Biochemical and Biophysical Research Communications 427 (2012) 343–348

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Matrigel Basement Membrane Matrix influences expression of microRNAs in cancer cell lines

Karina J. Price [a,b], Anna Tsykin [c,d], Keith M. Giles [a], Rosemary T. Sladic [c], Michael R. Epis [a], Ruth Ganss [e], Gregory J. Goodall [c,d,f], Peter J. Leedman [a,b,*]

[a] Laboratory for Cancer Medicine, Western Australian Institute for Medical Research and University of Western Australia Centre for Medical Research, Perth, WA 6000, Australia
[b] School of Medicine and Pharmacology, University of Western Australia, Nedlands, WA 6008, Australia
[c] Centre for Cancer Biology, SA Pathology, Adelaide, SA 5000, Australia
[d] School of Molecular and Biomedical Science, University of Adelaide, Adelaide, SA 5005, Australia
[e] Laboratory for Cancer Medicine Angiogenesis Unit, Western Australian Institute for Medical Research and University of Western Australia Centre for Medical Research, Perth, WA 6000, Australia
[f] Department of Medicine, University of Adelaide, Adelaide, SA 5005, Australia

ARTICLE INFO

Article history:
Received 24 August 2012
Available online 18 September 2012

Keywords:
MicroRNA
Matrigel
Cancer
Cell culture

ABSTRACT

Matrigel is a medium rich in extracellular matrix (ECM) components used for three-dimensional cell culture and is known to alter cellular phenotypes and gene expression. microRNAs (miRNAs) are small, non-coding RNAs that regulate gene expression and have roles in cancer. While miRNA profiles of numerous cell lines cultured on plastic have been reported, the influence of Matrigel-based culture on cancer cell miRNA expression is largely unknown. This study investigated the influence of Matrigel on the expression of miRNAs that might facilitate ECM-associated cancer cell growth. We performed miRNA profiling by microarray using two colon cancer cell lines (SW480 and SW620), identifying significant differential expression of miRNAs between cells cultured in Matrigel and on plastic. Many of these miRNAs have previously been implicated in cancer-related processes. A common Matrigel-induced miRNA signature comprised of up-regulated miR-1290 and miR-210 and down-regulated miR-29b and miR-32 was identified using RT-qPCR across five epithelial cancer cell lines (SW480, SW620, HT-29, A549 and MDA-MB-231). Experimental modulation of these miRNAs altered expression of their known target mRNAs involved in cell adhesion, proliferation and invasion, in colon cancer cell lines. Furthermore, ITGA5 was identified as a novel putative target of miR-32 that may facilitate cancer cell interactions with the ECM. We propose that culture of cancer cell lines in Matrigel more accurately recapitulates miRNA expression and function in cancer than culture on plastic and thus is a valuable approach to the *in vitro* study of miRNAs.

1. Introduction

The architecture of biological tissues permits the interaction of cells with an extracellular matrix (ECM). The ECM influences cells mechanically and chemically, altering cell phenotypes and behaviour [1–3]. In turn, cells produce, regulate and remodel their extracellular microenvironment [1]. The ECM is composed of proteoglycans, fibrous proteins, glycoproteins such as fibronectin, and contains various growth factors [1]. The ECM promotes cell differentiation, invasion and migration and can alter gene expression, cell morphology, proliferation and chemosensitivity [1,3,4]. These processes are mediated by signalling through Integrins and other cell surface adhesion site-associated molecules following their activation by ECM components and growth factors [3,5].

Conventional *in vitro* monolayer cell culture lacks the physiological input of an ECM. Alternative cell culture media containing ECM components have been utilised and promote altered cell behaviours [6]. *In vivo*, epithelial cells associate with a specialised ECM, the basement membrane. Matrigel Basement Membrane Matrix (BD Biosciences) is a commercial cell culture medium comprised of the basement membrane secretions of Engelbreth-Holm-Swarm mouse sarcoma cells [7,8]. Cancer cell culture in Matrigel is characterised by multicellular three-dimensional growth and altered cell morphology, proliferation, differentiation, chemosensitivity and gene expression [7,9–11]. Matrigel is also widely used as an injection medium for cancer cells, enhancing tumourigenicity for the establishment of xenograft tumour models

* Corresponding author at: Western Australian Institute for Medical Research, Level 6, MRF Building, Rear 50 Murray Street, Perth, WA 6000, Australia. Fax: +61 8 9224 0322.
E-mail address: peter.leedman@waimr.uwa.edu.au (P.J. Leedman).

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http://dx.doi.org/10.1016/j.bbrc.2012.09.059

Table 1
Ten most significantly up- and down-regulated miRNAs in Matrigel culture relative to growth on plastic for SW480 and SW620 cell lines.

		SW480		SW620	
MicroRNA	Direction of change	Absolute fold change	Adjusted *p*-value	Absolute fold change	Adjusted *p*-value
miR-1275	Up-regulated	14.56	3.84×10^{-16}	25.16	1.19×10^{-17}
miR-3180–3p	Up-regulated	4.39	6.35×10^{-10}	8.14	1.48×10^{-12}
miR-1290	Up-regulated	4.20	2.19×10^{-12}	7.98	2.58×10^{-15}
miR-210	Up-regulated	5.35	2.29×10^{-10}	7.38	7.87×10^{-12}
miR-943	Up-regulated	3.66	1.86×10^{-09}	6.02	6.10×10^{-12}
miR-1908	Up-regulated	3.20	1.52×10^{-10}	5.34	1.90×10^{-13}
miR-518e-5p[a]	Up-regulated	3.02	5.01×10^{-09}	4.16	4.45×10^{-11}
miR-675	Up-regulated	2.18	2.34×10^{-07}	3.97	1.97×10^{-11}
miR-584	Up-regulated	1.80	4.01×10^{-05}	3.62	2.92×10^{-10}
miR-29b	Down-regulated	5.31	3.40×10^{-09}	1.29	0.160
miR-32	Down-regulated	5.17	1.56×10^{-07}	3.21	6.21×10^{-06}
miR-33a	Down-regulated	3.61	1.05×10^{-06}	1.53	0.031
miR-886–5p	Down-regulated	1.89	1.69×10^{-06}	3.21	1.26×10^{-10}
miR-886–3p	Down-regulated	1.72	4.76×10^{-07}	3.03	6.46×10^{-12}
miR-574–3p	Down-regulated	1.64	1.34×10^{-06}	2.71	2.58×10^{-11}
miR-138–1*	Down-regulated	1.90	1.52×10^{-06}	2.64	1.91×10^{-09}
miR-576–3p	Down-regulated	1.68	7.18×10^{-06}	2.38	2.79×10^{-09}
miR-19a*	Down-regulated	1.97	2.12×10^{-04}	2.33	6.56×10^{-06}
miR-122*	Down-regulated	1.63	8.19×10^{-07}	2.20	3.30×10^{-10}

[a] Entry represents miR-518e-5p, miR-519a-5p, miR-519b-5p, miR-519c-5p, miR-522-5p and miR-523-5p.

in vivo [9,12]. Thus, Matrigel is a useful tool for studying cancer cell growth, cancer-associated gene expression and chemosensitivity [7,10,13].

While the influence of Matrigel on gene expression has been recognised [10,11], the influence of Matrigel on expression of small non-coding RNAs is largely unknown. microRNAs (miRNAs) are a class of short, endogenous, non-coding RNA molecules that bind to and repress expression of specific target mRNAs [14,15]. Bioinformatic tools to identify putative miRNA–mRNA interactions have been developed [16], and miRNAs are believed to regulate expression of over a third of all protein-coding genes [17]. miRNAs regulate a broad range of cellular processes including differentiation, proliferation and apoptosis [18,19] and aberrant expression of specific miRNAs in cancer underpins their roles as oncogenes and tumour suppressors [20].

As critical regulators of cellular and oncogenic signalling pathways there is potential for miRNAs to participate in ECM-driven changes in cell activity. We hypothesised that the culture of cancer cell lines within an ECM would provide a more representative model for the study of miRNA-regulated signalling networks *in vitro* than conventional culture on plastic. This study aimed to identify miRNAs specifically associated with cancer cell culture within Matrigel and to define potential roles of these miRNAs in ECM-associated processes relating to cell growth and oncogenesis.

2. Methods

2.1. Cell culture

The SW480, SW620, HT-29, A549 and MDA-MB-231 human cancer cell lines were purchased from the American Type Culture Collection (ATCC). Cells were grown in monolayer on standard cell culture plastic at 37 °C in 5% CO_2 with Dulbecco's modified Eagle's medium supplemented with 10% foetal calf serum, or in Matrigel as described by Lee et al. [12]. Cells were cultured in Matrigel at 37 °C with 5% CO_2 for 10 days.

2.2. RNA isolation

Total RNA was isolated from monolayer and Matrigel cultures using QIAzol lysis reagent (QIAGEN) according to manufacturer's instructions. Cells in Matrigel were first incubated in QIAzol for 15 min to permit complete dissociation.

2.3. miRNA microarray

miRNA expression profiling of cells grown in monolayer and embedded in Matrigel was performed with spotted miRNA microarrays containing the miRCURY Locked Nucleic Acid (LNA) probe library, version 11 (Exiqon), with duplicate probe spots. Biological duplicates of each RNA sample were used and a dye-swap implemented for each contrast. 5 μg of RNA was ligated with Cy3 or Cy5 fluorescent dinucleotide labels (Dharmacon) as previously described [21] and competitive hybridisation of RNA performed overnight at 56 °C. Slides were manually washed using the LNA microRNA Array Kit (Exiqon) stringency wash protocol and reagents. Arrays were scanned and digital images created using a GenePix 4000B Scanner (Axon) with GenePix Pro 4.0 Software (Molecular Devices). Spot identification and intensity extraction from digital images was performed (Spot; http://experimental.act.cmis.csiro.au/Spot/index.php, CSIRO, Australia) using a seeded region growing algorithm. A morphological opening background correction was applied to mean spot signal intensities [22] [Richie ME (2004), unpublished data].

2.4. Transfection of miRNA molecules

Monolayer cultured cancer cell lines were transfected with synthetic pre-miRNA precursor molecules (Ambion) corresponding to human miR-210 (Product ID: PM13679) and a negative control miRNA (pre-miRNA Precursor Negative Control #1, Product ID: AM17110) or with miRCURY LNA miRNA inhibitor molecules (Exiqon) against miR-29b (Product ID: 410005-00), miR-32 (Product ID: 410006-00) or a scrambled control molecule (Product ID: 199002-00) using Lipofectamine 2000 (Invitrogen). RNA was extracted from cells at 24 h.

2.5. Reverse transcription and quantitative Polymerase Chain Reaction (RT-qPCR)

For analysis of miRNA expression TaqMan miRNA RT-qPCR assays (Applied Biosystems, part number 4427975) were performed for miR-210 (assay ID 000512), miR-1290 (assay ID 002863),

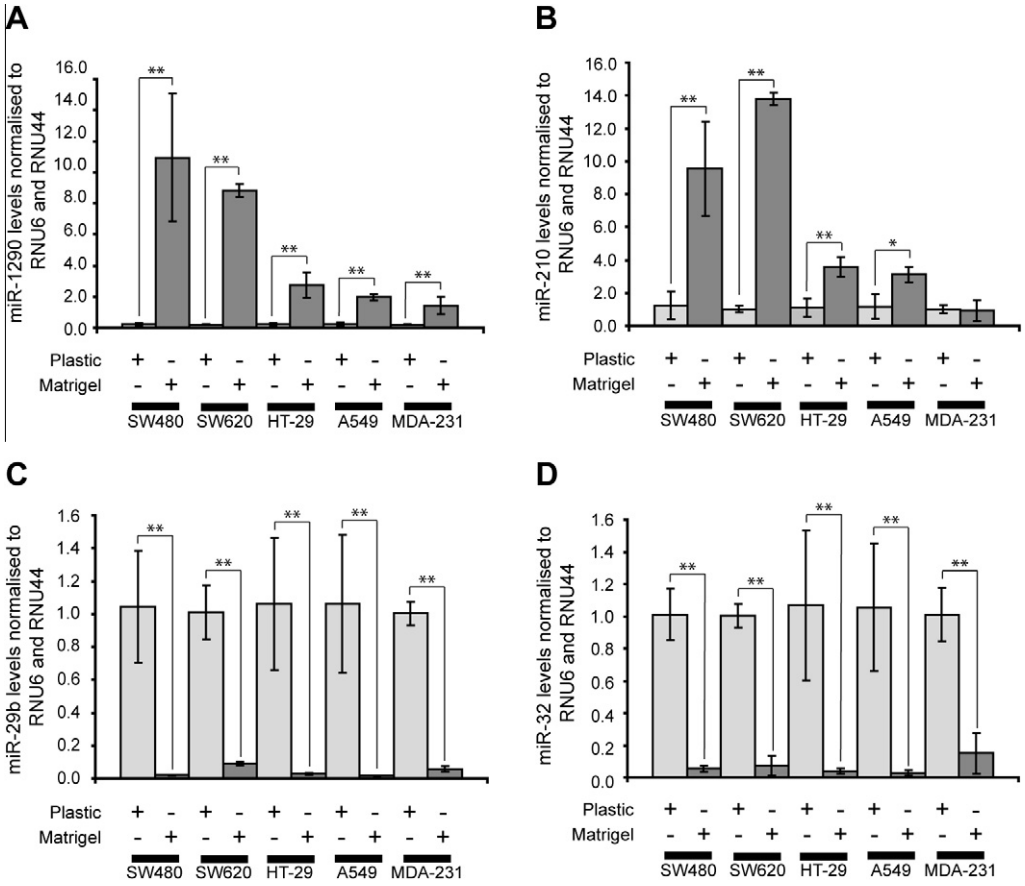

Fig. 1. A Matrigel-induced miRNA signature is consistent across five epithelial cancer cell lines. RT-qPCR analysis of (A) miR-1290, (B) miR-210, (C) miR-29b and (D) miR-32 expression in five epithelial cancer cell lines: SW480, SW620, HT-29, A549 and MDA-MB-231 (indicated as MDA-231) grown in Matrigel relative to expression with growth on plastic. miRNA expression was normalised to RNU6 and RNU44 snRNA, and indicated as a ratio of Matrigel:plastic-grown cells (±SD). Asterisks indicate a significant difference in expression from plastic-grown cells (** = $p < 0.01$. * = $p < 0.05$).

miR-32 (assay ID 002109), miR-29b (assay ID 000413), and two reference RNAs, RNU6B (assay ID 001093) and RNU44 (assay ID 001094) small nuclear RNA (snRNA). Biological duplicates for each condition were used. Duplicate RT-PCRs were performed using 10 ng of sample RNA and qPCR reactions set up in duplicate.

For analysis of mRNA expression, 250 ng of total RNA was reverse transcribed in triplicate using the QuantiTect Reverse Transcription kit (QIAGEN). Biological RT duplicates for each condition were prepared. qPCR primer sequences were sourced from PrimerBank (http://pga.mgh.harvard.edu/primerbank) [23] (Supplementary Table 1) and qPCR reactions prepared in duplicate using the SensiMix SYBR Hi-ROX kit (Bioline). geNorm and NormFinder utilities in GenEx software (MultiD) were used to select optimal reference genes from a set of five (HPRT1, PPIA, β-actin, B2M and GAPDH).

2.6. Ingenuity Pathway Analysis (IPA) and miRNA target prediction

Datasets of significantly altered (adjusted p-value < 0.01) miRNAs from microarray comparisons were analysed using Ingenuity Pathway Analysis (IPA; Ingenuity Systems, http://www.ingenuity.com) to identify putative miRNA functions. IPA Core Analyses were performed using default settings.

TargetScan (Release 6.2: June 2012; http://www.targetscan.org/) [17], PicTar (March 2007 release; http://pictar.mdc-berlin.de/) [24] and miRanda-mirSVR (August 2010 release; http://www.microrna.org/) [25] algorithms were used to identify putative mRNA targets for miRNAs of interest.

2.7. Statistical analysis of microarray and RT-qPCR data

Analysis of microarray data was performed in R: A Language and Environment for Statistical Computing (R Foundation for Statistical Computing, Vienna, Austria, http://cran.r-project.org) with the LIMMA (Linear Models for Microarray Data; WEHI, Australia) [26] package of BioConductor (http://www.bioconductor.org) [27]. Intensity-dependent global loess normalization was used to correct for dye-bias relative to spot intensity and data in individual arrays were scaled to each other [28]. Within-array replicate spots were treated as technical replicates [29]. Differentially expressed miRNAs were identified using a moderated t statistic approach [30]. p values were calculated from t statistics and adjusted for multiple testing by controlling the false discovery rate [31]. A mean over median quality control was implemented. See Supplementary Table 2 for full, processed array data.

Statistical analysis of RT-qPCR data was performed using GenEx Software (MultiD). Normality of data was confirmed by Kolmogorov–Smirnov (KS) test, where appropriate. Significance was assessed using Student's t-test. All analyses were performed at a minimum confidence interval of 95% (CI = 0.95).

3. Results and discussion

3.1. A Matrigel-induced miRNA signature in epithelial cancer cell lines and its implications for the behaviour of cancer cells within the ECM

Using a microarray approach we profiled miRNA expression in SW480 and SW620 colon cancer cell lines from monolayer or

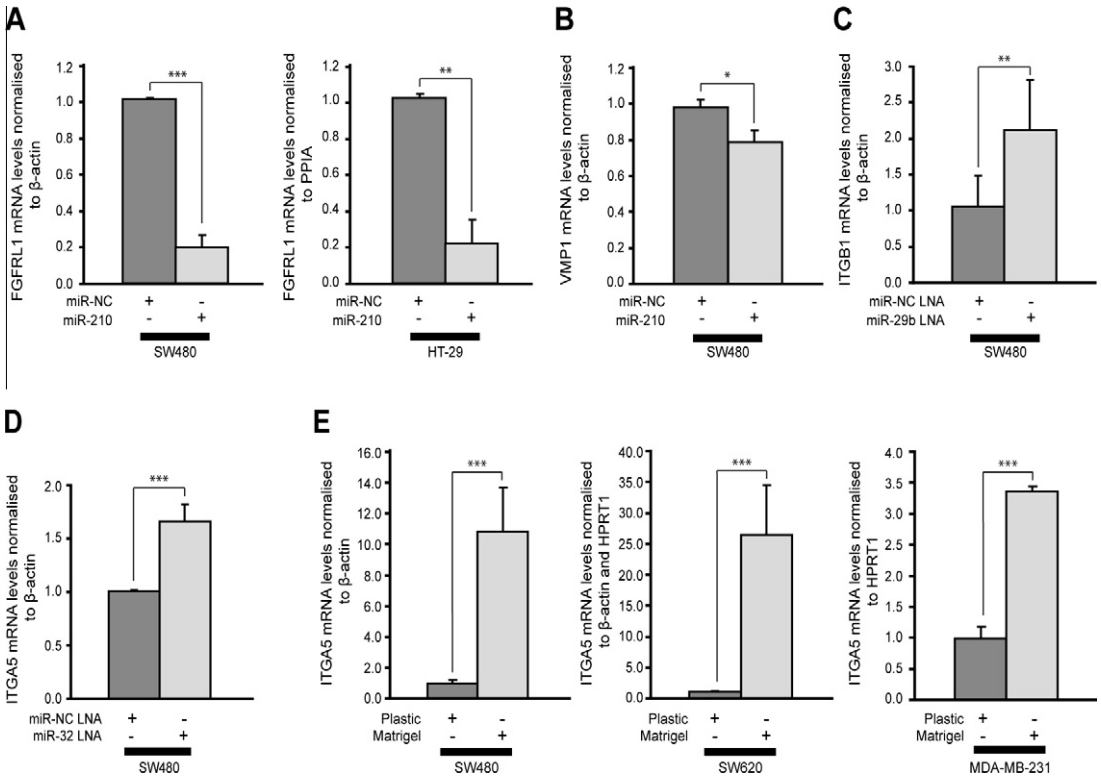

Fig. 2. Matrigel-regulated miRNAs control mRNA expression of genes involved in cancer cell adhesion, proliferation and invasion. RT-qPCR analysis of (A) FGFRL1 mRNA expression in SW480 and HT-29 cells and (B) VMP1 mRNA expression in SW480 cells post-transfection with 10 nM miR-210 precursor (miR-210) or negative control precursor (miR-NC) molecules. RT-qPCR analysis of (C) ITGB1 and (D) ITGA5 mRNA expression in SW480 cells post-transfection with 10 nM miR-29b LNA inhibitor (miR-29b LNA) and 1 nM miR-32 LNA miRNA inhibitor (miR-32 LNA), respectively, compared to mRNA levels following transfection with an equivalent concentration of negative control LNA inhibitor (miR-NC LNA). (E) RT-qPCR analysis of ITGA5 mRNA expression in three epithelial cancer cell lines (SW480, SW620 and MDA-MB-231) grown in Matrigel relative to growth on plastic. Target mRNA expression was normalised as indicated. Results are indicated as a ratio of miRNA:miR-NC-transfected cell expression or Matrigel:plastic-grown cells (±SD). Asterisks indicate a significant difference in expression from miR-NC-transfected cells or cells grown on plastic ($^{***} = p < 0.001$. $^{**} = p < 0.01$. $^{*} = p < 0.05$).

Matrigel culture. For each cell line a significant change in expression of a number of miRNAs was observed when comparing the two growth conditions (Table 1. See Supplementary Table 2 for full, processed array data). Many of the changes in miRNA expression due to Matrigel culture were common to both cell lines. For example, miR-1275 was the most significantly up-regulated miRNA between Matrigel and monolayer culture in both cell lines (~15 and ~25-fold, respectively; Table 1). A number of significantly down-regulated miRNAs were also common to the two cell lines, including miR-32 (Table 1).

We investigated putative biological functions of miRNAs with significantly altered expression (adjusted p-value < 0.01) between Matrigel and monolayer culture for SW480 and SW620 cell lines using IPA. Based on predicted and known miRNA targets both datasets of Matrigel-regulated miRNAs from SW480 and SW620 cells were enriched for those associated with "Cancer (Carcinoma)" (Supplementary Table 3). miRNAs associated with biological functions with specific relevance to ECM-associated cancer cell growth, including "Growth and Proliferation of Tumour Cell Lines", "Migration of Tumour Cell Lines" and "Contact Growth Inhibition" were also identified from the Matrigel-regulated datasets (Supplementary Table 3), suggesting that miRNAs regulated by Matrigel-based cell culture may, in part, mediate the characteristic behaviour of cancer cells seen with three-dimensional growth.

We next hypothesised that culture of cancer cell lines in Matrigel might induce a characteristic miRNA signature across different types of epithelial cancer cell lines. We focused on the expression of several miRNAs most significantly altered by Matrigel culture of both SW480 and SW620 cell lines; miR-1290, miR-210, miR-29b and miR-32, using TaqMan RT-qPCR to determine their expression in Matrigel culture relative to monolayer growth for SW480, SW620 and three additional human epithelial cancer cell lines: HT-29 (colon cancer), A549 (non-small cell lung cancer) and MDA-MB-231 (breast cancer). We observed a Matrigel-regulated miRNA signature across the five epithelial cancer cell lines, with significant up-regulation of miR-1290 in all cell lines (Fig. 1A) and miR-210 in SW480, SW620, HT-29 and A549 (Fig. 1B) and down-regulation of miR-29b and miR-32 in all five cell lines (Fig. 1C and D respectively) following culture in Matrigel. Taken together, these data indicate a consistent pattern of Matrigel-regulated miRNA expression across different epithelial cancer cell types.

3.2. Matrigel-regulated miRNAs control mRNA expression of genes involved in cell adhesion, proliferation and invasion

We next investigated whether the Matrigel-induced miRNA signature we identified could influence expression of downstream target mRNAs that are associated with growth in an ECM. miR-210 has a number of validated target mRNAs that regulate stress responses and growth [32], including Fibroblast Growth Factor Receptor-Like 1 (FGFRL1), which regulates cell–cell adhesion and cancer cell proliferation [33,34], and Vacuole Membrane Protein 1 (VMP1), which participates in tumour cell migration, invasion and metastasis [35]. The miR-29 miRNA family mediates ECM homeostasis, regulates production of a number of structural ECM proteins [36] and has been implicated in control of cell proliferation, migration, and invasion [37]. It has previously been

demonstrated that miR-29b regulates expression of Integrin subunit, beta 1 (ITGB1) [38], which is involved in cell adhesion and migration, and promotes metastasis [39]. miR-32 is frequently dysregulated in cancer [40–42], but has few proven targets. We identified human Integrin subunit, alpha 5 (ITGA5) as a strongly predicted miR-32 target using the TargetScan, miRanda and PicTar prediction algorithms. Interestingly, the ITGA5 Integrin subunit and the miR-29b target ITGB1 are known to form a heterodimeric Integrin α5/β1 fibronectin (FN) receptor that mediates cell adhesion and migration [43].

We first assessed the capacity of miR-210 to regulate FGFRL1 and VMP1 mRNA in colon cancer cell lines and found that transient transfection of miR-210 to mimic its Matrigel-induced up-regulation significantly decreased FGFRL1 mRNA expression in SW480 and HT-29 cells (Fig. 2A) and VMP1 mRNA in SW480 cells (Fig. 2B). We then used LNA inhibitors to block the activity of endogenous miR-29b and miR-32 in SW480 cells and observed increased expression of ITGB1 (Fig. 2C) and ITGA5 (Fig. 2D) mRNA, respectively. To complement our finding that endogenous miR-32 has the capacity to regulate ITGA5 mRNA levels we used RT-qPCR to demonstrate that SW480, SW620 and MDA-MB-231 cells grown in Matrigel express higher levels of ITGA5 mRNA than cells grown on plastic (Fig. 2E). Taken together, these results suggest that endogenous miR-32 may regulate ITGA5 expression and that miR-210 up-regulation and miR-29b and miR-32 down-regulation by Matrigel culture has the capacity to alter expression of specific target mRNAs, such as FGFRL1, VMP1 and ITGB1, which have been implicated in cancer cell adhesion, proliferation and invasion.

3.3. A proposed model of miRNA involvement in interactions between cancer cells and the ECM

Based on literature and our current findings we propose a model in which expression of Matrigel-regulated miRNAs might be controlled by the extracellular environment and, in turn, how these miRNAs might function to influence the behaviour of cells and their interaction with the ECM (Fig. 3). miR-210 is known to be transcriptionally induced by Hypoxia Inducible Factor 1 alpha HIF1α in response to cellular stress and hypoxia in cancer [44]. Furthermore, Matrigel induced up-regulation of HIF1α has been demonstrated [45] and ECM-related properties relevant to Matrigel culture have been shown to influence HIF1α expression; for example, activity of Prolidase, a collagen-degrading enzyme [46]. Together, these findings suggest a possible mechanism for Matrigel-induced up-regulation of miR-210 via HIF1α. miR-29b expression is reported to be suppressed by Transforming Growth Factor, beta (TGFβ) [36,47], a growth factor found in both Matrigel and the ECM *in vivo* [7]. The Matrigel-induced down-regulation of miR-29b could in part be mediated by the TGFβ within Matrigel. Interestingly, miR-29b has been shown to decrease expression of TGFβ *in vivo* [47], suggesting the possibility of a regulatory feedback loop that maintains the reciprocity between cells and the surrounding ECM. Matrigel-regulated miRNAs may co-operate in regulatory networks to influence multiple targets with related functions in order to facilitate cell survival and growth within the ECM, such as the potential convergence of miR-29b and miR-32 on Integrin subunits ITGB1 and ITGA5, respectively. Our model highlights the potential networking of miRNA regulatory pathways to achieve a coordinated cellular outcome and suggests that miRNAs may be regulated by the cellular context and environment, fine-tuning gene expression to facilitate cell growth and survival under these conditions. We propose that the concept of "dynamic reciprocation" [2] between cells and the ECM be revised to include miRNAs as regulatory molecules controlling processes relevant to this interaction, with further studies needed to define the specific roles of Matrigel-regulated miRNAs in ECM-associated cancer cell growth.

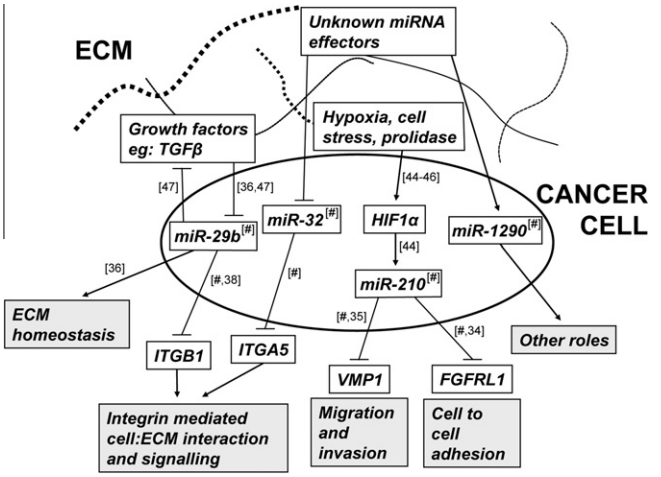

Fig. 3. A proposed model of miRNA involvement in cancer cell–ECM interactions. Structural components of the ECM, factors contained within it (e.g. TGFβ) and inherent conditions (e.g. those inducing cell stress) influence a cell's miRNA expression. For example, HIF1α may act as an intermediate between the ECM and miR-210, regulating miR-210 expression in response to ECM conditions. ECM-regulated miRNAs in turn influence the ECM and cell behaviour. For instance, a feedback loop exists between miR-29b and TGFβ. Targets of Matrigel-regulated miRNAs may regulate processes facilitating cell growth and survival within an ECM, such as miR-210 targets FGFRL1 and VMP1 which mediate cell adhesion, migration and invasion. Similarly, miR-29b and miR-32 converge on Integrin subunits ITGB1 and ITGA5, respectively, to mediate Integrin signalling and cell interaction with the ECM. The model exemplifies the potential networking of ECM-influenced miRNA regulatory pathways to achieve a coordinated cellular outcome and facilitate cell growth and survival within the ECM. # indicates a finding from this study.

Prior studies have shown distinct clustering of cancer cell lines and tumour samples of the same type based on their miRNA expression profiles [48,49]. The regulation of a set of cancer-associated miRNAs by Matrigel *in vitro* suggests that the ECM influence may induce a shift in miRNA expression to one that is more representative of miRNA expression *in vivo*, and may provide a more relevant cell culture system than traditional monolayer culture for the study of specific miRNAs involved in cell–ECM interaction and of miRNA-mediated signalling networks in cancer. The potential for Matrigel to alter miRNA expression may also have implications for the study of miRNAs *in vivo*, where Matrigel is often used as an injection medium for tumour cells in animal studies. Interpretation of such experiments should take into consideration the effect of Matrigel itself on miRNA expression, given the capacity for miRNAs to broadly influence gene expression and their involvement in diverse cellular processes.

Acknowledgments

KP is the recipient of an Australian Postgraduate Award (APA) and also funding from the University of Western Australia via a Richard Walter Gibbon scholarship.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.09.059.

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