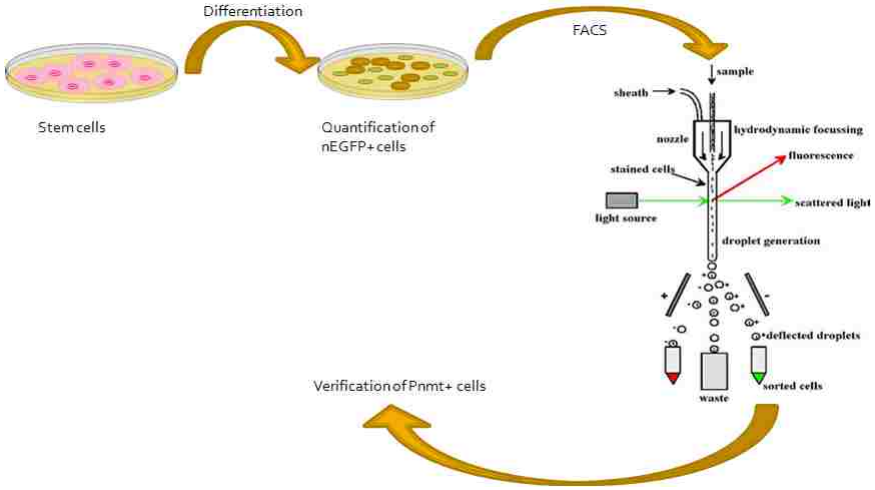

Figure 2.4: Scheme for isolation of Pnmt+ progenitor cells via FACS

The mESCs will be differentiated into cardiomyocytes via hanging drop method. The nEGFP+ cells in the differentiated cardiac progenitor cells will be identified and quantified. In the next step, differentiated cardiomyocytes were sorted via FACS and analysed for Pnmt expression at the end. http://missinglink.ucsf.edu/lm/molecularmethods/flow.htm

2.2 Material and Methods

2.2.1 Materials

Anti-GFP rabbit antibody and Alexafluor 594-conjugated donkey anti-rabbit secondary antibody were from Invitrogen, Inc. (Carlsbad, CA) Vectashield mounting medium for fluorescence with DAPI were from Vector Laboratories (Burlingame, CA). All the media cell culture media (Dulbecco's Modified Eagle Medium) and supplements were obtained from Invitogen (Carlsbad, CA). Fetal bovine serum was obtained from Hyclone (Logan, UT). BD Sciences FACS Aria Sorter was used for sorting of cells by FACS. FBS used for MEF was from Atlanta Biologicals (Flowery Branch, GA). All the other ingredients and reagents used for the experiments were and Sigma Aldrich (St. Louis, MO).

2.2.2 Methods

2.2.2.1 Identification of nEGFP+ cells

The Pnmt-nEGFP mESCs were differentiated using the hanging drop method. During 7+5d the differentiated cardiomyocytes were stained. The cells were grown on 8 chambered glass dishes. The cells were rehydrated with PBS for 20 mins followed by incubation with blocking solution (0.3% Triton X-100, 5% powdered nonfat dry milk, and 0.02% sodium azide in PBS) for 20-30 mins at room temperature. The blocking solution was replaced with blocking solution containing primary antibody (anti-GFP rabbit antibody) with dilution of 1:100. Cells were incubated with primary antibody for 2 hrs at room temperature and then at $4\,^{\circ}\mathrm{C}$ overnight. After incubation the cells were washed thrice with PBS and stained with Alexafluor 594 conjugated donkey anti-rabbit secondary antibody in a dilution of 1:200 for 2 hrs. The cells were given successive washes of PBS three times, and were mounted using Vectashield mounting medium with DAPI. The images were captured using 63X objective of Perkin Elmer laser scanning confocal miscroscope. The controls were stained the same way as described above.

2.2.2.2 RS1 cells transfected with EGFP plasmid

The RS1 cells were transfected with EGFP plasmid using lipofectamine 3000. 30μg of plasmid DNA was used for a 100mm dish, 43.4μl of lipofectamine and 60μl of p3000 (enhancer reagent). The media was changed after 4 hours. The cells were harvested about 48 hrs after transfection and isolated using FACS technique.

2.2.2.3 FACS of RS1 cells transfected with EGFP plasmid

The RS1 cells were harvested 48hrs after transfection and were centrifuged twice and passed through a 75μm cell strainer to ensure single cell suspension. The cells were washed with PBS and re-suspended in flow buffer (1% FBS, 2mM EDTA, PBS)

2.2.2.4 Microscopic analysis

All the images were captured using 63x objective on the Perkin Elmer laser scanning confocal microscope. The lasers used for capturing images were 488nm for GFP, 561nm for RFP and 405nm for DAPI.

2.3 Results

2.3.1 Identification and quantification of nEGFP+ cells in differentiated beating cardiomyocytes

We used the pluripotent Pnmt-nEGFP mESCs and differentiated them into cardiomyocytes by the hanging drop method to verify EGFP expression in them. The nEGFP expression was seen at 7+5d in small clusters of cells. The images were captured in red filter set to avoid any disturbance of autofluorescence. Figure 2.5 shows nEGFP expression in differentiated cardiomyocytes co-localized with anti-GFP red fluorescence and stained with DAPI to identify the nuclei. If observed carefully, only 2-3 cells are fluorescently labeled out of the cluster of cells. We counted the number of nEGFP+ cells out of the total 72,000 cells analyzed and found only 18 of them showed nEGFP expression or representing 0.025% of the total number of differentiated mESCs. The controls for these experiments were the undifferentiated pluripotent mESCs. As seen in figure 2.6 there was no nEGFP expression nor red fluorescence in the pluripotent mESCs.

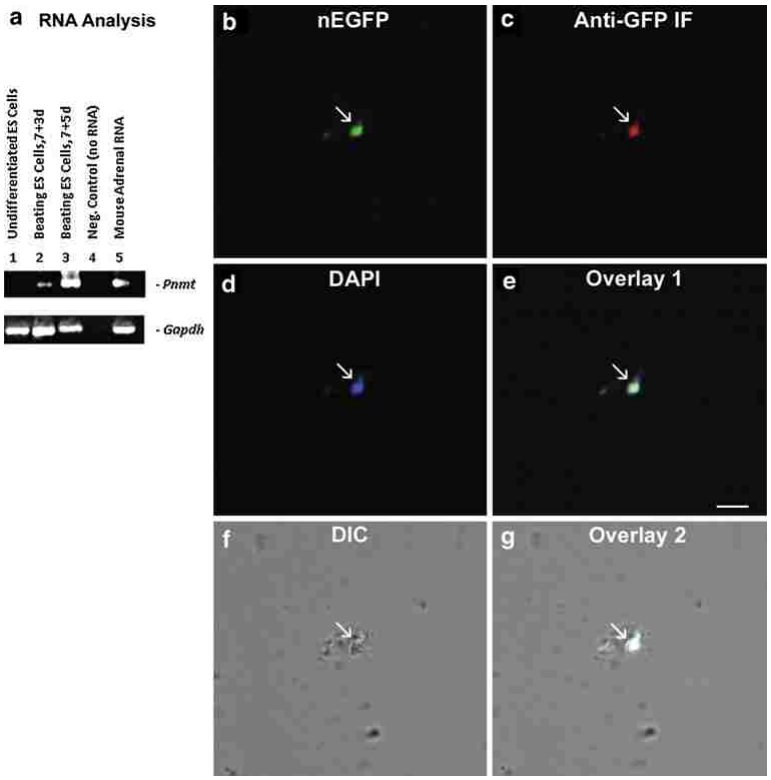

Figure 2.5: Identification of nEGFP$^+$ cells in differentiated cardiomyocytes

(a) Endogenous mouse Pnmt gene expression as detected by RT-PCR before and after induction of cardiac differentiation in mESCs. This procedure takes (7+5d) days. b-g Images of nEGFP$^+$ cells in 7+5d cultures of cardiacdifferentiated mESCs as visualized in green fluorescence for nEGFP (b), red fluorescence for anti-GFP IF (c), blue fluorescence for DAPI (d),Overlay of bd images (e), DIC (f), and overlay of bd plus f (g). Arrow indicates the same positively stained mESC in bg. Scale bar, 10 μm. Reprinted from [25].

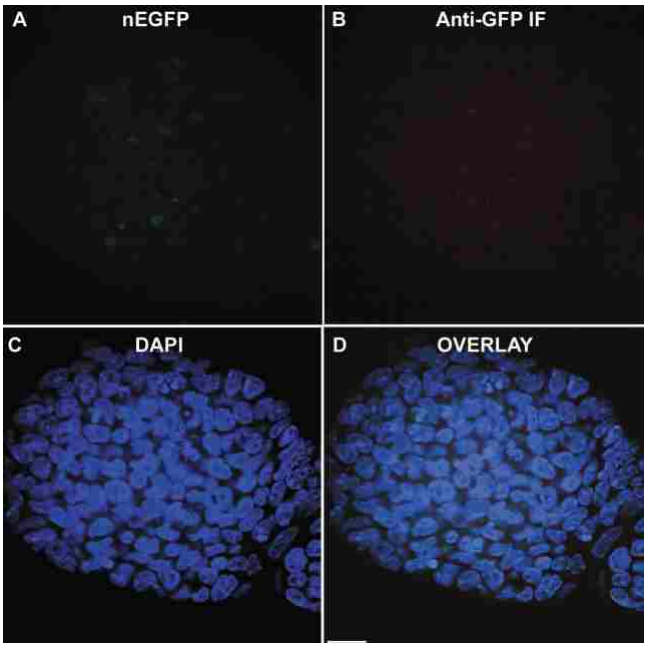

Figure 2.6: Fluorescence expression and staining in control undifferentiated mESCs

(A) nEGFP expression (green). (B) Anti-GFP IF staining (red). (C) DAPI nuclear stain (blue). (D) Overlay of images obtained in panels A-C. Scale bar, 10 μm. Reprinted from [25].

2.3.2 Sorting of EGFP+ cells by FACS

RS1 cells are a type of pheochromocytoma derived adrenal medullary cells that are used as surrogate cells to establish the methods for isolation of [37]. The RS1 cells were transfected with EGFP plasmid and sorted by FACS Aria sorter. We analyzed the percent of EGFP+ cells before sort and found 6.22% of the population were positive for GFP as seen in the dot plot figure 2.7. The cells were sorted, and both the positive and negative fractions of the cells were collected. We did a flow analysis on the positive and negative fractions of the cells to verify efficiency of the method. As seen in the figure 2.7, 92% of the cells were EGFP+ in the positive fraction and absolutely none were EGFP+ when the negative fraction was analyzed. The controls for the experiment were transfected with (no DNA) lipofectamine and analyzed by flow cytometry. The results showed none of them were positive for EGFP expression. The same experiment was repeated two more times. Figure 2.8 shows that during the second trial there were 1.97% EGFP+ cells but none were positive in the controls. Post sorting approximately 93.7% of the positive fraction showed green fluorescence whereas 3.77% were EGFP+ in the negative fraction. During the third trial 2.25% cells were EGFP+ whereas 0.013% of the controls showed EGFP fluorescence. Post sorting the flow cytometry results showed 95.8% cells were green for the positive fraction whereas about 0.60% cells were showing green fluorescence in the negative fraction.

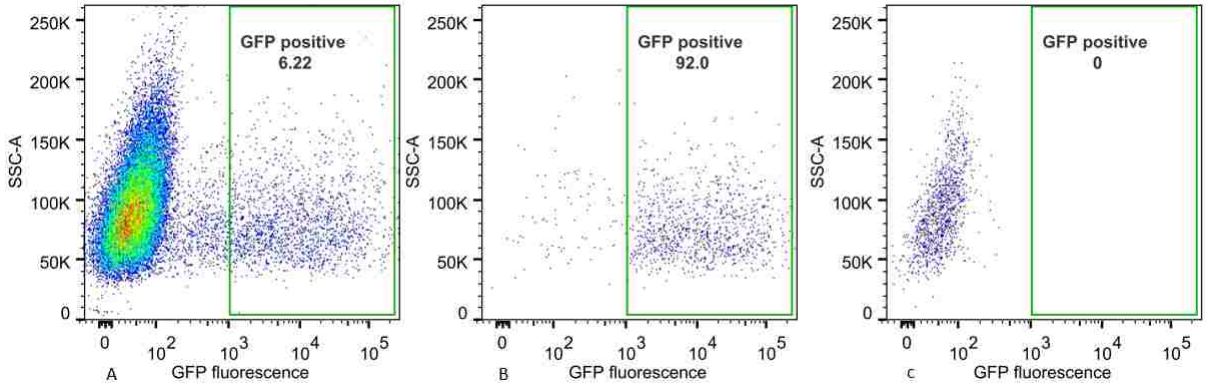

Figure 2.7: Dot Plots of RS1 cells (EGFP transfected) Pre and Post sort

Dot plots of the FACS results. A, Presort results. 6.22% of the total RS1 cells were GFP+. The left gate represents negative population. B,C Post sorting flow cytometric results. 92% were GFP+ in the positive fraction and none were positive in the negative fraction.

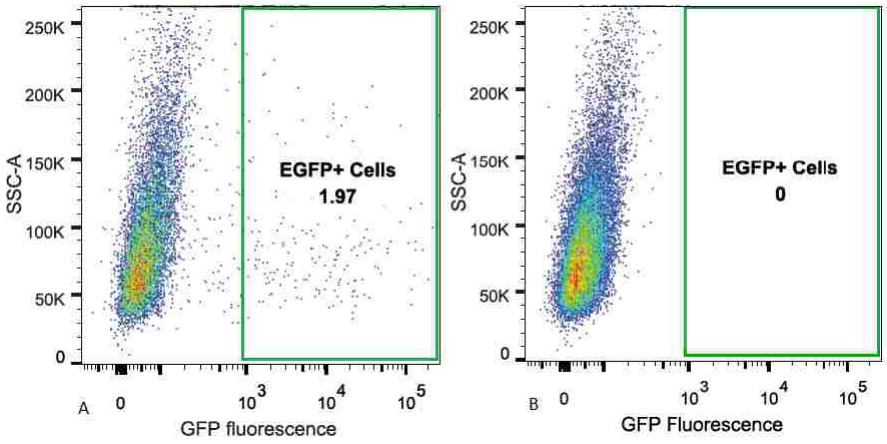

Figure 2.8: FACS(2) Dot Plots of RS1 cells (EGFP transfected) Pre sort

Dot plots of the 2nd trial FACS results. A,B Presort results. 1.97% of the total RS1 cells were GFP+ in A. The left gate represents negative population. B Presort results for controls show no EGFP+ cells

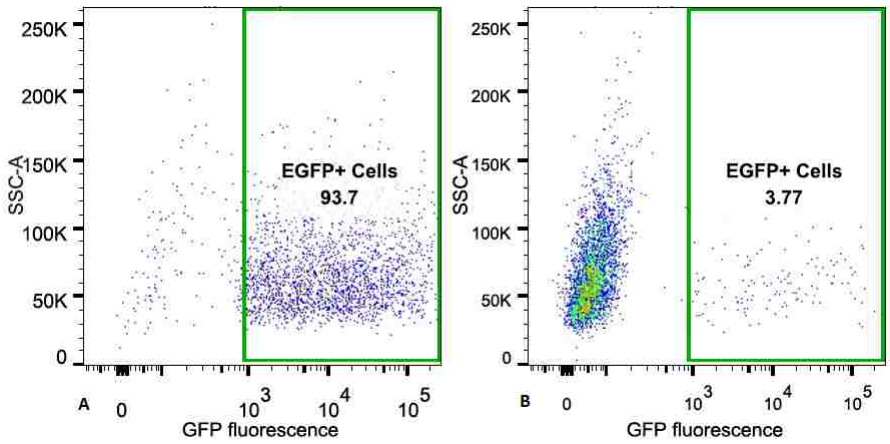

Figure 2.9: Dot Plots of RS1 cells (EGFP transfected) Post sort

A Dot plot of positive fraction shows 93.7% EGFP+ cells. B Representative dot plot of negative fraction shows 3.77% EGFP+ cells.

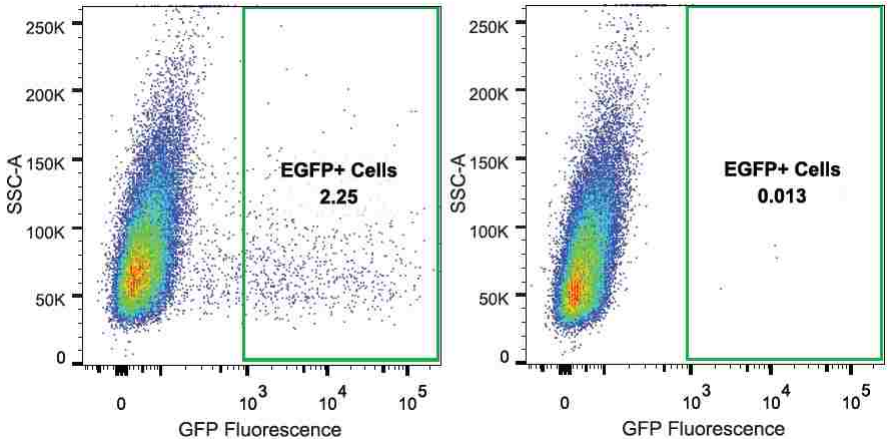

Figure 2.10: FACS(2) Dot Plots of RS1 cells (EGFP transfected) Pre sort

Dot plots of the 3rd trial FACS results. A,B Presort results. 2.25% of the total RS1 cells were GFP+ in A. The left gate represents negative population. B Presort results for controls show 0.013% EGFP+ cells

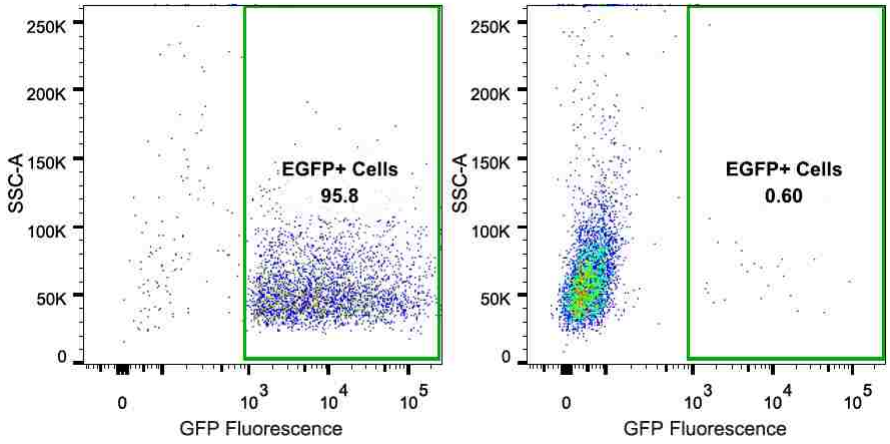

Figure 2.11: FACS (3) Dot Plots of RS1 cells (EGFP transfected) Post sort

A Representative dot plot of positive fraction shows 95.8% EGFP+ cells. B Representative dot plot of negative fraction shows 0.6% EGFP+ cells.

2.3.3 Microscopic analysis of RS1 cells

The RS1 cells transfected with EGFP plasmid were observed under 63X objective of confocal microscope pre and post sort. The cells were stained DAPI vectashield. As observed in figures 2.12 and 2.13 there were EGFP+ cells in pre sort and post sort. The controls for the experiment transfected with just lipofectamine (no DNA) showed no EGFP expression as seen in 2.15.

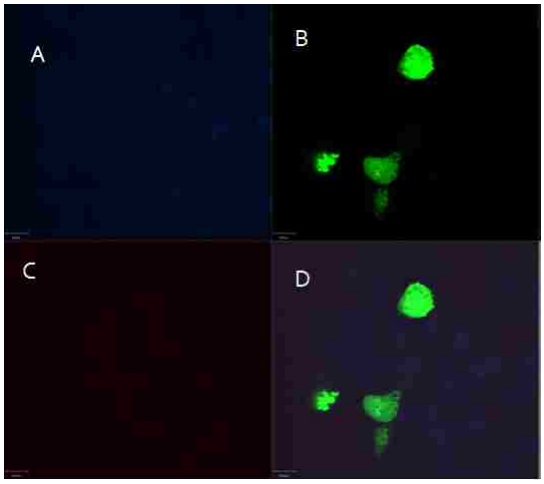

Figure 2.12: Microscopic Analysis of EGFP+ cells FACS presort

A: DAPI blue nuclear fluorescence, B: GFP fluorescence (green), C: RFP, no red fluorescence , D: overlay of panels A-C Scale Bar, 21 μm.

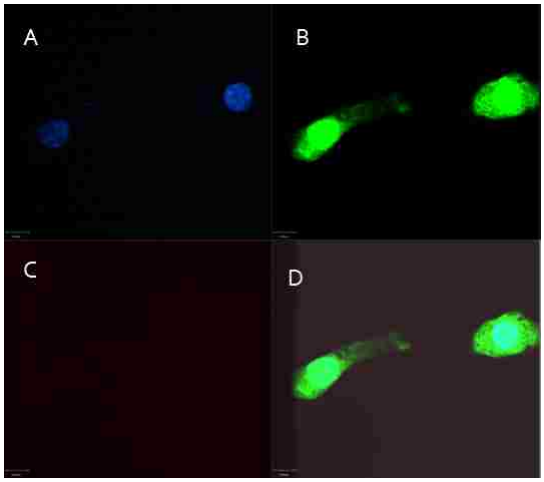

Figure 2.13: Microscopic Analysis of EGFP+ cells FACS postsort

Positive fraction, A: DAPI blue nuclear fluorescence, B: GFP fluorescence (green), C: RFP, no red fluorescence , D: overlay of panels A-C Scale Bar, 21 μm.

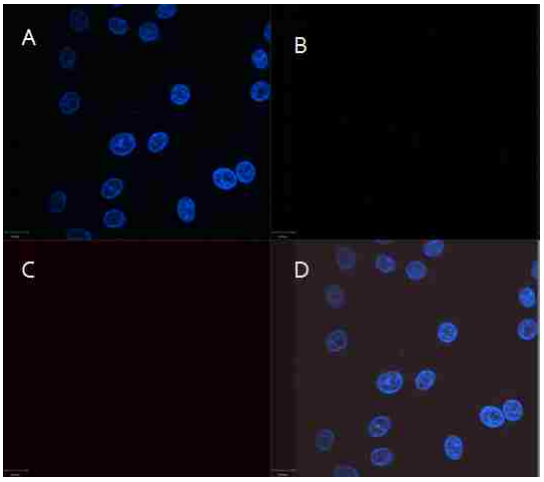

Figure 2.14: Microscopic Analysis of EGFP+ cells FACS postsort

Negative fraction, A: DAPI blue nuclear fluorescence, B: no GFP fluorescence (green), C: RFP, no red fluorescence , D: overlay of panels A-C Scale Bar, 21 μm

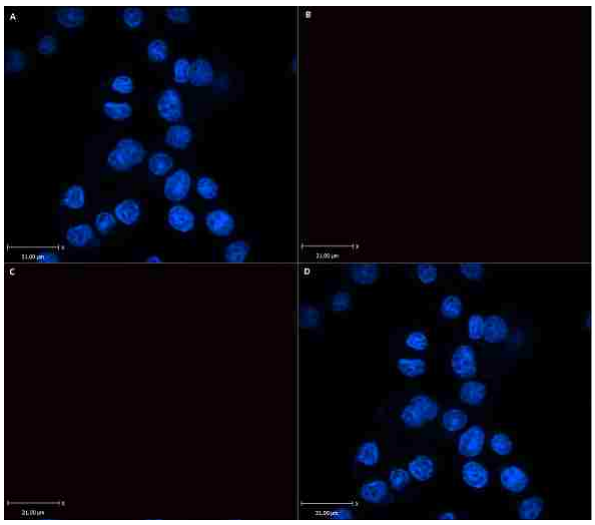

Figure 2.15: Microscopic Analysis of control(empty transfected) RS1 cells for FACS

A: DAPI blue nuclear fluorescence, B: no GFP fluorescence (green), C: RFP, no red fluorescence , D: overlay of panels A-C Scale Bar, 21 μm

2.4 Discussion

This chapter discusses about Pnmt-nEGFP model. The advantage of this mouse model over the Pnmt-Cre mouse model is that it tracks the actively Pnmt expressing cells as opposed to the ones that used to express Pnmt at earlier stages [26]. In this chapter, we induced cardiac differentiation in the pluripotent Pnmt-nEGFP mESCs by hanging drop method, however, while analysis of the differentiated cells it was observed that very few cells out of a cluster of cells were nEGFP+. The undifferentiated cells did not show any kind of nEGFP expression. Pnmt expression was also seen in these differentiating cells at 7+5d. But it was difficult to observe the green fluorescence due to autofluorescence and the limited number of cells expressing EGFP. Hence, to have a successful isolation of the differentiated beating cardiomyocytes we need to scale up the protocol to have numerous cells facilitating the detection of nEGFP+ cells.

We faced significant technical difficulties trying to differentiate the Pnmt-nEGFP mESCs owing to several reasons such as use of different lots of fetal bovine serum, inability of the mESCs to differentiate into cardiomyocytes etc. Therefore, we thought that establishment of the techniques for isolation of these Pnmt+ cardiac progenitor cells will be a pivotal step. We used RS1 cells as the surrogate cells for verifying the isolation techniques. RS1 cells are a type of cells derived from PC12 (pheochromocytoma of rat adrenal medulla) cell line [39, 38, 37, 36]. In a normal healthy tissue the adrenal medullary cells are of adrenergic type but these pheochromocytoma cells are thought to have ceased the expression of Pnmt. The RS1 cells were chosen as they are easily transfectable. We transfected the RS1 cells with EGFP plasmid to establish the FACS method of isolation. The controls for the experiment were RS1 cells transfected with empty transfection. The controls and target cells were treated exactly the same, and were sorted by FACS. The positive and negative fractions of the cell populations were collected and analyzed once the sorting was completed. It was observed that the positive fraction contained 92% EGFP+ cells whereas the

negative fraction contained absolutely no EGFP+ cells. The controls for the experiment showed absolutely no EGFP expression as expected. Both the positive and negative fraction of the cells were plated post sorting. The cells were microscopically analyzed pre as well as post sorting and the images were captured under the confocal microscope. The number of EGFP+ cells post sorting were more as compared to pre sort. FACS was run thrice to verify the sorting technique and efficiency of the FACS sort. It was observed that during first sort 6.22% of the cells were positive for EGFP whereas 2.5 and only 2.0% for the next two sorts. The variation in the percentage of EGFP+ cells was due to the varying transfection efficiencies of the transfection regents used.

2.5 Limitations

As discussed in the previous section a major technical challenge in identifying the EGFP+ cells was autofluorescence. Since the EGFP+ cells were found in low abundance it made it further difficult to observe and identify them. But these challenges can be overcome by taking certain steps. The disturbance due to autofluorescence was minimized by using a red fluorescent anti-GFP antibody which facilitated the identification cells showing EGFP expression. The low abundance of cells expressing EGFP can be overcome by increasing the number of differentiating cells. We have been exploring newer methods of differentiation which would help improve the efficiency of cardiac differentiating cells. Another limitation was the expression of EGFP in Pnmt+ cells. It was seen in adrenal glands isolated from the Pnmt-nEGFP mouse model that only a few number of Pnmt+ cells expressed EGFP protein. There might be several reasons of this limited expression of EGFP in Pnmt expressing cells.

Another major technical difficulty observed before and after FACS technique was the viability and survival rate of the cells. Since the cells were exposed to such high pressures and high intensity lasers the health of the cells was compromised. It took almost 2-2.5 hours for each sort to get

completed which made the cells devoid of media and fetal bovine serum hence decreasing their viability. It was also observed that only about 50% of the total number of fluorescent cells in reality get sorted out. In addition to that, a very few number of the sorted cells remained actually viable post-sort.

2.6 Conclusions

This chapter primarily focuses on isolation of nEGFP+ cells based on the FACS technique. Pnmt-nEGFP mESCs were differentiated into cardiomyocytes, and nEGFP^{+} cells were identified in the differentiated cardiomyocytes. Figure 2.4 gives a general scheme for differentiation and isolation of Pnmt-nEGFP+ cells via FACS technique. The results show that the mESCs were successfully differentiated into cardiomyocytes and expressed nEGFP and Pnmt post differentiation. To establish the FACS technique we used the RS1 cells derived from the pheochromocytoma cells of adrenal medulla and transfected them with the EGFP plasmid and isolated the cells using FACS technique. The results confirm that EGFP+ cells were successfully isolated using the FACS technique and therefore, this technique could be used for isolation of Pnmt-nEGFP expressing differentiated cardiomyocytes. In the future, this isolation technique will enable us to isolate and study the characteristics of the cardiac progenitor cells.

CHAPTER 3: ISOLATION AND IDENTIFICATION OF LNGFR+ CELLS

3.1 Introduction

As we discussed in the previous chapter, Pnmt-nEGFP mESCs have several limitations when it comes to differentiation and isolation of Pnmt+ cardiomyocyte progenitor cells. Not only is it difficult to detect the nEGFP expression in differentiated cardiomyocytes, but the expression is not robust and was observed in low abundance.

Hence, another mouse embryonic stem cell line was generated containing a unique cell surface marker, ΔLNGFR. ΔLNGFR is a cell surface epitope, a type of neurotrophin receptor which is primarily expressed in the central and peripheral nervous system. ΔLNGFR is a C-terminal non-truncated version of the protein as well as human in origin which makes it an ideal and unique reporter gene as it will be expressed in cells only transfected with the above mentioned gene. It was seen that ΔLNGFR is already used a marker to enrich a specific population of cells by a an isolation technique called (Magnetic Activated Cell Sorting) MACS. It may serve as a better method than FACS as it is said to be less harsh than FACS and more efficient.

This Pnmt-ΔLNGFR cell line was created by Jixiang Xia (in his thesis) using a strategy similar to that need to develop the Pnmt-nEGFP cell line. The cDNA of ΔLNGFR and neomycin is inserted into the Pnmt 5' and 3' flanks under the Pnmt 5' regulatory sequences as seen in figure 3.1. Diphtheria toxin A fragment (DTA) was used a negative marker. Since the ΔLNGFR cDNA is inserted into the downstream regulatory sequences of Pnmt gene, it should be expressed only in the cells expressing Pnmt gene. This entire construct of ΔLNGFR cDNA along with Pnmt 5' and 3' flanks was inserted into the YFP mESCs by electroporation. The YFP mESCs is a cell line containing the yellow fluorescent protein in it.

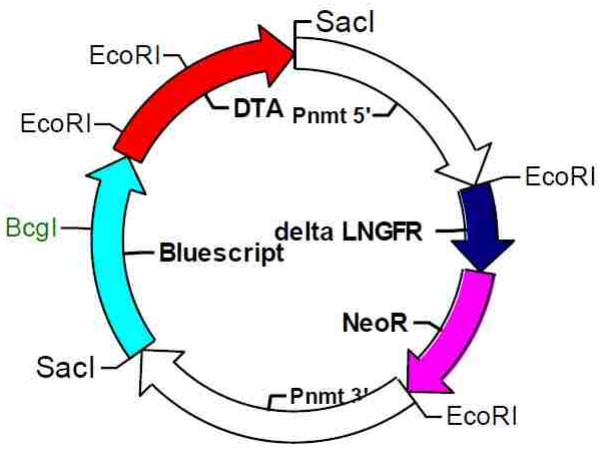

Figure 3.1: Schematic map of Pnmt-ΔLNGFR-Neo targeting construct (Created by Jixiang Xia

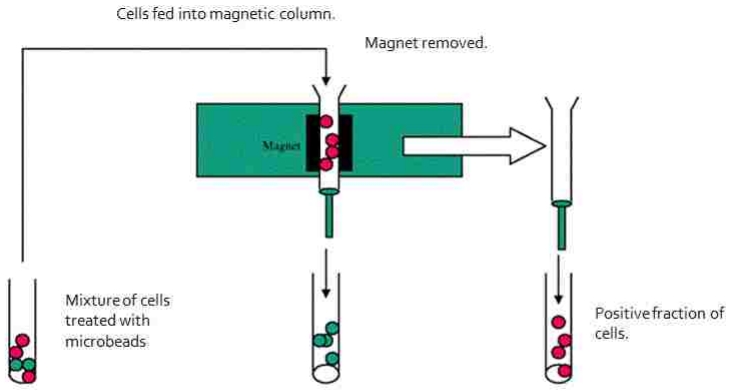

Figure 3.2: Scheme for isolation of LNGFR+ cells

Figure adapted from Milteyni Biotec

Figure 3.2 gives a schematic representation of the MACS technique. Once the cells express the

ΔLNGFR epitope on the surface they will be labeled with MACS anti-LNGFR microbeads (provided by Milteyni Biotech). The microbeads are 50μm in size have a magnetic component attached to the antibody. The cells expressing the surface epitope will have the magnetic microbeads attached to them. These cells will be passed on to a magnetic column which is attached to a magnetic separator. Since the microbeads have a magnetic component attached to it, the cells expressing the ΔLNGFR marker will get retained in the column whereas the others will elute out of the column. Both the positive and negative fractions of the cells can thus be analyzed for further experiments.

The purpose of generating this Pnmt-ΔLNGFR mouse embryonic stem cell line was to have an efficient technique for isolation of the Pnmt+ cardiac progenitor cells. The same principle of differentiation shall be applied to these mESCs for induction of cardiac differentiation.

In this chapter we will discuss about characterization of Pnmt-ΔLNGFR cell clones and establishment of the MACS technique. The first step is to characterize the ΔLNGFR cell line by genomic screening to verify if the construct was properly inserted into the mouse genome. Post characterization of the cell clones and establishment of the MACS technique this cell line could be effectively used to induce cardiac differentiation in the Pnmt-ΔLNGFR mESCs and isolate them using the MACS technique so as to further study the characteristics of the adrenergic progenitor cells for future cardiac regenerative applications.

3.2 Materials and Methods

3.2.1 Materials

Anti-LNGFR FITC antibody, anti-LNGFR APC antibody, pMACS LNGFR plasmid, anti-LNGFR magnetic microbeads, MS columns and the entire MACS apparatus was obtained from Milteyni Biotec Inc. (San Diego, CA). The primers for PCR and RT-PCR were obtained from Promega (Madison, WI). DMEM (Dulbecco's Modified Eagle Medium) and other supplements for cell culture were from Invitrogen, Inc.(Carlsbad, CA). All the other supplements were from Sigma Aldrich (St Louis, MO). Fetal bovine serum was from Atlanta Biologicals (Flowery Branch, GA. Lipofectamine 2000 and 3000 were obtained from Invitrogen, Inc. (Carlsbad, CA), BD Sciences FACS Aria sorter and FACS canto was used for sorting of cells and for flow cytometry respectively.

3.2.2 Methods

3.2.2.1 Characterization of ΔLNGFR: Genomic Screening by PCR

We designed primers L1, L2, L3, L4, L5, Neo, L6, LNGFR primers for verifying specific junction points for the fragment inserted in mouse genome and the for evaluating the presence LNFGR gene. Table 3.1 lists the primers used for genomic screening. We screened 18 Pnmt-ΔLNGFR cell clones of which three clones namely LN9, LN10, and LN43 showed positive results.

Table 3.1: Primers Used for Genomic Screening

Primer Name	**Primer Sequence**
L1	AGAAGTGTCCTCTCCCCTGC
L2	GGGGGACCCAGTGGTAGA
L3	CACCTGCCCCCATCGC
L4	GTGGTATGGCTGATTATAGCAGG
L5	CACTCCCTGTAGCGTGCTT
L6	GTGTCAGGTGAGCACTTGGG
Neo	CGATTGTCTGTTGTGCCCAG
LNGFR (F)	GGTGATAGCTTCGATCCAGACA
LNGFR (R)	CCGCCTCAGAAGCCATAGAG

3.2.2.2 Restriction Digestion of pMACS LNGFR plasmid

We performed digestion of pMACS LNGFR plasmid to verify the plasmid. The digestion was done by using 2.5μl buffer, 1μg DNA, 1-2μl restriction enzyme, about 0.5μl BSA and water in a 20μl reaction. The reaction was incubated for 1 hr at 37 $^{\circ}$C. Once the reaction was complete about 5μl dye was added and was observed on agarose gel.

3.2.2.3 Transfection of RS1 cells with pMACS LNGFR plasmid

The RS1 cells were transfected with pMACS LNGFR plasmid and control for the experiment were empty transfected RS1 cells. The cells were transfected with 30μg of plasmid, 60μl of P3000

(enhancer) and 43.4μl of lipofectamine 3000. The protocol was as specified in the manufacturer's kit. The transfection was verified by treating the transfected cells (both the control and the target) with anti-LNGFR antibody. 10μl of antibody was used for 10^7 cells in 100μl of MACS buffer (PBS containing 0.5% BSA)

3.2.2.4 Isolation of ΔLNGFR+ cells by MACS technique

The cells were trypsinized and centrifuged twice and passed through a 70μm cell strainer to ensure ingle cell suspension. The cells were then re suspended in 80μl of buffer. 20μl of anti-LNGFR microbead suspension was added to the cells and they were kept in dark for 15-20 mins at $4\,^{\circ}\mathrm{C}$. The cells were then washed with PBS. A minimum of 10^8 of total cells were resuspended in degassed MACS buffer. The MACS column was rinsed with 1ml of buffer, which was discarded. The remaining cell suspension was then fed into the column. The unlabeled cells were passed and collected in a tube. MS column was washed with 3x500 μl degassed buffer, adding buffer every time as the column got empty. The MS column was then removed from the separator and 1 ml of buffer pipetted into the column and plunged so as to collect the retained the cells. The flow-through was passed once again in a new column using the same protocol, and the flow-through and retained cells were collected. The protocol was as specified in the manufacturer's kit (Milteyni Biotec).

3.2.2.5 Isolation of ΔLNGFR+ cells by FACS

We also isolated ΔLNGFR+LNGFR+ cells using FACS. In this experiment the control for the experiment were empty transfected RS1 cells. The control and target samples were harvested after 48 hrs of transfection and treated with anti-LNGFR antibody FITC (provided by Milteyni Biotec, Inc). The cells were suspended in 100μl MACS buffer with 10μl antibody and incubated in the dark for 15 mins at $4\,^{\circ}\mathrm{C}$. They were washed twice with PBS, passed through 75μm cell strainer and

resuspended in flow buffer. Both the positive and negative fractions were collected and analyzed by flow cytometry to verify the percentage of LNGFR+ cells (green cells). The controls were also analyzed for green fluorescence by flow cytometry. In addition, all the populations of cells including the controls, positive and negative fractions post FACS and pre sorted cells were analyzed microscopically before and post sorting to confirm green fluorescence.

3.2.2.6 RT-PCR

RNA was extracted from both the positive and negative fractions of the controls as well as the target cells (cells transfected with pMACS LNGFR plasmid) by TRIzol reagent and cDNA was generated using MuLV Reverse Transcriptase. LNGFR primers used for RT-PCR were obtained from operon and a 1:20 dilution was used for analysis. They were as follows: forward 5' CCGTTGGATTACACGGTCCA 3' and reverse 5' TGTCTGGATCGAAGCTATCACC 3'. The product size was 265 bp which was observed on agarose gel. The RT-PCR analysis was done on all the four populations collected from MACS namely Lipo +ve (control retained), Lipo -ve (ontrol flow through), MACS positive or LN+ (MACS retained), MACS negative or LN- (MACS flow through). GAPDH expression was used as a control for the experiment. The primers used for verifying GAPDH expression were as follows: forward 5' AGAGATGATGACCCTTTTGGC 3' and reverse 5' CCATCACCATCTTCCAGGAGCG 3'.

3.2.2.7 Confocal Microscopy

All the images were captured on the 63x objective of Perkin Elmer laser scanning confocal microscope. The images were captured using the following lasers: 488nm for GFP, 563nm for RFP, 405nm DAPI and DIC.

3.3 Results

3.3.1 Genomic Screening of Pnmt-ΔLNGFR ES cells

Figure 3.3 shows the scheme used for genomic screening and characterization of the Pnmt-ΔLNGFR ES cells. Out of 18 clones that were screened, three clones showed the presence of LNGFR cDNA, the NeoR, and the Pnmt 5' and 3' flanks. The clone numbers were LN9, LN10 and LN43. Figure 3.4 provides the size of bands generated for each of these constructs.

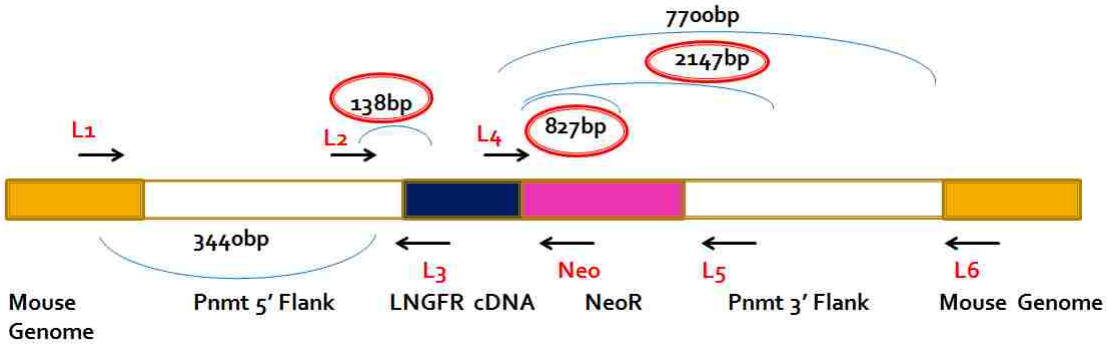

Figure 3.3: Scheme for Genomic Screening of Pnmt-ΔLNGFR ES cells

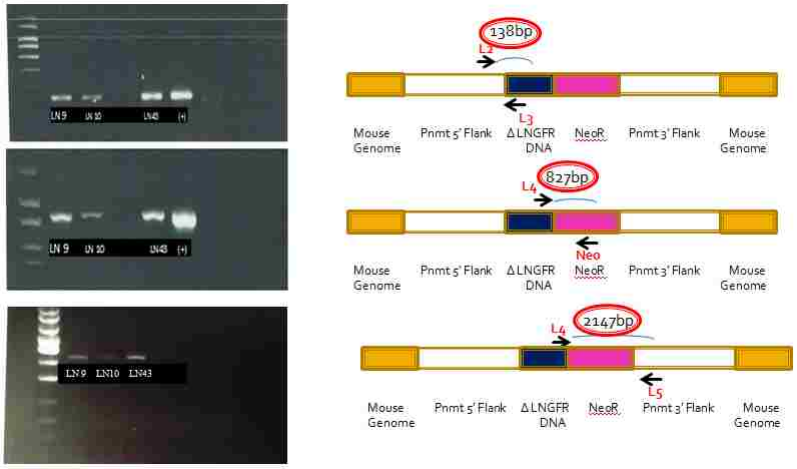

Figure 3.4: Results of Genomic Screening

Left panel: Gel Electrophoresis, Right panel: Schematic showing band sizes

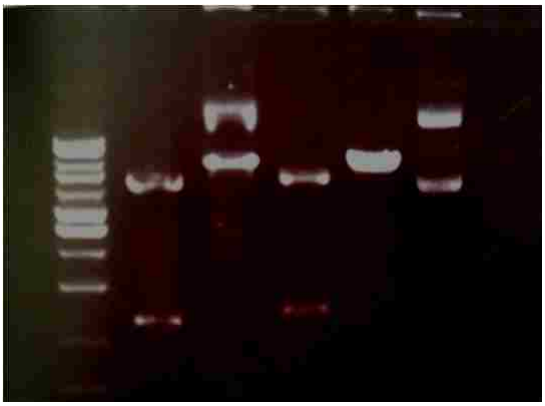

Figure 3.5: Restriction analysis of pMACS LNGFR plasmid

3.3.2 Restriction Digestion of pMACS LNGFR

Figure 3.5 shows the bands observed under agarose gel electrophoresis after restric digestion of the plasmid. Table 3.2 specifies the enzymes used for restriction digestion of the plasmid.

Table 3.2: Enzymes Used for Digestion of pMACS LNGFR

Lanes	**Enzyme Used**	**Band size (bp)**
1	Ladder	1kb
2	BamH1	1156, 4289
3	ECoR1	5445
4	Xho1	1178, 4269
5	Hind III	5445
6	No enzyme	

3.3.3 FACS of RS1 cells transfected with pMACS LNGFR and stained with FITC anti-LNGFR antibody FITC

The RS1 cells transfected with pMACS LNGFR plasmid were treated with anti-LNGFR antibody and the cells were sorted via FACS. This experiment was performed twice with the controls being the empty transfected RS1 cells. In the first sort 7.15% of the cells were FITC and hence ΔLNGFR+ whereas the controls showed absolutely no fluorescence. Once the cells were sorted the positive and negative fraction were analyzed by flow cytometry. 98.4% of the cells were ΔLNGFR+ in the positive fraction whereas 0% were ΔLNGFR+ in the negative fraction post sorting. During the second sort 2.34% were ΔLNGFR+ whereas 0.030% were positive in the control experiment. Post sorting the positive fraction showed 96% LNGFR+ cells whereas 0.012% cells were LNGFR+ for the negative fraction. The difference in the percentage of ΔLNGFR+ cells in FAC1 and FAC2 results pre sorting was because of the different types of lipofectamines used for transfection of RS1 cells. During first transfection lipofectamine 2000 was used whereas lipofectamine 3000 was used for the second transfection experiment.

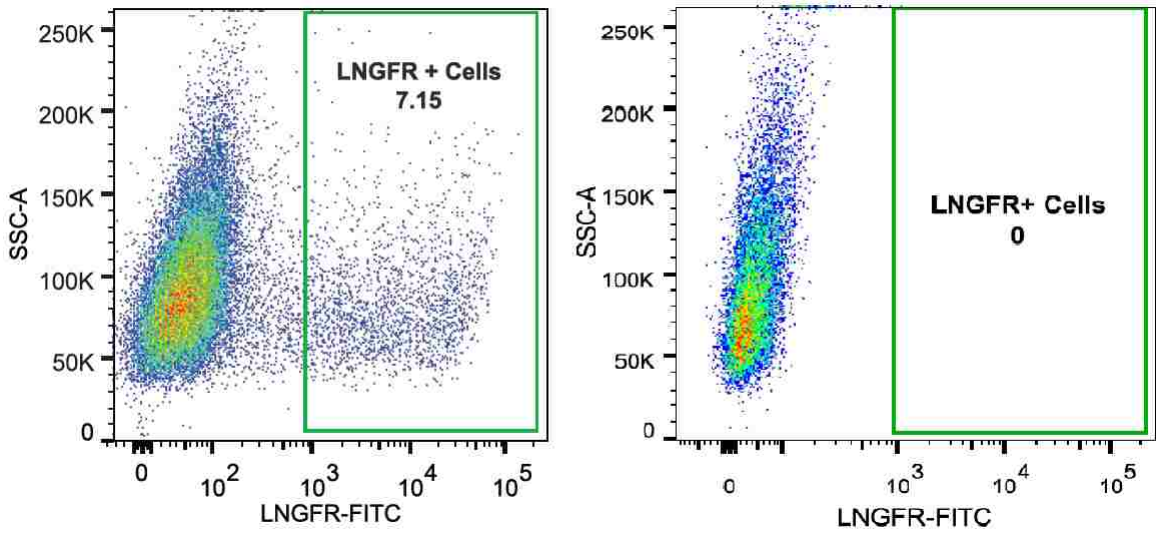

Figure 3.6: Dot plots of RS1 cells transfected with LNGFR plasmid and stained with anit-LNGFR FITC antibody

(A) Presort of LNGFR transfected RS1 cells B Presort of empty transfected RS1 cells. 7.15% of the total RS1 cells were LNGFR+ in A. The left gate represents negative population. (B) Presort results for controls show 0.0% LNGFR+ cells

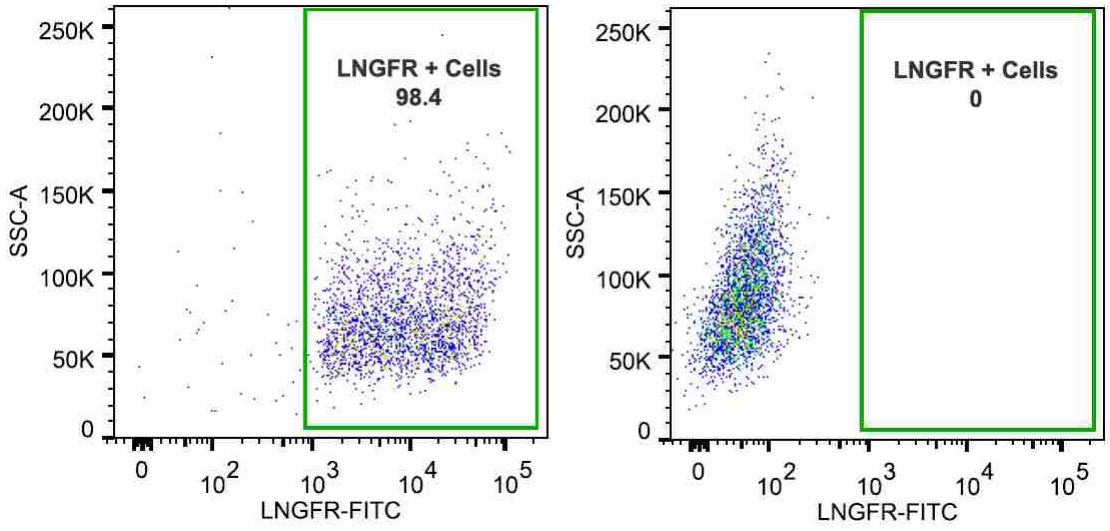

Figure 3.7: FACS Post Sort cytometry results

A Post sort positive fraction shows 98.4% ΔLNGFR+ cells. B Post sort negative fraction shows 0.0% ΔLNGFR+ cells.

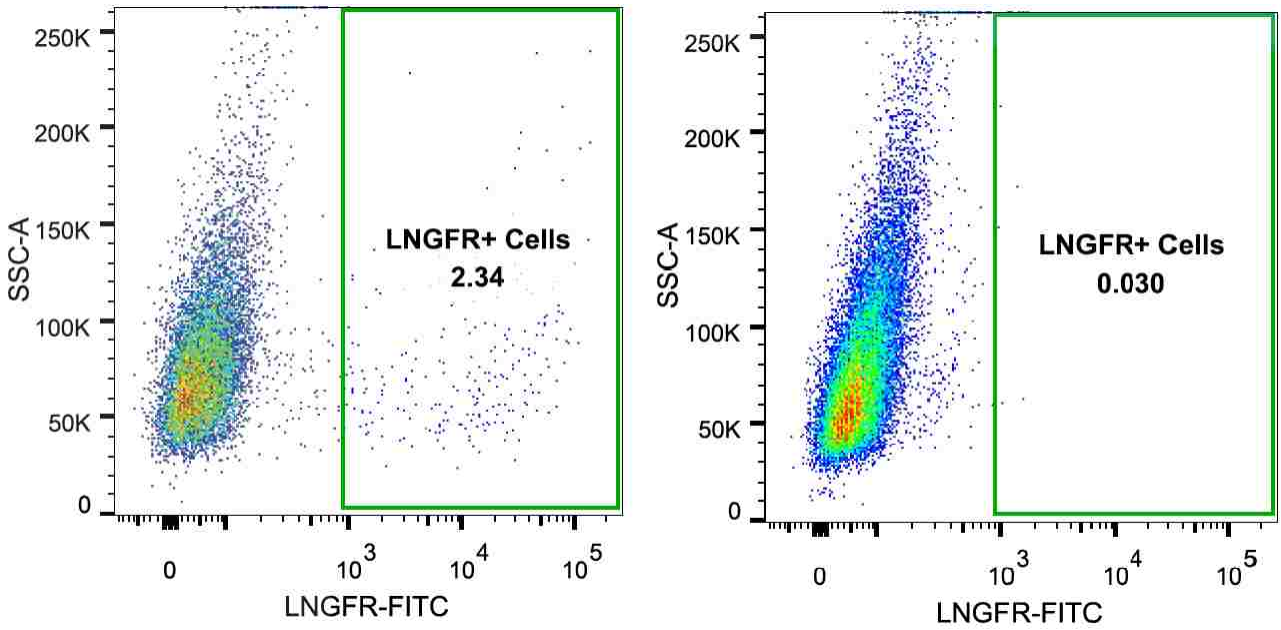

Figure 3.8: FACS of RS1 cells transfected with LNGFR plasmid and stained with anit-LNGFR FITC antibody

Post sort dot plots of FACS A,B Presort results. 2.34% of the total RS1 cells were ΔLNGFR+ in A. The left gate represents negative population. B Presort results for controls show 0.03% ΔLNGFR+ cells

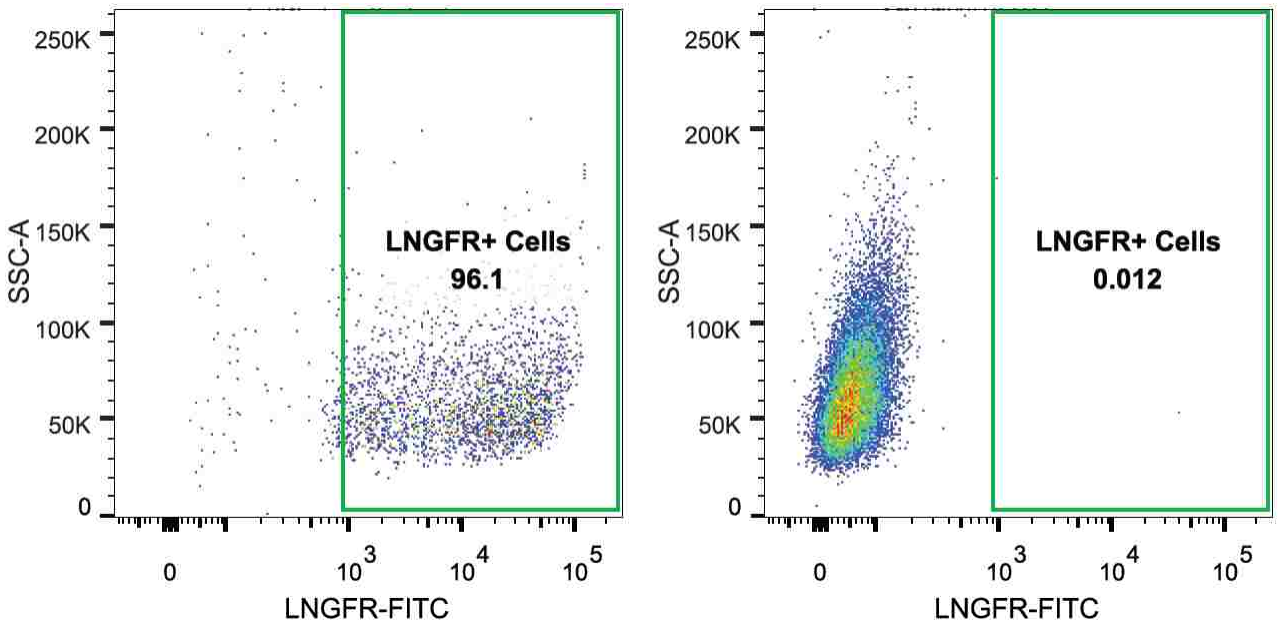

Figure 3.9: Post Sort cytometry results

(A) Post sort positive fraction shows 96.1% ΔLNGFR+ cells. (B) Post sort negative fraction shows 0.012% ΔLNGFR+ cells.

3.3.4 Isolation of LNGFR+ cells using MACS technology

Figure 3.10 gives a detailed method for isolation ΔLNGFR+ cells using MACS technique. The same experiment was performed three times to verify the validity of the experiment. The controls for the experiment were empty transfected RS1 cells. The transfection with LNGFR plasmid was confirmed by staining the cells with anit-LNGFR antibody.

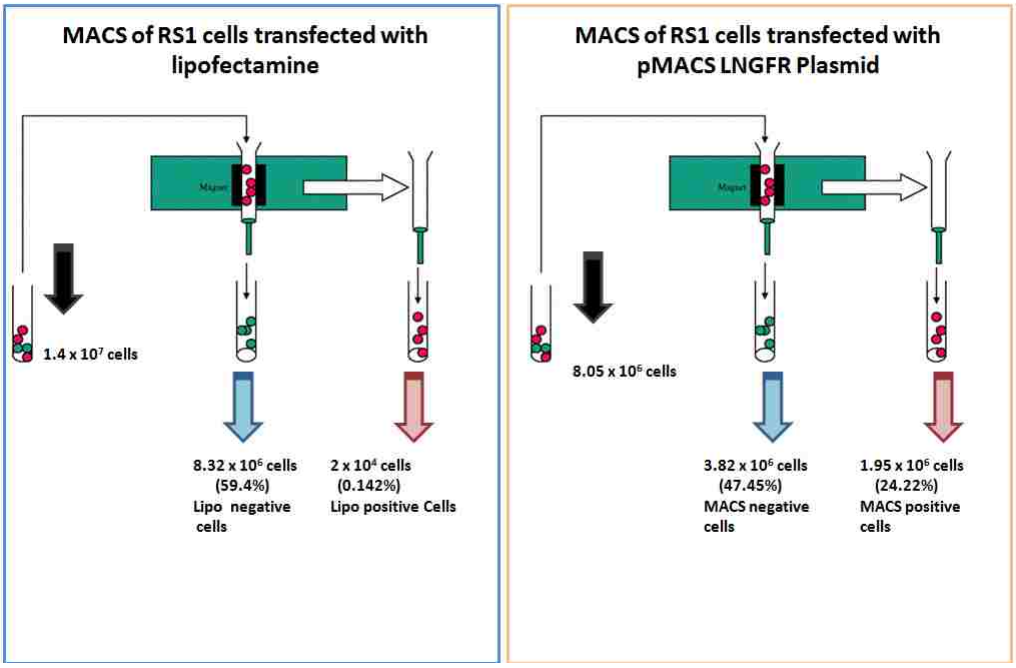

Figure 3.10: Magnetic separation of RS1 cells transfected with pMACS LNGFR plasmid

The cells were transfected with LNGFR plasmid and treated with anti-LNGFR microbeads and fed into the MACS column. The flow though and retained cells were collected and counted. The controls (empty transfection) were treated similary. Figure adapted from Milteyni Biotec

3.3.5 Analysis of MACS technique

Tables 3.3, 3.4 & 3.5 show the initial count of cells and number of cells retained and in flow through for both the control and the target cells (pmacs LNGFR transfected). This data is plotted in the form of graphs in figures 3.11 and 3.12.

Table 3.3: Analysis of MACS1 Technique: A

Target (ΔLNGFR RS1 cells) transfected with pMACS LNGFR plasmid treated with anti-LNGFR microbeads and isolated using MACS technique. Analysis was done by counting the initial number of cells, cells retained in the column and the flow through fractions. The controls (empty transfection) were treated similary.

	Control (Lipofectamine RS1 cells)	**Target (ΔLNGFR RS1 cells)**
Initial number of cells	1.4×10^7 cells	8.05×10^6 cells
Cells Retained	2×10^4 cells (0.142%)	1.95×10^6 cells (24.22%)
Flow Through	8.32×10^6 cells (59.4%)	3.82×10^6 cells (47.45%)

Table 3.4: Analysis of MACS2 Technique: B

Target (ΔLNGFR RS1 cells) transfected with pMACS LNGFR plasmid treated with anti-LNGFR microbeads and isolated using MACS technique. Analysis was done by counting the initial number of cells, cells retained in the column and the flow through fractions. The controls (empty transfection) were treated similar.

	Control (Lipofectamine RS1 cells)	**Target (ΔLNGFR RS1 cells)**
Initial count of cells	4.02×10^6 cells	5.6×10^6 cells
Cells Retained	0 cells (0.0%)	2.1×10^5 cells (3.75%)
Flow Through	1.81×10^6 cells (45.0%)	2.34×10^6 cells (41.7%)

Table 3.5: Analysis of MACS3 Technique: C

Target (ΔLNGFR RS1 cells) transfected with pMACS LNGFR plasmid treated with anti-LNGFR microbeads and isolated using MACS technique. Analysis was done by counting the initial number of cells, cells retained in the column and the flow through fractions. The controls (empty transfection) were treated similar.

	Control (Lipofectamine RS1 cells)	**Target (ΔLNGFR RS1 cells)**
Initial count of cells	7.44×10^6 cells	6.44×10^6 cells
Cells Retained	0 cells (0.0%)	8.722×10^5 cells (13.477%)
Flow Through	6.34×10^6 cells (85.2%)	4.95×10^6 cells (76.7%)

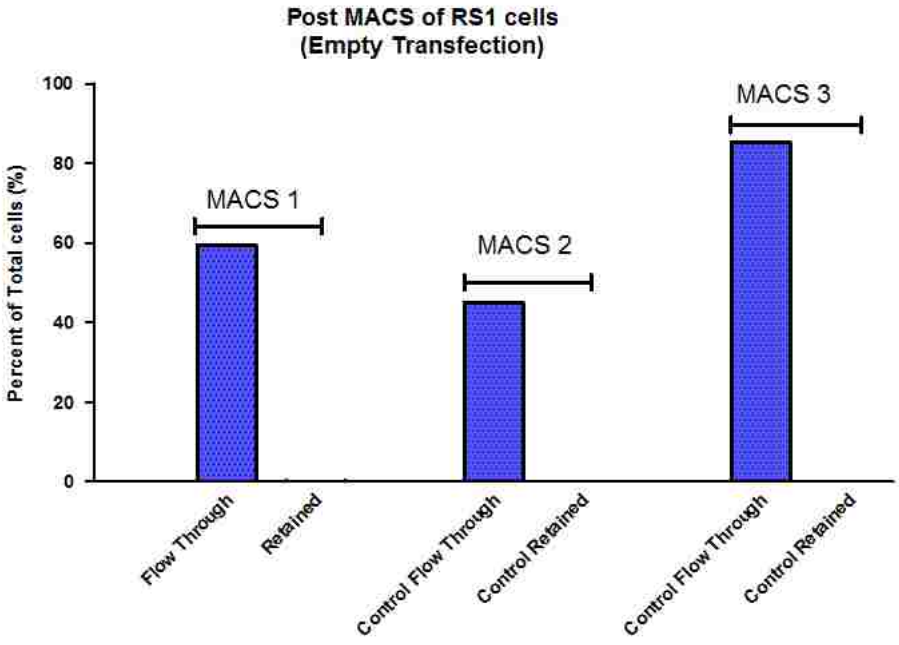

Figure 3.11: Percent of Total RS1 cells in flow through and retained.

The results show percent of total cells (empty transfection) in the flow-through and retained. For MACS1, 2 and 3: 60, 45 and 85% of the cells were flow through whereas almost zero or negligible were retained.

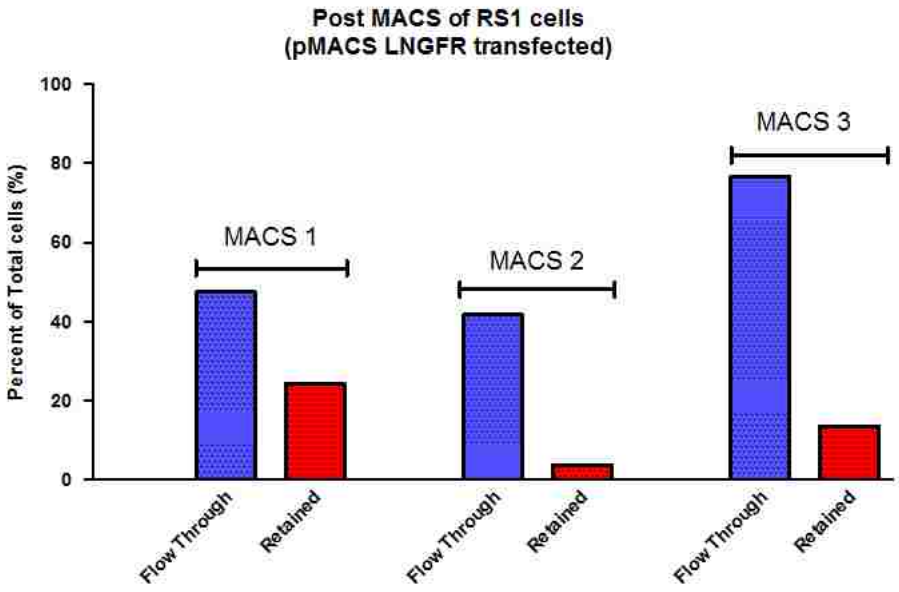

Figure 3.12: Percent of Total RS1 cells in flow through and retained.

The results show percent of total cells (pMACS LNGFR transfected) in the flow-through and retained. For MACS1, 2 and 3: 47.45%, 41.7% and 76.7% of cells were flow through whereas 24.22%, 3.75% and 13.477% of the cells were retained.

3.3.6 RT-PCR Analysis

Relative fold-change in the expression of ΔLNGFR gene was evaluated in MACS-retained, MACS flow- through and control-retained, and control flow through as the reference for calculation according to the Δ ΔCt method. ΔLNGFR expression was observed in both MACS retained and flow through fractions of the cells. This shows that some ΔLNGFR+ cells are present in the eluted fraction of the cells as well. However, the ΔLNGFR expression was almost double in the case of MACS retained population. The RT-PCR was run and respective bands of LNGFR and GAPDH (control) were observed on the agarose gel electrophoresis. Figures 3.14 and 3.16 are the gel images showing GAPDH and LNGFR bands. Figure 3.15 shows there were no bands in control retained and flow-through fractions.

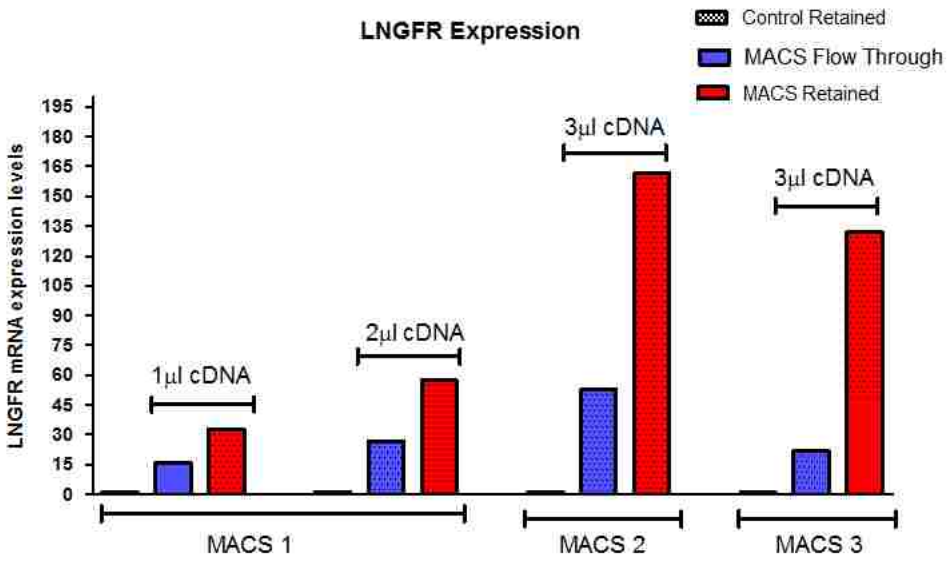

Figure 3.13: RT-PCR Analysis: ΔLNGFR expression in RS1 Cells Isolated by MACS Technology

MACS 1, MACS2, MACS3 are the three trials of MACS. Control flow-through is eluted fraction of the control cells. MACS retained: Positive fraction of RS1 cells tranfected with pMACS LNGFR plasmid and retained in MS column. MACS flow through: Negative fraction of RS1 cells tranfected with LNGFR plasmid. Relative fold change expression of ΔLNGFR was $\geq$ double in case of MACS retained to that of MACS flow-through.

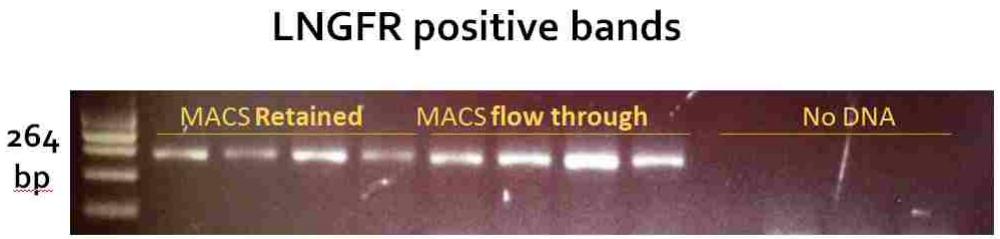

Figure 3.14: RT-PCR Analysis: LNGFR positive bands

RT PCR run on all four population of cells post MACS isolation. MACS retained, MAcs flow through, Control retained, control flow through. Bands (264bp) were observed for MACS retained and MACs flow through. No bands were seen for negative control (no DNA).

Figure 3.15: RT-PCR Analysis:Negative controls

No LNGFR bands seen in Lipo negative and Lipo positive populations. Lanes 2-5: Lipo +ve,

Lanes 6-9: Lipo -ve, Lanes 10-13: Null controls, no cDNA

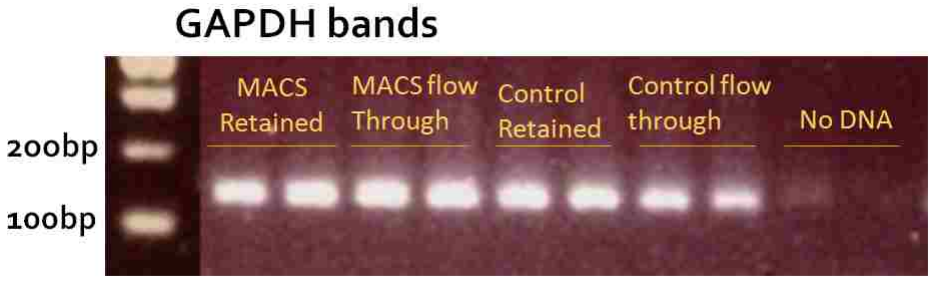

Figure 3.16: RT-PCR Analysis: GAPDH gel

GAPDH used as a control for RT-PCR. Gel shows bands for all the samples including LN+, LN-, Lipo +ve, Lipo -ve. No bands are seen for the null controls, Lanes: 10-11. Expected size is 147bp.

3.3.7 Fluorescence Microscopy Analysis

Figure 3.17, figure 3.18, figure 3.19 and figure 3.20 correspond to microscopic analysis of cells pre and post isolation of the ΔLNGFR+ cells via FACS technique.

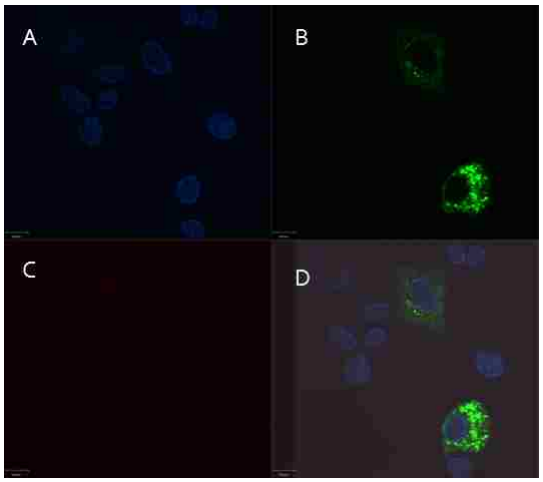

Figure 3.17: Fluorescence Microscopy Analysis of ΔLNGFR+ cells presort

Cells stained with anti-LNGFR FITC antibody. A: DAPI blue nuclear fluorescence, B: FITC fluorescence (green) around the nucleus, C: RFP, no red fluorescence , D: overlay of panels A-C. Scale bar, 21μm.

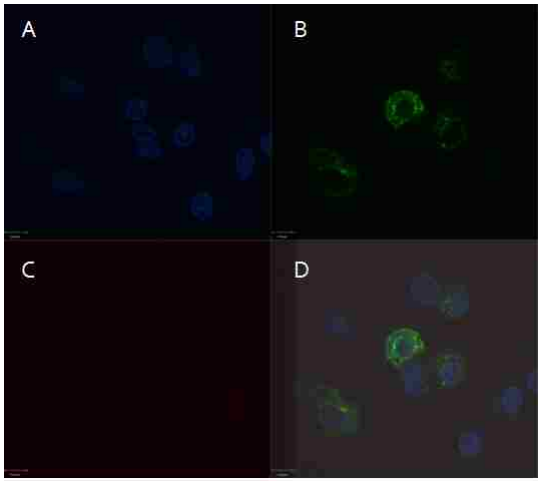

Figure 3.18: Fluorescence Microscopy Analysis of ΔLNGFR+ cells

Cells stained with anti-LNGFR FITC antibody, Post sort positive fraction. A: DAPI blue nuclear fluorescence, B: FITC fluorescence (green) around the nucleus, C: RFP, no red fluorescence , D: overlay of panels A-C Scale bar, 21μm.

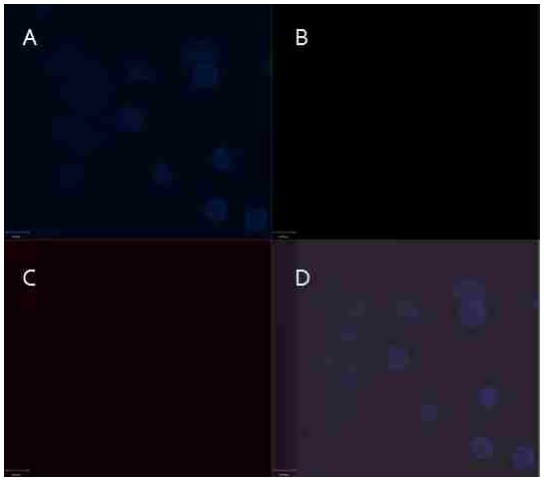

Figure 3.19: Fluorescence Microscopy Analysis of ΔLNGFR+ cells

Cells stained with anti-LNGFR FITC antibody, Post sort negative fraction. A: DAPI blue nuclear fluorescence, B: no FITC fluorescence (green) around the nucleus, C: RFP, no red fluorescence , D: overlay of panels A-C Scale bar, 21μm.

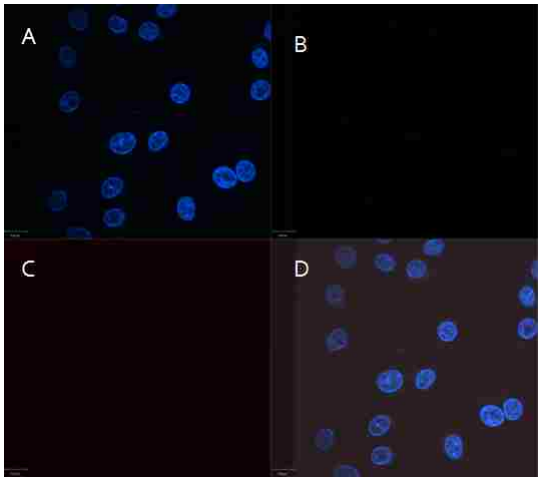

Figure 3.20: Microscopic Analysis of control cells

Cells stained with anti-LNGFR FITC antibody, A: DAPI blue nuclear fluorescence, B: no FITC fluorescence (green) around the nucleus, C: RFP, no red fluorescence , D: overlay of panels A-C Scale bar 21μm.

3.4 Discussion

ΔLNGFR is a very useful cell surface marker used for isolation of cell populations. A study conducted by Jianping Zhang showed effective isolation of LNGFR+ neural crest-derived stem cells from rat embryonic mandibular processes by using the MACS technique [27]. The results clearly showed that LNGFR antibodies are very specific and enabled enriched and pure population of these stem cells. Not only did it help in isolation of ΔLNGFR+ cells but in this particular study they differentiated these LNGFR+ cells into muscle, bone and melanin secreting cells by supplementing them with some special growth factors [27]. This shows that MACS is effective in enrichment of LNGFR+ cells and does not compromise on viability of the cells. It is less tough on the cells, and it is less time-consuming. Another advantage is the isolation of about 10^7 cells in a single isolation is possible in about 5-7 mins whereas it takes 2-4 hours for isolation via FACS. Another study showed staining of bone marrow mesenchymal cells using monoclonal antibodies against α-LNGFR [28]. These stem cells post isolation were assessed for their proliferative capacity and various other properties after differentiation [28]. This shows that in future once the Pnmt+ cardiac progenitor cells are isolated on basis on LNGFR expression, they could be readily characterized for numerous characteristics such as proliferative potential, differentiation potential, transplantation etc.

There are several other studies where MACS technology is effectively used for isolation of rat hair follicle stem cells, purification of differentiated ES cells expressing CD4 as surface marker. All these studies suggest effective purification of stem cells or other type of specialized cells which were cultured post MACS isolation and proved to be efficient in enrichment of the cells [29, 30, 31]. The same principle was used in this study to isolate LNGFR+ cells. Since the viability and proliferative potential of the enriched cells was not compromised this technique of isolation has great potential to generate enriched and pure populations of Pnmt+ cardiac progenitor cells based

on the expression of ΔLNGFR as as surface receptor.

3.5 Limitations

Though we were able to identify the specific junction points of the Pnmt-Δ LNGFR mESCs, we could not verify if the construct was homologously recombined with the mouse genome. We tried long-range PCR technique but since the products were about 3kb and 8kb, it was difficult to amplify such a huge construct. But the homologous recombination could also be verified by southern blot technique.

We were able to successfully isolate the ΔLNGFR+ cells by using the MACS technique. However, there were some minor technical difficulties we faced during the actual process of the MACS technique. When the RS1 cells expressed LNGFR on the surface of the cells, an aliquot of the cells was stained with anti-LNGFR anti-body to confirm transfection. During this step the cells were suspended in about 100μl of MACS buffer and 10μl of antibody was added and they were kept in the dark at 4C for 10-15 mins. It is very important to have a high population of cells in a specified volume of buffer. If the anti-LNGFR antibody is less or the population of the cells is less, it becomes difficult to stain the LNGFR expressing cells.

Another drawback of the MACS technique is verification. Once the cells are labeled with anti-LNGFR magnetic microbeads, there is no way to confirm that the microbeads have attached to the surface antigen of the cells. Miltenyni Biotec has provided us with a MACS control FITC antibody but we are facing some difficulties in getting the microbeads stained with the antibody. The only way to test the efficiency of MACS and labeling of the cells with the antibody is after the isolation of ΔLNGFR+ cells by RT-PCR analysis. The anti-LNGFR antibody could be eventually used for verifying ΔLNGFR expression in the differentiated cardiomyocytes as well as pluripotent Pnmt-

ΔLNGFR ES cells. Since the ES cells have a YFP gene, it is difficult to use the FITC conjugated antibody to verify ΔLNGFR expression as the excitation emission peak of YFP is in the range of FITC absorption peak. But the Milteyni Biotec provide an APC conjugated anti-LNGFR antibody which could be used to stain the ES cells as well as the differentiating cardiomyocytes.

3.6 Conclusions

We successfully identified ΔLNGFR cDNA in 3 out of 18 clonees flanked by Pnmt 5' and 3' flanks. We could isolate the ΔLNGFR+ cells via MACS technique and verify the expression of LNGFR in the isolated samples as well as in the controls. This shows that the MACS technique is efficient in isolation of ΔLNGFR cells. We also noticed that the ΔLNGFR cells could be isolated by FACS technique by staining them with the anit-LNGFR FITC antibody. The isolation of ΔLNGFR cells by FACS technique could serve as an alternative technique.

Hence, MACS can be effectively used for isolation of the Pnmt-ΔLNGFR cardiac progenitor cells and it could serve to be a better and efficient technique for isolation of Pnmt+ cardiac progenitor cells.

CHAPTER 4: SUMMARY AND CONCLUSIONS

The purpose of this thesis was to establish the methods of isolation for Pnmt+ cardiac progenitor cells. This was shown using both FACS and MACS techniques.

In chapter 2, we described about the Pnmt-nEGFP mice cell lines and the initial characterization of the Pnmt-nEGFP differentiated cardiomyocytes. We could clearly identify the number of nEGFP+ cells in a population of differentiated cardiomyocytes. There were about 0.025% nEGFP+ cells found on 7+5d. The Pnmt expression in these cells was verified by RT-PCR analysis. The controls for this experiment were the undifferentiated pluripotent mouse embryonic stem cells which clearly did not show any kind of green fluorescence or red fluorescence of the anti-GFP antibody. We also discussed about the technical difficulties faced during differentiation of these mESCs. We have been working hard to increase the percentage of differentiated cardiomyocytes so as to facilitate the nEGFP fluorescence. Also, while identifying and counting the number of nEGFP+ cells, it was difficult to observed and identify them owing to the disturbance of autofluorescence and low abundance of nEGFP+ cells. The nEGFP expression was not robust amongst the differentiated cells which could be attributed to the variability of green fluorescence expressed by the EGFP reporter gene. However, we were able to successfully isolate the surrogate RS1 cells transfected with EGFP plasmid via the FACS technique. The cells were sorted efficiently and verified by laser scanning confocal microscopy and flow cytometry. The technique is efficient but compromises health of the cells. It was seen that only 50% of the effective green fluorescent cells were sorted by FACS technique. Once the cells were sorted, the health of the cells is highly compromised due to which most of the cells perish due to the harsh conditions of FACS technique since the cells are exposed to so much pressure and are without the media for about 2-3 hours. This shows that to efficiently sort the Pnmt+ cardiac progenitor cells via FACS technique we need to have an abundant population of cells and we should also work on the technical specifications of FACS technique so

that technique is less rough on the cells.

In chapter 3, we described other cells lines where Pnmt-ΔLNGFR was developed. Instead of the nEGFP reporter gene, we have the ΔLNGFR gene inserted in the exon 1 of Pnmt gene. Our first step in this chapter was to characterize the cell clones to evaluate the construct is inserted in the respective exon in the mouse genome flanked by the Pnmt flanks. We were able to successfully identify the ΔLNGFR cDNA flanked by the Pnmt flanks by using PCR genomic screening. We were unable to verify if the 3 clones expressing this contruct were homologously recombined with the mouse genome due technical difficulties faced owing to the large amplicon sizes required to be generated. We could successfully isolate the ΔLNGFR+ cells via MACS technique. The only problems faced for this technique was the verification of labeling of the ΔLNGFR cells with the magnetic microbeads. But we were able to successfully verify LNGFR expression in both the positive and negative fraction of the cells. It was found that the ΔLNGFR expression was almost double for positive fraction as compared to the negative fraction cells eluted out. This shows that the MACS technique could efficiently isolate ΔLNGFR+ cells. Hence this technique could be effectively used in the future for isolation of Pnmt+ cardiac progenitor cells. LNGFR could also be used as an efficient marker for isolation of other Pnmt+ cells found in other regions of the body such as brain, kidney, liver, eyes etc.

The main purpose of the study was to establish the isolation techniques that would enable isolation of Pnmt+ cardiac progenitor cells in future studies. From the above discussion we could conclude that the MACS technique is technique of choice for isolation of Pnmt+ cardiac progenitor cells. There are several reasons for this conclusion which could be summarized as follows: we found that FACS technique was much harsher on the health of the cells which hampered the viability of cells resulting in very few cells being actually isolated via the technique. On the other hand, the MACS technique is much easier to handle and use. It facilitates effective isolation of ΔLNGFR+ cells without compromising the viability of the cells. Other important points to consider are time

and money. MACS technique is a much quicker and less time consuming technique as compared to FACS. The apparatus used for MACS is easy to use and relatively inexpensive. In case of FACS, if about 2% of the cell population was fluorescent during presort, it was observed that about 50-70% of this 2% population was actually sorted out. Of these 50% about 10-20% of the cells were dead because of the through-put pressure that the cells are exposed to during FACS technique. Though during successive trials of MACS technique we observed that about 50% of the cells were getting lost. This could be due to decrease in the viability of the cells or a large number of cells must be retained in the column. But this was overcome during the third trial where we washed the MS column three times with the MACS buffer so as to get as many cells as possible from the MS column. We also passed the enriched fraction of the ΔLNGFR cells once more through a new MS column so as to enrich the ΔLNGFR+ cells. Following precautions and measure enabled us to retrieve as many cells as possible in addition to an enriched ΔLNGFR population. Exactly same scenario was seen in a study conducted by Li et al where they try to compare these two isolation techniques to effectively isolate third generation bone marrow mesenchymal stem cells. The viability of these stem cells was much lower than the stem cells isolated by MACS technique [32]. A study showed the comparison of two methods for isolation of $CD4^+$ $CD25^+$ regulatory T (Treg) cells. The cells were isolated by both the techniques and analyzed by trypan blue and propidium iodide for evaluating the cell viability. For this particular study they observed MACS took more time and was challenging to sort the cells as compared to FACS. However, they suggest using both the methods simultaneously for higher purity of cells.

However, there are certain criteria which should also be taken into consideration while selecting the best possible method of isolation. FACS is an ideal method of enrichment when you desire highest purity of cells and recovery. Also, FACS could be used for isolation of proteins based on their intracellular characteristics whereas MACS is solely based on expression of cell surface proteins [32]. Another important point to consider is the magnet used for isolation of cells in

MACS is very powerful which isolates anything and everything that is attached to the microbeads [32]. Another drawback of using MACS technique is the lack of purity [32]. We noticed LNGFR expression in the flow through fractions as well which concludes presence of LNGFR+ cells in this population of cells. Another point to be considered is with MACS you could isolate only one type of cell population as opposed to FACS which enables collection of atleast 2-4 different cell populations [33]. The shape, granularity and viability of cells is also an important criteria. With FACS you easily set gates so as to avoid collecting the dead cell population whereas MACS is unable to distinguish the dead cells from the live ones. If the cells are sticky and and irregular shape they tend to stick to the MS column resulting in loss of cells [34]. Considering all the points the most favorable technique to be employed for isolation of Pnmt+ cardiac progenitor cells is MACS followed by FACS. This would help in achieving a highly pure population of enriched cells. Otherwise, one could also use MACS to facilitate enhanced viability of cells.

In the introduction we discussed that Pnmt+ cells were found during early embryonic development of heart. It was also observed that these Pnmt+ cells contributed to a major chunk of the left ventricular chamber of heart which is most susceptible to cardiac damage during several cardiovascular diseases. There could be a question as to why these Pnmt+ cells could be useful for cardiac regenerative applications. We are yet to confirm if these Pnmt+ cells could serve as an important tool for regenerative applications or not. They might also serve as a tool for destruction of cardiac myocardium owing to their association with the sympathetic system of the body. Nevertheless, isolation of these Pnmt+ cells will definitely enable us to study the characteristics of these cardiac progenitor cells enable us to conclude if these Pnmt+ cells could be useful for regenerative applications.

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