Original Research

The cell cycle- and insulin-signaling-inhibiting miRNA expression pattern of very small embryonic-like stem cells contributes to their quiescent state

Magdalena Maj[1], Gabriela Schneider[1], Janina Ratajczak[1], Malwina Suszynska[1], Magda Kucia[1,2] and Mariusz Z Ratajczak[1,2]

[1]Stem Cell Institute, James Graham Brown Cancer Center, University of Louisville, Louisville, KY 40202, USA;
[2]Department of Regenerative Medicine, Warsaw Medical University, Warsaw 02-091, Poland
Corresponding author: Mariusz Z Ratajczak. Email: mzrata01@louisville.edu

Abstract

Murine Oct4$^+$, very small embryonic-like stem cells (VSELs), are a quiescent stem cell population that requires a supportive co-culture layer to proliferate and/or to differentiate *in vitro*. Gene expression studies have revealed that the quiescence of these cells is due to changes in expression of parentally imprinted genes, including genes involved in cell cycle regulation and insulin and insulin-like growth factor signaling (IIS). To investigate the role of microRNAs (miRNAs) in VSEL quiescence, we performed miRNA studies in highly purified VSELs and observed a unique miRNA expression pattern in these cells. Specifically, we observed significant differences in the expression of certain miRNA species (relative to a reference cell population), including (i) miRNA-25_1 and miRNA-19 b, whose downregulation has the effect of upregulating cell cycle checkpoint genes and (ii) miRNA-675-3 p and miRNA-675-5 p, miRNA-292-5 p, miRNA-184, and miRNA-125 b, whose upregulation attenuates IIS. These observations are important for understanding the biology of these cells and for developing efficient *ex vivo* expansion strategies for VSELs isolated from adult tissues.

Keywords: MicroRNA, *p57^{Kip2}*, very small embryonic-like stem cells, quiescence, insulin/insulin-like growth factor signaling

***Experimental Biology and Medicine* 2015; 240: 1107–1111. DOI: 10.1177/1535370215584940**

Introduction

First identified by our group in murine bone marrow (BM), Oct4$^+$SSEA-1$^+$Lin$^-$CD45$^-$, very small embryonic-like stem cells (VSELs) can, under favorable conditions, differentiate into cells from all three germ layers.[1] These small cells are mobilized into peripheral blood (PB) during organ injury, which suggests that they contribute to the regeneration of damaged tissues.[2,3] Interestingly, unlike embryonic stem cells (ESCs) or induced pluripotent stem cells, VSELs do not grow teratomas *in vivo* and do not proliferate *in vitro* if cultured without feeder-layer support.[4,5]

The molecular characterization of VSELs has enabled the discovery of several factors that may contribute to the quiescent state of these cells. VSELs exhibit very high expression of the cell cycle kinase inhibitors *p57^{Kip2}* and *p21^{CIP}*, which are involved in inhibiting exit from the cell cycle.[6,7] In addition, evidence has accumulated that epigenetic changes of certain imprinted genes related to insulin and insulin-like growth factor 1 (IGF-1) and 2 (IGF-2) signaling (IIS) also contribute to the VSEL quiescent state.[8–10] As reported more recently, some VSELs share molecular characteristics with primordial germ cells (PGCs), and epigenetic changes in some of the imprinted genes involved in exit from the cell cycle and IIS that regulate the quiescence of VSELs are also responsible for the quiescence of PGCs.[11,12]

The expression of several genes involved in IIS is regulated in VSELs and PGCs at the epigenetic level by imprinting within differentially methylated regions (DMRs). The *Igf-2-H19* locus, imprinted both in mice and humans, plays the most important role in the regulation of IIS gene expression. While the *Igf2* gene encodes the autocrine/paracrine mitogen IGF-2, the *H19* gene gives rise to a non-coding RNA, which is a precursor of several microRNAs (miRNAs) that negatively affect cell proliferation.[9,13]

Mounting evidence indicates that miRNAs contribute to the pluripotency, self-renewal, and differentiation of ESCs.[14] In support of these roles, global loss of miRNAs results in defects in proliferation and differentiation of ESCs *in vitro*,[15] and Dicer-deficient animals die in the early stages of development.[16] Thus, since several genes are regulated at the

post-translational level by non-protein-coding miRNAs, we became interested in the expression of miRNAs that regulate genes involved in cell cycle exit and IIS in VSELs.

We report here that VSELs exhibit differences in miRNA levels (relative to a reference cell population) that have the effect of regulating the cell cycle kinase inhibitor $p57^{Kip2}$ and display a unique miRNA expression pattern that attenuates IIS. These miRNAs may in future be targeted by antagomirs to facilitate *ex vivo* expansion of these cells for purposes of regenerative medicine.

Materials and methods

Isolation of VSELs, HSCs, and mononuclear cells from murine BM

This study was performed in accordance with the guidelines of the Animal Care and Use Committee of the University of Louisville, School of Medicine and with the Guide for the Care and Use of Laboratory Animals (Department of Health and Human Services, Publication No. NIH 86-23).

BM was isolated from pathogen-free C57BL/6 mice (4–6 weeks old; Jackson Laboratory, Bar Harbor, ME, USA). The preparation of mononuclear cells (MNCs) from BM and the isolation of VSELs ($Sca\text{-}1^+Lin^-CD45^-$) and HSCs ($Sca\text{-}1^+Lin^-CD45^+$) by multiparameter live-cell sorting (MoFlo, Dako) were performed as previously described.[17]

MicroRNA expression profiling

VSELs and MNCs were obtained from the BM of WT C57BL/6 mice. Total RNA was isolated from freshly purified cells from both populations using TRIzol reagent (Life Technologies). We used MNCs as a reference population for miRNA profiling analyses as in our previous gene expression studies.

The miScript PCR System was employed following the manufacturer's instructions (Qiagen, Valencia, CA). For reverse transcription of total RNA containing miRNA, the miScript II RT kit was used, followed by real-time quantitative PCR detection of miRNA using the miScript.

Results and discussion

Murine VSELs were purified by FACS from murine BM, and their purity was confirmed by enrichment for Oct-4 mRNA expression as described.[1] Initial screening for miRNA expression employing an miRNA PCR array specific for cell differentiation and development revealed miRNA species significantly upregulated in VSELs relative to MNCs. Based on our miRNA assay results, the literature, and existing bioinformatics databases (miRBase, Target Scan), we focused on miRNAs that contribute to cell cycle control, cell proliferation, and IIS. Accordingly, quantitative RT-PCR was employed to evaluate expression of miRNAs that are well-established participants in these processes, both in VSELs and in BM-derived MNCs.

As demonstrated in Figure 1, we observed several differences in expression pattern for miRNA genes that inhibit the cell cycle in murine BM-purified VSELs relative to MNCs. One of the most important genes that inhibits exit from the cell cycle and is regulated by paternal imprinting is the cell cycle kinase inhibitor $p57^{Kip2}$, and it is known that this gene is negatively regulated by miRNA-25_1.[18] We found that miRNA-25_1 was significantly downregulated in VSELs compared with MNCs ($P < 0.05$), which could explain the previously reported relatively high expression level of $p57^{Kip2}$ in VSELs.[11] Furthermore, miRNA-25 not only inhibits several G1 cyclin/Cdk complexes but also targets two ubiquitin ligases that may regulate Oct4, c-Myc, and Klf5.[19] Hence, miRNA-25_1 may regulate the cell cycle in VSELs and may be additionally involved in their re-programming and self-renewal.

In contrast to $p57^{Kip2}$, there are many genes involved in cell cycle regulation and proliferation that are unregulated by paternal imprinting, and we also analyzed their expression. For example, miRNA-19 b and miRNA-92 b, which together with miRNA-17-5 p belong to the miRNA-17 family, are known to regulate cell cycle entry and self-renewal and to target proteins that suppress Wnt-β-catenin signaling.[20–22] In particular, miRNA-19 b has an important role in cell proliferation by regulating expression levels of its downstream proteins, including PTEN, p-AKT, p-MDM2, p53, and PCNA.[23] Fan *et al.*[24] determined that high expression of miRNA-19 b in human cancer cells promotes the cell cycle by diminishing levels of p53 protein, which subsequently decreases levels of Bax and p21. We found that both miRNA levels were significantly changed in VSELs ($P < 0.05$), although miRNA-19 b was downregulated, while miRNA92b was upregulated.

Our initial miRNA microarray analysis also revealed high expression of miRNA-429-3 p in VSELs, which was confirmed by RT-qPCR analysis ($P < 0.05$). It has been reported that c-Myc in ESCs upregulates expression of miRNA-429-3 p that is involved in self-organizing network that maintains pluripotency of ESCs by inhibiting genes involved in cell differentiation.[25] Significant upregulation of miRNA-429-3 p in VSELs corroborates with high expression of c-Myc in the cells.[11]

Our miRNA studies also revealed some changes in expression of several miRNA species, such as miRNA-221 and miRNA-222 (regulators of $p27^{Kip1}$ and $p57^{Kip2}$),[26,27] the miRNA let-7 a (regulator of cyclin D1 and pS2),[28] miRNA-129-5 p (inhibitor of *Cdk6* expression and G_1 phase progression),[29] and miRNA-16 (regulator of cancer cell proliferation)[30]; however, these changes were not statistically significant.

Furthermore, we also analyzed the signature of several miRNAs involved in IIS. It is well established that inhibition of IIS promotes the VSEL quiescent state.[9,10] In particular, VSELs highly express the *H19* non-coding RNA that gives rise to miRNA-675-3 p and miR-675-5 p, both of which negatively affect expression of the IGF-1 receptor[31] and INS R,[32] which, in turn, plays an important role in IGF-1 and insulin signaling.[9] As expected, we confirmed high expression of both miRNAs in murine BM-purified VSELs by RT-qPCR ($P < 0.05$). Furthermore, another gene regulated by parental imprinting in mouse is *RasGRF1*, which encodes a small GTP exchange factor for H-Ras that is associated with postnatal growth and is involved in IIS.[33] In support

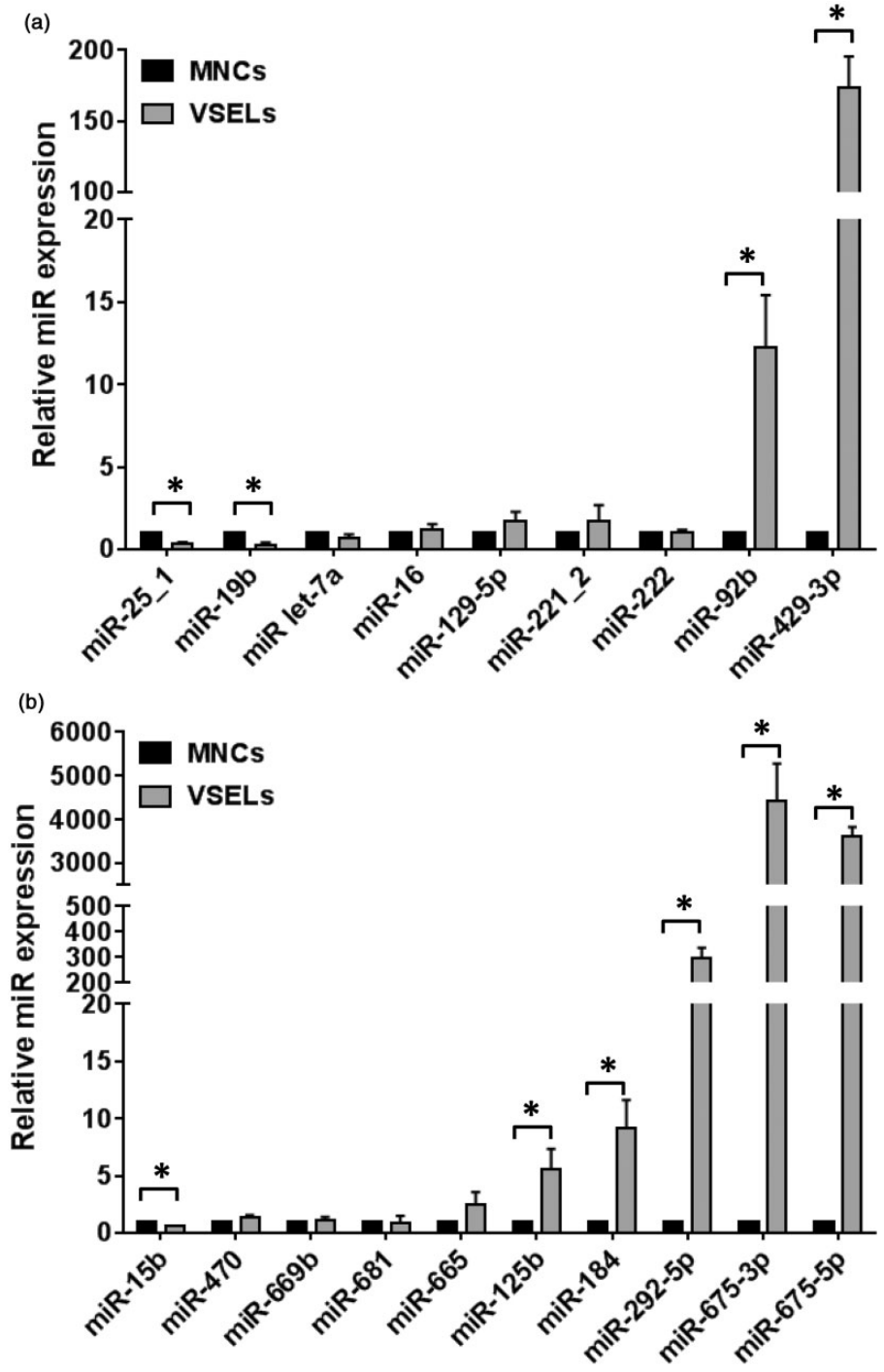

Figure 1 Changes in expression of miRNAs regulating the cell cycle (panel A) and insulin/insulin-like growth factor signaling (panel B). Combined data from four independent experiments are pooled together. *$p < 0.05$

of changes in miRNA expression that may additionally contribute to attenuation of IIS, we found that miRNA-184, which exhibits a complementary sequence to the mRNA sequence of the *RasGRF1* gene, is upregulated in murine VSELs ($P < 0.05$).

Due to erasure of paternal imprinting at the *Igf2-H19* locus, murine VSELs also display very low levels of IGF-2 expression.[11] Since expression of IGF-2 may be additionally regulated at the miRNA level,[34,35] we evaluated the expression of miR-292-5 p, miR-125 b, and miR-665, all of which negatively regulate IGF-2 expression. Murine VSELs exhibited upregulation of all three miRNAs, and changes in expression of miR-292-5 p and miR-125 b were significant ($P < 0.05$).

While IGF-1 R is the signaling receptor for IGF-1 and IGF-2, the IGF-2 receptor (IGF-2 R) is a non-signaling protein expressed on the cell surface that binds IGF-2 and prevents its binding to IGF-1 R. Thus, high expression of

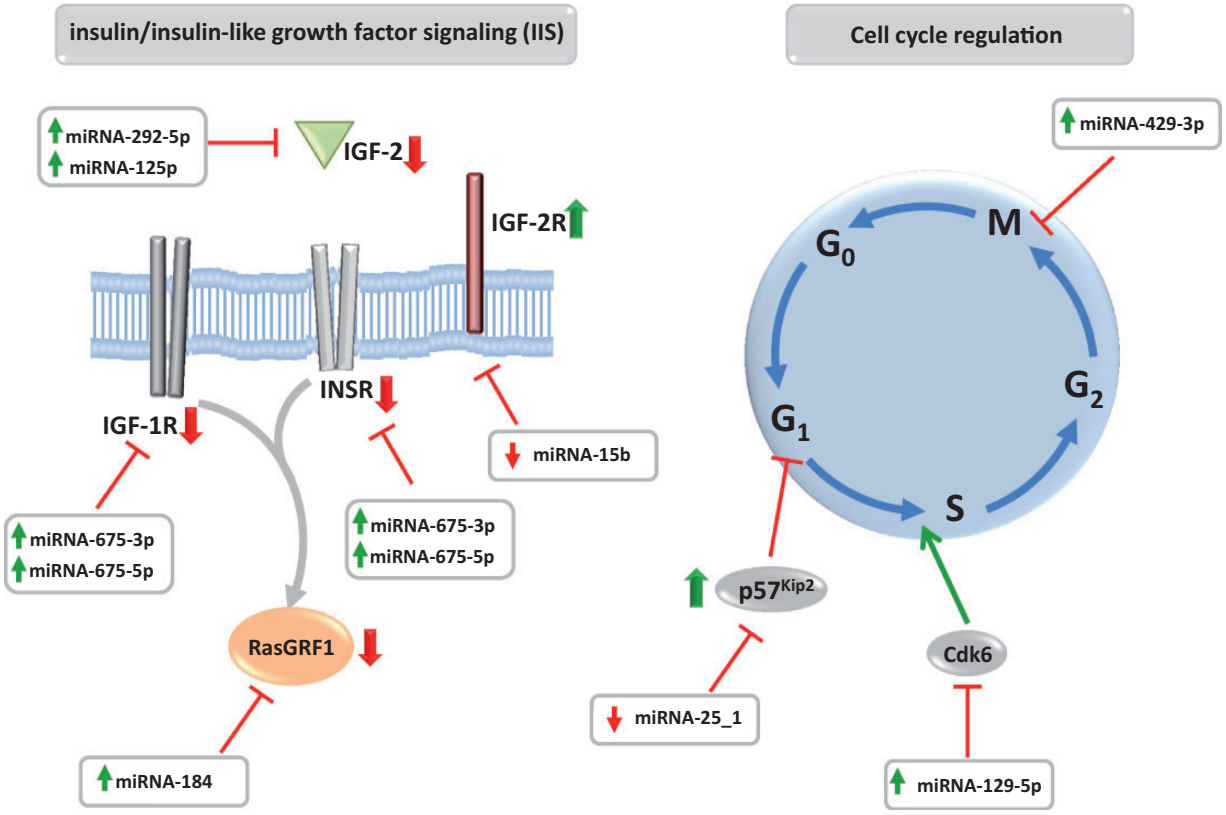

Figure 2 Genes that regulate the cell cycle and insulin/insulin-like growth factor signaling as potential targets for miRNA species identified in this study. (A color version of this figure is available in the online journal.)

IGF-2 R inhibits IIS. In fact, IGF-2 R is an imprinted gene, and VSELs highly express it on their surface as this receptor binds IGF-2 and prevents IGF-2 binding to signaling IGF-1 R and INSR.[9] As reported, miRNA-15 b plays an inhibitory role in *Igf2R* expression, and our RT-qPCR studies revealed that miR-15 b is strongly downregulated in VSELs ($P < 0.05$).

Finally, we also tracked changes in expression of certain other miRNA species that are involved in regulation of IIS. First, we found changes in expression of miRNA-470, miRNA-669 b, and miRNA-681, which are involved in IGF-1/growth hormone signaling.[36] As reported, these miRNAs negatively regulate IGF-1, IGF1R, and PI3 kinase expression,[36] and upregulation of these three miRNAs in growth hormone receptor knockout mice led to a decrease in downregulation of *IGF1*, *IGF1R*, and *PI3* kinase genes.[36] We also found that miRNA-470 and miRNA-669 b are slightly upregulated in VSELs; however, our predetermined level of significance was not reached. This suggests that this group of miRNAs contributes to attenuation of IIS but is unlikely to play a major role in regulating IIS in murine VSELs.

In conclusion, our results defined an miRNA signature contributing to the quiescent state of VSELs (Figure 2). We demonstrate significant changes in the expression level of several miRNAs species that (i) upregulate cell cycle checkpoint genes (e.g. miRNA-25_1) and (ii) attenuate IIS (e.g. miRNA-675-3 p and miRNA-675-5 p, miRNA-292-5 p, miRNA-125 b, and miRNA-184). In future, these miRNAs may be targeted using antagomirs in order to facilitate *ex vivo* expansion of these cells for the purposes of regenerative medicine.

Author contributions: All authors participated in the design, interpretation of the studies and analysis of the data and review of the manuscript; MM, GS, JR, and MS conducted the experiments; MZR and MM wrote the manuscript; GS prepared figures; MZR and MK provided funds.

ACKNOWLEDGEMENTS

This work was supported by NIH grants 2R01 DK074720 and R01HL112788 and the Stella and Henry Endowment to MZR.

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