KDR expression enhances commitment toward the cardiac lineage of hESC- and iPS- derived CD15$^+$ progenitors

E. Di Pasquale[1], P. Portararo[2], R. Rizzi[2,3], M. Mancino[2] and G. Condorelli[3,4]

1. Multimedica Foundation, Milan, Italy; 2. IRCCS Multimedica Institute, Milan. Italy; 3. Department of Medicine, National Research Council, Italy; 4. Department of Medicine, University of California San Diego, USA

ABSTRACT:

Cell-based therapies represent exciting therapeutic options for myocardial regeneration after ischemic injury. Although many cell types have been proposed as a source of cardiac myocytes, the type of progenitor as well as the optimal conditions for their stimulation still represent major challenges. Moreover, molecular characterization of cardiac progenitors (CP) *in vitro* is still unsatisfactory: the identification of novel surface markers for their selective isolation is of utmost importance.

In our study we employed different human ESCs and induced pluripotent stem (iPS) cells lines to set up a simple, reliable and efficient protocol to selectively induce cardiac fate *in vitro*.

At first, we comparatively applied different methods and monitored the differentiation process by FACS analysis, RT-PCR and immunofluorescence for early and late markers. Our results show that sequential treatments with ActivinA and BMP4 or BMP2, ascorbic acid and TGFβ1 in a specific media formulation induce CD15$^+$ cardiac progenitor population with an enhanced cell vitality compared to other methods. While we confirmed that CD15 is among the earliest markers upregulated after differentiation, the definition of other associated markers could better specify progenitors committed toward cardiomyogenesis. For this purpose, we systematically evaluated induction of other markers already shown associated with cardiac differentiation or heart development, that is PDGFRa, KDR, Sca1, CXCR4, cKIT. Our result demonstrate that a specific population expressing both KDR and CD15 surface markers is more committed toward cardiovascular lineages.

The induced KDR$^+$/CD15$^+$ cell population exhibits higher levels of Gata4 and Isl1 expression (2-fold induction), compared to the KDR$^-$/CD15$^+$ counterpart and, after isolation, gives rise to 66.15% Troponin I positive cells (43 out of 65 in a single representative experiment), against the 26.5% (18 out of 68) detectable in the KDR$^-$/CD15$^+$ population.

In conclusion, our studies, tough preliminary, strongly indicate that KDR in CD15$^+$ progenitor cells is an earlier marker of cardiomyogenesis.

INTRODUCTION:

Cell-based therapies constitute a promising therapeutic option for myocardial repair due to heart failure (HF). HF represents the final consequence of a vast set of cardiovascular diseases and it is one of the major causes of morbidity and mortality in the Western world (*Vinge et al., 2008*). So far, cell-based replacement therapy has been restricted by the paucity of sources of functional human cardiomyocytes (CMCs) and by the limited proliferation rate and survival of these cells *in vitro (Murry & Lee, 2009)*. Various cell types have been proposed as a source of CMCs, including cardiac stem cells, endothelial progenitors and mesenchymal stem cells, but data obtained using these cells are not unequivocal (*Wu SM et al, 2008*).

Due to their multilineage differentiation potential, pluripotent stem cells (PSCs), such as embryonic stem cells (ESCs) and induced pluripotent stem cells (iPS), constitute an ideal source for differentiating CMCs: both ESCs and iPS cells are able to give rise to all the cell derivatives of the three germ layers (ectoderm, mesoderm and endoderm), including CMCs (*Yuasa & Fukuda, 2008, Thomson JA et al, 1998, aggiungi ref diff cardio, (Mauritz et al, 2008, Narazaki et al, 2008, Gai H et al, 2009)*. Additionally, employment of iPS cells offer some advantages, being a promising source of patient-specific cells that can be used for autologous therapies and allowing to overcome the ethical issues related to the use of embryonic material.

In the last few years, the effect of specific growth factors, small molecules and culture conditions on the differentiation of PSCs into CMCs has been extensively studied and improved methods of induction have been proposed. In particular, specific progenitor populations, which are able to give rise to a highly enriched CMC population, have been identified, both in mouse and in human (i.e. KDR^{low}/cKIT$^-$, CD15$^+$, PDGFRα$^+$, KDR$^+$/CXCR4$^+$, Sca1$^+$) (*Laflamme et al. 2007; Leschik et al. 2008; Nelson TJ et al. 2008; Yang et al, 2008; Kattman et al. 2010; Moretti et al. 2010).*

Nonetheless, identifying the type of progenitor as well as the optimal conditions for their selective induction still represent major challenges of the stem cell cardiovascular biology field. Moreover, molecular characterization of cardiac progenitors (CPs) *in vitro* is still unsatisfactory: the identification of novel surface markers for their selective isolation is of utmost importance.

From these consideration, the main purpose of our study aims to further dissect the molecular events driving cardiac differentiation of human PSCs, defining a reliable and efficient protocol to induce them toward the cardiac fate *in vitro* and identifying markers of cardiac commitment to selectively isolate cardiac progenitors.

RESULTS:

Cardiac induction protocol design and validation

In order to ameliorate methods for cardiac differentiation from both human ESCs and iPS cells, we comparatively analyzed the most recent induction strategies with a method based on treatments with defined factors in two specific media formulations, first one was based on KO-DMEM, Serum Replacement and pro-survival factors (non-CM), whereas the second one was specific for culturing cardiovascular progenitors (*Smits et al. 2009*). The detailed protocol is represented in the Fig. 1A. Among all the available methods, we focused on one recently published by Leschik and colleagues (2008), showing BMP2 treatment in RPMI-B27 medium was sufficient to induce a progenitor cell population expressing CD15 and committed to the cardiac lineage. Accordingly, our results showed sequential treatments of PSCs with Activin A for 24 hours and BMP2 for 4 days promote the induction of a CD15$^+$ population that was greater than 40% and expressing key cardiac genes (Fig.1C,D), confirming CD15 is among the earliest markers upregulated during differentiation.

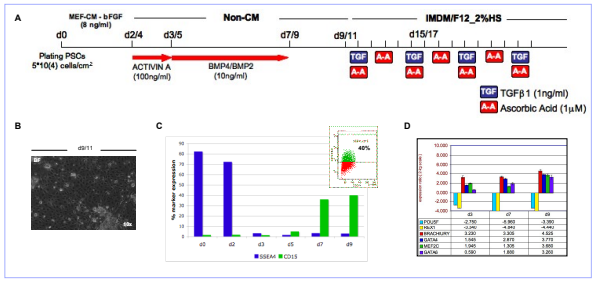

Figure 1. (A) Schematic representation of the proposed differentiation protocol: PSCs were seeded onto 6-well Matrigel coated dishes and maintained up to 2/4 days in MEF-CM in presence of bFGF. Treatments started when cells reached about 50-70% confluence: ActivinA and BMP2 or BMP4 in non-CM medium were initially used. Later in differentiation TGFβ1 and ascorbic acid were added, in presence of IMDM/F12 and 2% horse serum (HS). (B) Morphology of differentiating ESCs at day 9/11 of differentiation. (C) Schematic diagram summarizing FACS analyses for the surface markers SSEA4 and CD15 and showing a progressive induction of a CD15+ cell population during differentiation, while the stemness marker is downregulated. The fluorescence scatter specific for CD15 at day 9 is also represented, as example. (D) Realtime PCR analysis, showing a downregulation of the typical stemness markers (Pou5f - Oct4 and Rex1) and induction of specific cardiac genes (Brachiury, GATA4, MEF2C and GATA6). cDNA from untreated cells was used as reference condition.

Next we compared the cardiomyogenic potential of our strategy, which represents an alternative approach to the method recently published by the Puceat's group (*Leschik et al. 2008*), to this last protocol. Our results showed the proposed method was effective in activating TGFβ and BMP pathways, by mediating the phosphorilation of the SMAD effectors (Fig. 2A), and it was able to induce the expression of cardiac specific transcription factors, both earlier and later in differentiation (Fig. 2B,C and D); however no relevant differences between the two methods were detected.

On the other hand, FACS analysis for AnnexinV and 7-AAD, markers of apoptosis and cell death respectively, revealed the media formulation used in our protocol ameliorated cell survival and vitality of the induced cells. CD15 marker detection was included and showed ActivinA supplement is necessary for its efficient induction if non-CM medium is used (Fig 2E).

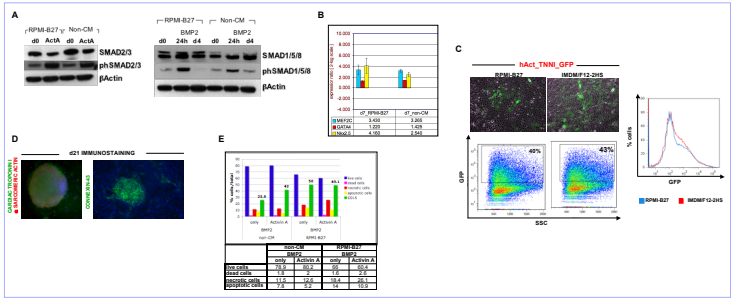

Figure 2. ***Differentiation protocols comparation evaluation.*** (A) Western Blot analysis of protein extracts from cells treated with ActivinA and BMP2 showing non-CM medium do not interfere with the activation of both the SMAD2/3 and SMAD1/5/8 pathways. (B) Realtime PCR showing the upregulation of MEF2C, GATA4 and Nkx2.5 genes after differentiation induction using the two media formulations (RPMI-B27 and Non-CM). cDNA from untreated cells was used as reference. Data were normalized using GAPDH housekeeping gene. (C) Cardiac Troponin I (TNNI) expression was monitored later in differentiation to track cardiac induction efficiency. A lentiviral construct, in which the GFP expression is driven by the TNNI promoter (hAct_TNNI_GFP - *Gallo et al 2008*) followed by FACS analysis was used and showed no significant differences between the two strategies. Images of the infected cells and the respective fluorescence scatters are represented. (D) Immunostaining on representative samples at day 21 of differentiation and showing the expression of TNNI, Sarcomeric Actin and Connexin-43 cardiac markers, preferentially in the clusterized cells; (E) Schematic representation summarizing FACS analyses performed for cell vitality determination: differentiating cells were harvested at different time during differentiation and evaluated for the expression of markers of death and apoptosis, 7-AAD and AnnexinV. Cells positive for both markers are indicated as necrotic, while live cells did not express any. Obtained data indicated non-CM medium is associated with increased survival and cell vitality during differentiation and showing a halved percentage of necrotic and apoptotic cells. CD15 expression was also monitored and indicated the requirement of ActivinA for its efficient induction if non-CM medium is used. The table indicating the specific values is included.

KDR$^+$/CD15$^+$ progenitor cells: isolation and characterization

CD15 is one of the earliest gene induced during differentiation and has been demonstrated to specifically mark cardiac commitment. However, other associated markers could better specify commitment toward the cardiac fate. To this end, we systematically evaluated, within the CD15$^+$ progenitor population, the induction of other markers that have already been shown associated with cardiac differentiation or heart development (*Leschik et al. 2008; Nelson TJ et al. 2008; Yang et al, 2008; Kattman et al. 2010).* Specifically, induction of PDGFRα, KDR, Sca1, CXCR4 and cKIT were considered. Our results showed a specific sub-population expressing both KDR and CD15, that revealed to be more prone to differentiate toward the cardiac fate.

KDR$^+$/CD15$^+$ cells were isolated by FACS sorting (Fig 3A) and cardiac differentiation evaluated by following expression of specific cardiac genes: KDR$^+$/CD15$^+$ population exhibits higher levels of Gata4 and Isl1 expression (2-fold induction by quantitative RT-PCR)(Fig. 3B) and, after isolation, give rise to TNNI positive cells more efficiently compared to the KDR$^-$/CD15$^+$ counterpart. TNNI expression was determined by immunostaining, flow cytometry and quantitative PCR methods (Fig. 3C,D,E,F,G). Altogether our data indicate that KDR expression enhances commitment of CD15$^+$ progenitor cells toward the cardiac fate.

On the contrary, we cold not detect any PDGFRα induction during differentiation (data not shown). Data on CXCR4 and cKIT exhibit instead high variability within the different experiments and need to be further evaluated.

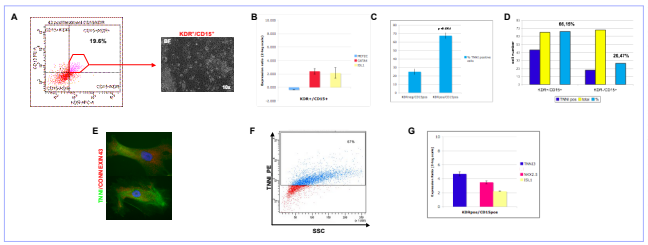

Figure 3. ***KDR$^+$/CD15$^+$ progenitors: isolation and characterization.***
(A) Fluorescence scatter specific for CD15 and KDR surface antigen at day 9 of differentiation showing the sub-population cells represent the 19.6% of the total cell. On the left, a optic microscope 10x resolution of the isolated cells 9 day post-sorting is included. (B) Realtime PCR analysis, showing the upregulation of Gata4 and Isl-1 genes in the KDR+/CD15+, compared to the KDR negative counterpart. The analyzed was performed on samples harvested immediately after sorting. (C) Later after isolation, cardiac commitment was determined by monitoring TNNI expression induction. The diagram summarizes the results obtained by counting TNNI positive cells in the two cell population after immunostaining with anti/-TNNI antibody. (D) A representative experiment showing KDR+/CD15+ cells give rise to 66.15% TNNI positive cells (43 out of 65), against the 26.5% (18 out of 68) detectable in the KDR$^-$ population. (E) Immunofluorescence staining indicating KDR+/CD15+ are able to generate cells expressing TNNI and Connexin-43 (Magnification 40x). (F) FACS analysis confirmed expression of TNNI in >60% KDR+/CD15+ at day 9 after sorting. (G) Quantitative PCR also showed a 4 fold-induction of TNNI expression in KDR+/CD15+ cells and upregulation of the other two cardiac genes, Nkx2.5 and Isl-1. KDR-/CD15+ sample was used as reference.

CONCLUSIONS:

Cardiac induction has been extensively studied and important steps forward have been done in defining culture conditions, factors and stimuli involved in the differentiation process in the last years *(Laflamme & Murry, 2011; Nuri D et al. 2009)*. However, many challenges remain and strategies for selectively induce cardiac fate need to be further improved. Similarly, the characterization of CPs and the surface markers for their selective isolation is still unsatisfactory.

In our study we suggests another method to induce cardiac phenotype from human PSCs, confirming CD15 is a key marker for CPs identification and isolation. Additionally we also found KDR specify a subpopulation, within the CD15$^+$ cells, that is more prone to differentiate toward the cardiac lineage.

Even though further investigations are required to fully characterize KDR$^+$/CD15$^+$ progenitor cell population both molecularly and functionally, our data, together with the previously published evidence (Yang et al. 2007), strongly indicate that KDR is an earlier marker of cardiomyogenesis and might better select for cardiac commitment in the CD15$^+$ cell compartment.

METHODS:

iPS cell: Generation and Maintenance

iPS lines were generated from human skin fibroblasts of healthy subjects, by a slightly modified Yamanaka protocol (*Takahashi et al, 2007*), using either three (Oct4, Sox2 and Klf4) or four (Oct4, Sox2, Nanog and Lin28) pluripotency factors in presence of valproic acid (VPA) *(Huangfu et al, 2008)*. iPS lines were validated for their stemness markers expression (RT-PCR, IF, and FACS analysis of endogenous Oct4, Nanog, Sox2, Rex1, DNMT3B, TRA1-60, SSEA4, and alkaline phosphatase), and for their developmental competence *in vitro* (EB formation and presence of derivatives from the three germ layers) and *in vivo* (teratoma formation assay). The analysis of the karyotype was performed by QFQ banding.

Maintenance of human PSCs

HUES3 line was cultured onto inactivated MEF (mouse embryonic fibroblasts) feeder as previously described (*Cowan et al., 2004*). RUES-2 (Rockefeller University Embryonic Stem cells -2) and iPS lines was maintained onto hESC-qualified Matrix (BD) in mTESR-1 medium (Stem Cell Technologies) and passaged weekly by Dispase (1mg/ml) treatment. For single cell dissociation, pre-treatment with the Rock inhibitor Y-27632 was applied.

Cardiac induction

For differentiation, cells were seeded onto Matrigel coated dishes (Growth Factor Reduced - BD) and grown as the protocol represented in the Fig. 1A. Two media formulations have been tested for their cardiomyogenic activity, as discussed in the results section.

Molecular Analyses

For expression studies, total RNA was isolated using Trizol Reagent and treated with DNAseI; cDNAss was then obtained using Superscript Reverse Transcriptase III (all from Invitrogen). Realtime PCR was carried out on ABI7900HT (Applied Biosystems) using the Gene Expression Master Mix and the Taqman Gene Expression Assays for stemness and cardiac specific genes. Data analysis was performed using SDS2.4, REST and PCRCalc softwares. Immunostaining was carried out using the following primary antibodies: a-Troponin I (Chemicon), a-Connexin-43) and a-Sarcomeric Actin (both from SIGMA). Anti-mouse IgG-Alexa488 and anti-rabbit IgG-Alexa555 (Molecular Probes) were used for detection. Cytofluorimetric analyses were performed on FACS Canto Instrument (BD) and data analyzed using DIVA and the FlowJo softwares. For intracellular markers, cells were fixed and stained with specific antibodies in 0.5% saponin-PBS. Surface antigens were detected on fresh samples. a-CD15-PE and a-SSEA4-FITC were from BD Pharmingen, a-KDR-APC was from Miltenyi. Anti-mouse PE from Jackson ImmunoResearch was used as secondary antibody.

Isolation and analysis of the KDR+/CD15+ cell population

For the sorting experiments, differentiated cells were detached using TrypLe Express (Invitrogen) and stained with antibodies against CD15-PE and KDR-APC. Cells were then resuspended in colture medium in presence of EDTA (2mM), after removal of clumps on 100μm cell strainer. Sorting was performed on BD FACSAria II Cell Sorter using 130 μm nozze, 10PSI pressure and PBS. Sorted cells were then plated onto 35mm dished coated with gelatin/fibronectin (5*104cells/cm2)in IMDM/F12 medium, supplemented with 2% horse serum.

Acknowledgments:

We are grateful to Dr Douglas Melton and Dr. Dr Ali Brivanlou for the HUES3 and RUES-2 (Rockefeller University) human embryonic cells lines. Lentiviral vectors for iPS generation have been provided by Dr. Luigi Naldini and Addgene for Dr. A. Thomson. We also thanks Prof. Vezzoni and Dr. S. Scaramuzza for the teratoma assay and Prof. Dal Pra' and Dr. S. Redaelli for the karyotype analyses.