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KLK6-regulated miRNA networks activate oncogenic pathways in breast cancer subtypes

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ABSTRACT

KLK6 is expressed in normal mammary tissues and is aberrantly regulated in breast cancer. At physiological levels of expression, *i.e.* those found in normal mammary tissues, KLK6 acts as a tumor suppressor in human breast cancer. However, aberrant overexpression of KLK6 (*i.e.* 50–100-fold higher than normal), a characteristic of a subset of human breast cancers is associated with increased tumorigenicity (Pampalakis et al. Cancer Res 69:3779–3787, 2009). Here, we stably transfected KLK6-non-expressing MDA-MB-231 breast cancer cells with the full-length *KLK6* cDNA to overexpress KLK6 at levels comparable to those observed in patients, and investigated potential oncogenic miRNA networks regulated by these abnormally high KLK6 expression levels and increased activity of this serine protease. A number of miRNAs that are upregulated (*e.g.* miR-146a) or downregulated (*e.g.* miR-34a) *via* KLK6-induced alterations in the miRNA biogenesis machinery were identified. Integrated experimental and bioinformatics analyses identified convergent miRNA networks targeting the cell cycle, MYC, MAPK, and other signaling pathways. In large clinical datasets, significant correlations between *KLK6* and downstream MAPK and MYC targets at both the RNA and protein levels was confirmed, as well as negative correlation with GATA3. It was also demonstrated that KLK6 overexpression and likely its proteolytic activity is associated with alterations in downstream miRNAs and their targets, and these differ with the molecular subtypes of breast cancer. The data partly explains the different characteristics of breast cancer subtypes. Importantly, we introduce a combined KLK6-CDKN1B+MYC+CDKN1C score for prediction of long-term patient survival outcomes, with higher scores indicating poor survival.

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1. Introduction

Breast cancer is the most commonly diagnosed cancer, and the leading cause of death by cancer worldwide for women, comprising 23% of total cancer incident and 14% of deaths by cancer in 2008 (Jemal et al., 2011). In addition to the well-known histological subtypes that are recognized in breast cancer, distinct molecular and "biological" subtypes with different clinical behaviors have been documented (Reis-Filho and Pusztai, 2011). Recent literature has demonstrated higher metastatic potential and reduced survival outcomes of the luminal B subtypes (Creighton, 2012; Engstrom et al., 2013). Interestingly, despite lower histological tumor grades, luminal A and B subtypes have worse outcomes and positive nodal status compared to basal-like subtypes (Creighton, 2012; Engstrom et al., 2013; Voduc et al., 2010). An in-depth understanding of the molecular mechanisms that underlie breast tumor phenotypes is vital in our move to an era of personalized medicine (Pasic et al., 2013).

Kallikrein-related peptidases (KLKs) constitute a family of 15 serine proteases mapped to chromosome 19q13.4 (Sotiropoulou et al., 2009a; Yousef et al., 2005) that are expressed in many tissues including breast, ovary, prostate, testis, skin, and kidney (Diamandis and Yousef, 2002). The expression of KLKs is dysregulated in various cancers including prostate, breast, and ovarian cancers (Yousef and Diamandis, 2002). Also, KLKs represent enzymes which can be targeted pharmacologically for various disorders (Sotiropoulou and Pampalakis, 2012). KLK6 is an active trypsin-like serine protease auto-catalytically regulated (Ghosh et al., 2004; Bayés et al., 2004; Sotiropoulou et al., 2003). KLK6 expression is significantly increased in lactating women, while also associated with TGFβ signaling (Qin et al., 2012). KLK6 was originally cloned based on its altered expression during breast cancer progression and was named protease M (Anisowicz et al., 1996). It was found significantly down-regulated or completely inactivated in metastatic breast cancers (Anisowicz et al., 1996) due to methylation of CpGs at the *KLK6* proximal promoter (Pampalakis et al., 2009; Pampalakis and Sotiropoulou, 2006). Nonetheless, although a subset of neoplastic mammary tumors display highly induced (50–100-fold higher than normal) levels of KLK6 resulting from promoter hypomethylation (Pampalakis et al., 2009; Anisowicz et al., 1996), elevated *KLK6* expression in early-stage ovarian tumors is due to gene amplification (Ni et al., 2004).

MiRNAs are small non-coding RNAs, which negatively regulate gene expression by binding to the 3′ untranslated region of complementary mRNAs, leading to degradation of the mRNA and/or inhibition of their translation. It is well-established that miRNAs are dysregulated in many cancers, and depending on the context, they can function either as oncogenes or as tumor suppressors (Zhang et al., 2007; Sotiropoulou et al., 2009b). Recent evidence pinpointed that miRNA networks synergistically control biological pathways (Peter, 2010). On the other hand, KLKs were shown to be regulated by miRNAs (Chow et al., 2008; Pasic et al., 2012) in kidney, prostate and ovarian cancers (White et al., 2012; White et al., 2010a,b). Recent studies suggest that miRNAs also constitute downstream targets of KLKs (Sidiropoulos et al., 2014).

The role of miRNAs as downstream effectors of dysregulated KLK6 expression was investigated and groups of miRNAs were identified that are differentially expressed in response to KLK6 induction at levels observed in breast tumor patients. Target prediction and pathway analyses indicated that these miRNAs are involved in divergent and convergent miRNA networks that regulate cell signaling, MYC and cell cycle pathways which contribute to aggressive breast cancer behavior. We also demonstrate that KLK6 is differentially expressed in the distinct breast cancer subtypes, resulting in alterations of miRNA-mediated pathways and their downstream cell signaling, contributing to tumor phenotype. Furthermore, our results show that KLK6-miRNA-cell signaling among the different phenotypic molecular groups of breast cancer leads to altered survival outcomes.

2. Materials and methods

2.1. Cell lines

The MDA-MB-231 breast cancer cell line was stably transfected to over-express KLK6 as described previously (Pampalakis et al., 2009).

2.2. Total RNA extraction and quantitative real-time PCR

Total RNA was extracted from cells using RNeasy (Qiagen, Mississauga, Canada). The concentration of RNA was determined spectrophotometrically and the quality was assessed using the Agilent 2100 Bioanalyzer (Agilent, Santa Clara, USA). Samples were stored at −80 °C and used only if they had a RIN ≥ 8.0. One μg total RNA was reverse-transcribed using high capacity cDNA reverse transcription (Life Technologies, Grand Island, USA). *LGALS1* and peptidylprolyl isomerase A (*PPIA*) gene expressions were used as endogenous controls. qPCR reactions were carried out on the Step One Plus Real-Time PCR System (Life Technologies, Grand Island, USA).

2.3. MiRNA profiling

Global miRNA expression changes between KLK6 over-expressing cells and mock-transfected cells were analyzed by miRNA microarrays. RNA was extracted and cDNA was synthesized from 200 ng of total RNA using the TaqMan® MicroRNA Reverse Transcription (Life Technologies) and the Megaplex™ RT primers (Life Technologies) as per the manufacturer's instructions. Briefly, 200 ng total RNA was mixed with 10×Megaplex RT primers, 100 mM dNTPs, 10×RT Buffer, 25 mM $MgCl_2$, and 20 U/μL RNase inhibitor. Samples underwent 40 cycles of 16 °C for 2 min, 42 °C for 1 min and 50 °C for 1 s. The reaction was stopped by heating at 85 °C for 5 min. Microarray analyses were performed using TaqMan® Array Human MicroRNA A + B Cards Set v3.0 (Applied Biosystems®). The cDNA was added to TaqMan Master mix and loaded onto miRNA arrays A and B. Arrays were run on the Applied Biosystems ViiA™ 7 real-time PCR System and data were analyzed using the Expression Suite Software (Life

Technologies). All miRNA expressions on array A were normalized with hsa-let-7d (present on array A) and those on array B were normalized using hsa-miR-151-3p (present on array B). All reported expression levels were determined using the mock cells as calibrator.

2.4. MiRNA target prediction and validation

Target prediction analysis was performed using: TargetScan-Human 6.2 (http://www.targetscan.org/), DIANA-mirPath (http://diana.cslab.ece.ntua.gr/pathways/), PITA database (http://genie.weizmann.ac.il/pubs/mir07/mir07_prediction.html), MiRanda (http://www.microrna.org/microrna/home.do), DIANA-microT (http://www.diana.pcbi.upenn.edu/cgi-bin/micro_t.cgi), RNA22 (http://cbcsrv.watson.ibm.com/rna22.html), and miRDB (http://mirdb.org/miRDB/). The altered miRNAs were probed for their gene targets. Predicted gene targets were confirmed to have altered expression in KLK6 overexpressing cells compared to mock cells by mRNA microarray expression analysis.

2.5. Pathway enrichment analysis

Pathway enrichment analysis was performed using DIANA miRPath v2.1 (Papadopoulos et al., 2009). DIANA miRPath pathway enrichment software analysis (http://diana.cslab.ece.ntua.gr/) was employed to gain insight into global molecular networks and canonical pathways related to differentially expressed miRNAs between KLK6 over-expressing and mock cells. The miRPath identified multiple miRNA target genes using enrichment analysis by comparing each set of miRNA gene targets to all known KEGG pathways. Pathways with $p < 0.05$ were considered significantly enriched.

2.6. MAPK mRNA profiling

The cDNA was synthesized as previously and was added to the MAPK Signaling Pathway RT^2 Profiler™ PCR array (Qiagen/SuperArray Biosciences) as per the manufacturer's protocol. Data was analyzed using the Expression Suite software.

2.7. SiRNA-mediated RNA interference mimic experiments

SiRNA mimics of miR-34a and non-targeting siRNAs were purchased from Santa Cruz Biotechnologies. The cells were transfected with specific or non-mimic siRNA using siPORT NeoFX transfection agent (AM4510) for 24 or 48 h, and were, then, used for subsequent experiments.

2.8. Clinical data validation

We validated our results on an independent set from The Cancer Genome Atlas (TCGA) databases (Cancer Genome Atlas Network, 2012). The breast cancer (BRCA) dataset was downloaded from Cancer Genome Atlas public data portal and from cBio Cancer Genomics at the Memorial Sloan-Kettering Cancer Center. Data was analyzed from approximately 1106 breast cancer cases, which comprised of tumor RNA Seq V2 RSEM (n = 904) or mRNA microarray expression data (n = 526) and tumor miRNA array expression data (n = 302). For expression data analysis, Z-scores or "level 3" normalized data from mRNA and miRNA microarrays were used. Molecular subtype data included luminal B (n = 132), luminal A (n = 236), basal-like (n = 81), and Her-2 enriched (n = 58). For microarray analysis, we employed the Genomics Browser for data visualization (Zhu et al., 2009). RPPA protein expression data was analyzed using patients with at least +0.5 Z-score (increase of more than +0.5 protein expression from the mean patient protein expression).

3. Results

3.1. Breast cancer associated KLK6 overexpression affects miRNA biogenesis and miRNA expression

We compared the expression levels of 754 human miRNAs between MDA-MB-231 breast cancer cell lines stably-transfected with the cDNA encoding the full-length KLK6 protein and mock-transfected pcDNA3.1 (+) counterparts. We identified 49 miRNAs that were upregulated and 14 miRNAs that were downregulated more than a 1.0-fold difference upon KLK6 overexpression (Figure 1A) with the top upregulated and down-regulated miRNAs shown in Table 1. Upregulated miRNAs included: miR-146a and miR-106a; and down-regulated miRNAs: miR-34a, miR-579, miR-324-5p, miR-10b, and miR-888. We validated altered expression of miR-34a by quantitative real-time PCR and verified that miR-34a was significantly reduced in KLK6 over-expressing cells (Figure 1B).

To explore the mechanism by which KLK6 can suppress miRNA expression, we examined the expression of a number of molecules involved in miRNA biogenesis (Figure 1C). KLK6 overexpression resulted in increased expression of multiple members of the miRNA biogenesis machinery including *Drosha*, which encodes the ribonuclease type III enzyme that acts in the first step of the miRNA maturation process. Also increased was the *Exportin5* mRNA, which enhances export of miRNAs from the nucleus to the cytoplasm, as well as *Dicer* and *Argonaute* (*Ago*) 1 and 2 mRNAs. Interestingly, analysis of the 3′ UTR of these miRNA biogenesis genes showed that most of the targets contained miRNA response elements for miRNAs, which were significantly downregulated upon KLK6 overexpression (Table 2). These results indicated that the effect of KLK6 on miRNAs were mediated, at least in part, by enhancing the expression of the genes involved in miRNA biogenesis.

3.2. The impact of KLK6 overexpression on signaling pathways

We performed target prediction of the most significantly dysregulated miRNAs followed by pathway analysis with the most significantly predicted gene targets of the top dysregulated miRNAs shown in Table 3. The most enriched pathways were the PI3K/Akt and the MAPK signaling pathways in both

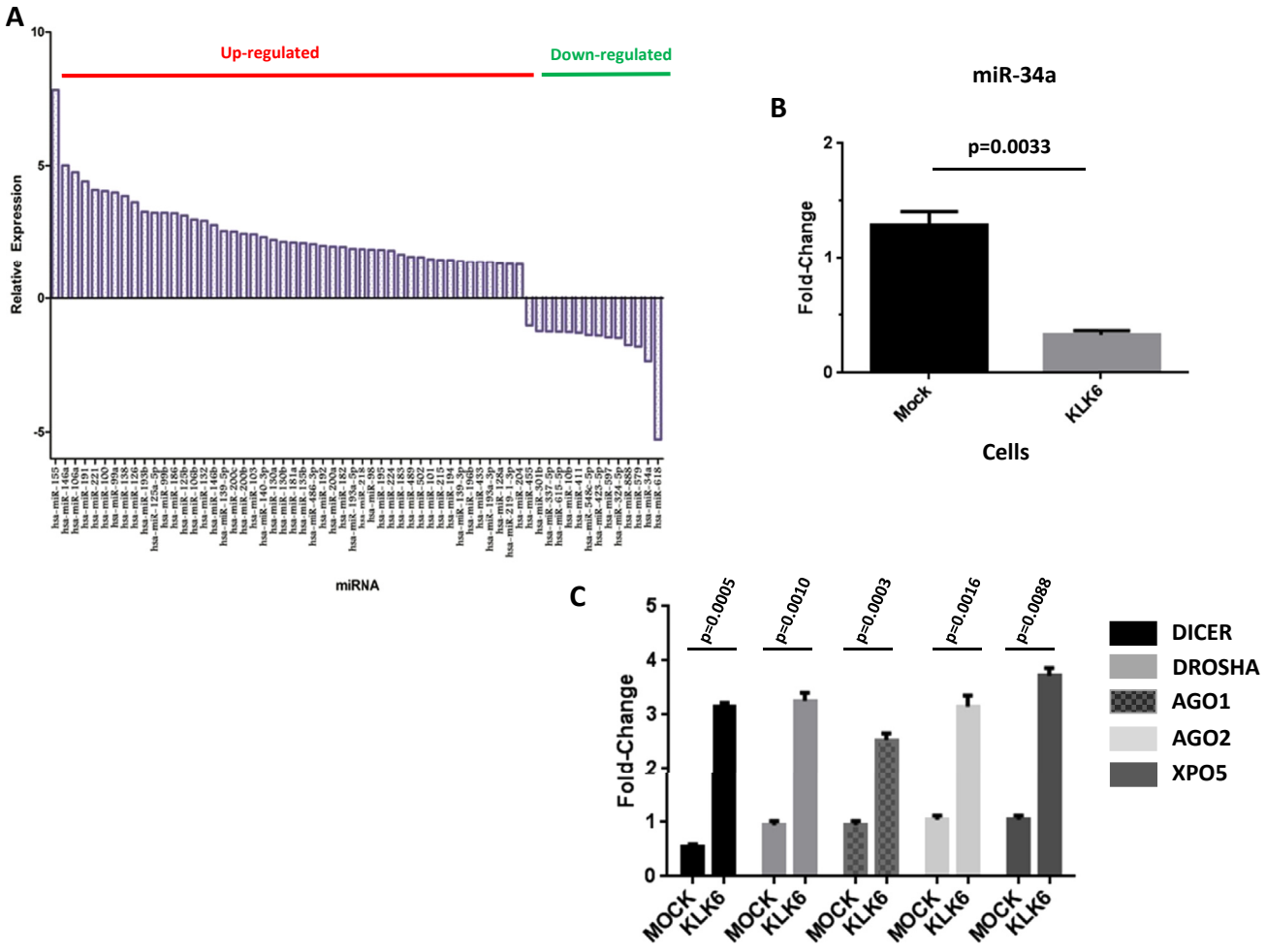

Figure 1 – A, KLK6 overexpression resulted in miRNA differential expression. 49 miRNAs were found to be upregulated and 14 miRNAs were downregulated as compared to mock-transfected cells. B, Confirmation of miR-34a downregulation in KLK6 overexpressing cells. Expression levels are shown as relative expression values to mock cells. C, Effect of KLK6 overexpression on key enzymes of miRNA biogenesis machinery. All experiments were performed in triplicates.

upregulated and downregulated miRNAs (52 miRNA targets in total). KLK6-related miRNAs were also enriched in Wnt signaling, signaling pathways in cancer, focal adhesion, and ErbB pathways.

Table 1 – Top dysregulated miRNAs in KLK6-overexpressing MB-MDA-231 cells compared to control cells.

Downregulated miRNAs		Upregulated miRNAs	
miRNA	Fold change	miRNA	Fold change
hsa-miR-618	−5.2818722	hsa-miR-155	7.828585
hsa-miR-34a	−2.3528504	hsa-miR-146a	5.005828
hsa-miR-579	−1.8131033	hsa-miR-106a	4.745738
hsa-miR-888	−1.7544025	hsa-miR-191	4.405273
hsa-miR-324-5p	−1.4891950	hsa-miR-221	4.083472
hsa-miR-597	−1.4657150	hsa-miR-100	4.040123
hsa-miR-423-5p	−1.3931675	hsa-miR-99a	3.985336
hsa-miR-548c-5p	−1.3781151	hsa-miR-138	3.859505
hsa-miR-411	−1.2890109	hsa-miR-126	3.626809
hsa-miR-10b	−1.2637239	hsa-miR-193b	3.270992
hsa-miR-615-5p	−1.2540911	hsa-miR-125a	3.230955

To validate our pathway analysis, we compared MAPK and cell cycle signaling between KLK6 overexpressing and mock cells using profiling arrays (see Methods and materials 2.3 & 2.6). We found significant alterations in the expression of genes associated with cell signaling pathway upon KLK6 overexpression. An overall increase in expression of transcription factors including *MYC* (Figure 2A), *MAPK* signaling molecules (Figure 2B–D), cell cycle genes with an increase in *CDKN1C*, *CCNA1* (*cyclin A*), *CCNB1* (*cyclin B*) and *CCNE1* (*cyclin E*) (Figure 2E), and *RAS* gene families (Figure 2F) was found. There was, however, a noticeable decrease in expression of *CDKN1B* (Figure 2E).

3.3. A *KLK6-miRNA-CDKN1C axis*

KLK6 overexpression resulted in a 2-fold increase in *CDKN1C* mRNA levels compared to mock cells (Figure 3A). A highly conserved 7 nucleotide miR-34a recognition site was identified in the *CDKN1C* 3′ UTR (Table 2). To examine if the effect of KLK6 overexpression on *CDKN1C* was mediated through downregulation of miR-34a, we transfected KLK6 overexpressing cells with miR-34a mimics which resulted in

Table 2 – Top KLK6-dysregulated miRNAs and corresponding binding sites at the 3′UTR of target genes.

miRNA	Gene	RefSeqID	Seed length	Start	Sequence	End	p-value	SPMS
hsa-miR-888	DICER1	NM_030621	9	6281	ACUCAAAAA	6273	0.0163	2
hsa-miR-888	DICER1	NM_177438	9	6337	ACUCAAAAA	6329	0.0163	2
hsa-miR-34a	XPO5	NM_020750	8	3914	UGGCAGUG	3907	0.0234	1
hsa-miR-548c-3p	RNASEN	NM_001100412	8	4642	AAAAAUCU	4635	0.0142	2
hsa-miR-548c-3p	RNASEN	NM_013235	8	4677	AAAAAUCU	4670	0.0142	2
hsa-miR-615	EIF2C1	NM_012199	10	5652	GGGGGUCCCC	5643	0.0045	1
hsa-miR-615	EIF2C1	NM_012199	9	5651	GGGGUCCCC	5643	0.0177	2
hsa-miR-34a	MAPK13	NM_002754.4	7		CACUGCC		0.0166	2
hsa-miR-34a	MAP2K1	NM_002755	8	2237	UGGCAGUG	2230	0.0143	1
hsa-miR-34a	MYCN	NM_005378	8	1725	UGGCAGUG	1718	0.0139	1
hsa-miR-34a	MYCN	NM_005378	8	2282	GGCAGUGU	2275	0.0139	2
hsa-miR-34a	CDKN1C	NM_000076	7	1873	CACUGCC	1879	0.0428	2
hsa-miR-146a	CDKN1B	NM_004064	8	1518	GACAGCTGA	1525	0.0203	2
hsa-miR-34a	MYCN	NM_005378	10–15		AGCCA–UAAUGUAAACUGCC			
hsa-miR-106A	CCND1	NM_053056	12–15		ACCAUUCCAUUUCCAAGCACUUU			
hsa-miR-579	CCNA1	NM_003914	7	1878	UUCAUUU	1872	0.0132	1
hsa-miR-106A	GATA3	NM_001002295	8	2171	UGCAAUGU	2164	0.0178	2
hsa-miR-146A	GATA3	NM_001002295	8	2519	CUCUGAAA	2512	0.0178	2

Table 3 – KLK6 overexpressing enriched miRNA-mediated pathways.

Downregulated miRNAs				Up-regulated miRNAs			
KEGG pathway	p-value	# genes	# miRNAs	KEGG pathway	p-value	# genes	# miRNAs
PI3K-Akt signaling	4.83E-17	131	21	MAPK signaling	7.49E-21	121	31
MAPK signaling	1.16E-10	100	21	Pathways in cancer	3.42E-25	162	30
Cholinergic synapse	2.73E-05	47	21	PI3K-Akt signaling	2.99E-30	157	30
Endocytosis	8.34E-30	96	20	Focal adhesion	2.16E-35	103	30
Wnt signaling	4.19E-21	72	20	Neurotrophin signaling	1.71E-25	71	30
Long-term potentiation	1.70E-19	37	20	Prostate cancer	2.88E-19	53	30
Dopaminergic synapse	1.11E-15	60	20	HTLV-I infection	7.41E-14	117	29
Pathways in cancer	1.75E-14	132	20	Chemokine signaling	2.54E-05	76	29
Calcium signaling	6.66E-14	76	20	Insulin signaling	5.83E-26	74	29
HIF-1 signaling	1.79E-08	46	20	ErbB signaling pathway	2.65E-22	58	29
HTLV-I infection	1.16E-06	93	20	Renal cell carcinoma	6.75E-18	43	29
Oocyte meiosis	1.47E-06	50	20	Regulation of actin cytoskeleton	8.32E-32	109	28
Tight junction	1.47E-06	53	20	Wnt signaling	6.03E-34	89	28
Amebiasis	0.01123572	38	20	Hepatitis B	1.28E-10	71	28

reduced expression of *CDKN1C* in a dose- and time-dependent manner compared to control (Figure 3B), thus confirming our hypothesis.

3.4. A *KLK6-miRNA-MYC axis*

Additionally, KLK6 overexpression resulted in a 1.6-fold increase in *MYC* mRNA levels compared to parental cells (Figure 3C; parental 1.00 ± 0.11 and KLK6 high 1.69 ± 0.10; $p = 0.01$). A highly conserved 10–15 nucleotide miR-34a response element (AGCCA–UAAUGUAAACUGCC) was identified in the *MYC* 3′ UTR (Table 2). To examine if the effect of KLK6 on *MYC* expression is mediated through miR-34a down-regulation, KLK6 overexpressing cells were transfected with miR-34a mimic which resulted in reversal of the inhibition of KLK6 on miR-34a, and reduced *MYC* expression in a dose- and time-dependent manner compared to a non-target mimic (Figure 3D).

3.5. A *KLK6-miRNA-MAPK13 axis*

KLK6 overexpression resulted in 3-fold induction of *MAP13K* mRNA (Figure S1A). Several miRNA-binding sites were identified in the *MAPK13* mRNA including a highly conserved miR-34a site (CACUGCC) in its 3′ UTR (Table 2). We hypothesized that the effect of KLK6 on *MAPK13* expression can be mediated by suppression of miR-34a. Upon transfection of KLK6 overexpressing cells with a miR-34a mimic, we found reduced *MAPK13* expression compared to a non-target mimic (Figure S1B).

3.6. A *KLK6-miRNA-MAP2K1 axis*

KLK6 overexpression resulted in 1.5-fold increase in *MAP2K1* expression compared to control cells (Figure S1C). A statistically significant and highly conserved miR-34a response element (UGGCAGUG) was identified in its 3′ UTR (Table 2). To test whether the effect of KLK6 on *MAP2K1* expression can be

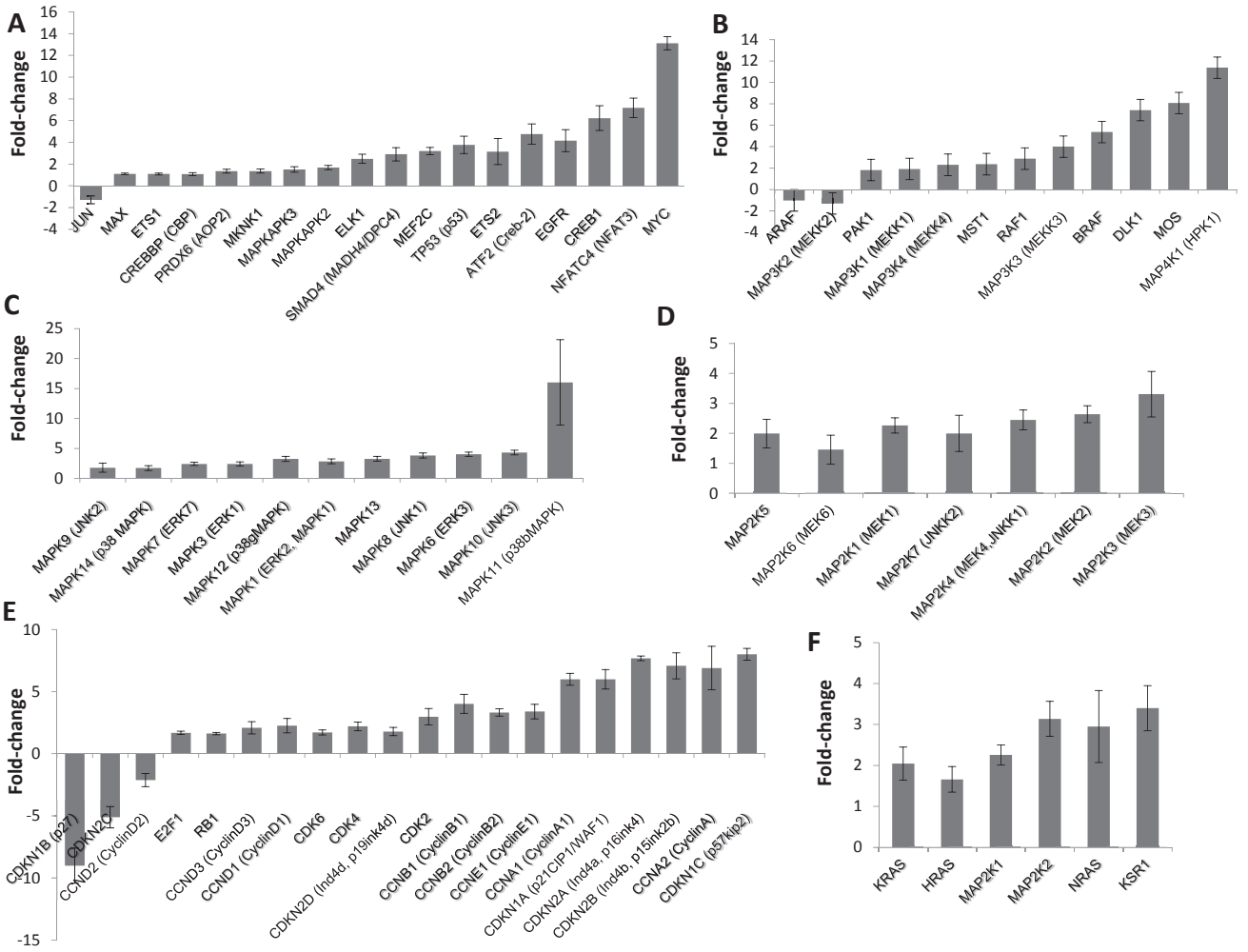

Figure 2 – The effect of KLK6 transfection on miRNA-mediated signaling pathways. Panels of MAPK signaling and related factors were assayed for expression changes between KLK6 overexpressing and mock cells. In general, expression of mRNAs encoding for transcription factors constituents, including MYC and EGFR, were increased with KLK6 overexpression (A). MAPK-KK and MAPK-K expression was also increased (B and C), as well as MAPK expression (D). Cell cycle signaling molecules were also generally increased (E), with CDKN1C experiencing the highest increase in mRNA expression with KLK6 overexpression. RAF regulating genes had increased mRNA expression with KLK6 (F), including KRAS, NRAS, and HRAS.

mediated through downregulating miR-34a, we transfected KLK6 overexpressing cells with the miR-34a mimic and found reduced *MAP2K1* expression in a dose- and time-dependent manner compared to a non-target control (Figure S1D).

3.7. *Clinical validation of the KLK6-miRNA correlations*

In order to explore the KLK6-miRNA network axis *in vivo*, we assayed the TCGA database for breast cancer patient expression correlations between KLK6 and its downstream miRNA targets. KLK6 expression was significantly reduced ($p < 0.0001$) in the luminal B (-0.64 ± 0.45; CI 95%: -0.7210 to -0.5643) breast cancer subtype as compared with basal-like breast cancers (1.34 ± 1.15; CI 95%: 1.081 to 1.590) (Figure 4A). All other comparisons were significant $p < 0.01$, with the exception of HER2 to luminal A. Expression of miRNA-34a showed the opposite pattern, with lower levels in basal-like compared to luminal B cancers (significance $p < 0.01$, mean of differences: 0.76) (Figure 4B). MiR-106a and miR-146a were confirmed to have a positive correlation with KLK6 levels with elevated expression in basal-like compared to luminal A or B cancers (Figure 4C and D), $p < 0.01$. We determined that miR-10b expression was inversely correlated to KLK6 expression with the lowest levels in basal-like subtype patients, as compared to luminal A or B (Figure 4E), $p < 0.01$. To further confirm our data in the clinical context, we visualized a larger clinical dataset (IlluminaHiSeq, $n = 1106$). The expression of miR-106a correlated positively with KLK6 expression in breast cancer subtypes, with both showing higher expression in basal-like subtypes, while the opposite pattern was seen for miR-34a (Figure 4F).

3.8. *Clinical validation of miRNA-cell cycle targets in breast cancer subtypes*

In order to validate miRNA network regulation of cell-cycle genes, we assayed the TCGA database. *CDKN1C* and *MYC*

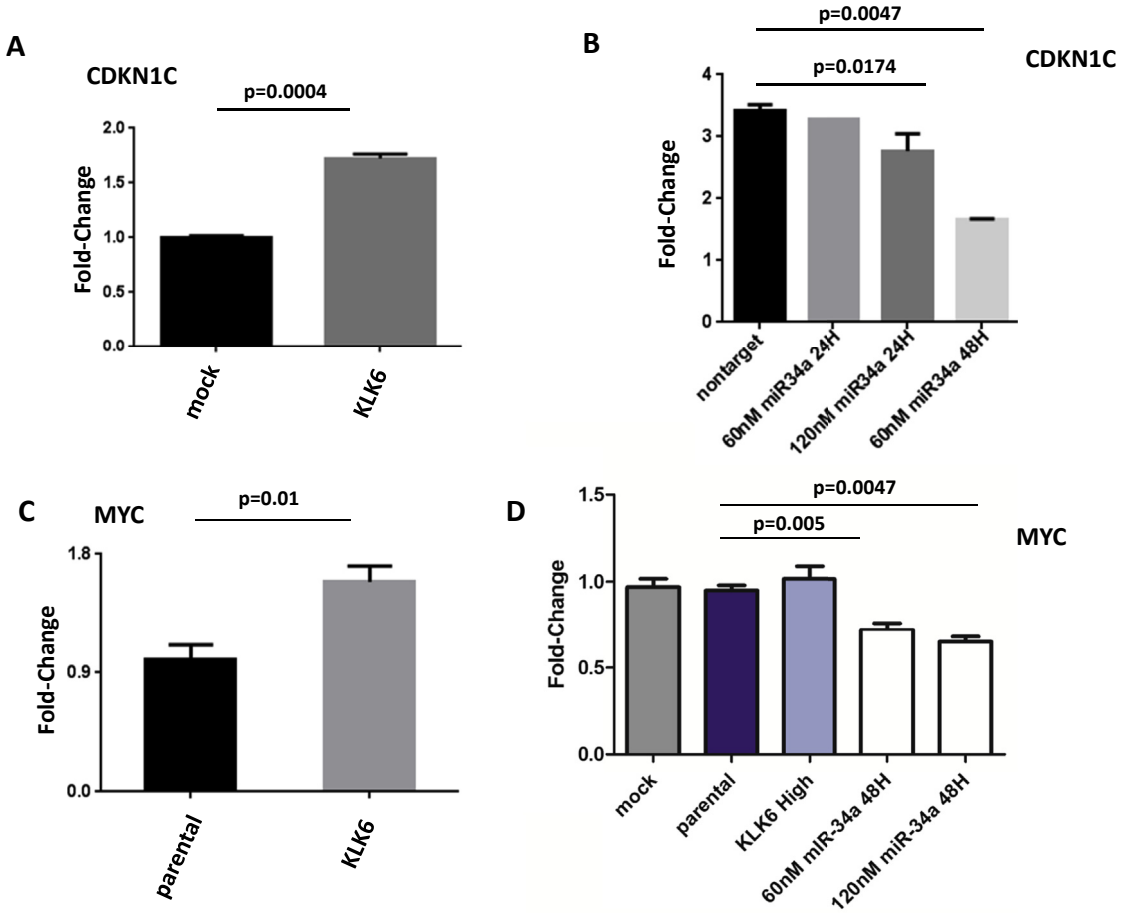

Figure 3 – Experimental validation of CDKN1C and MYC. A, There was significant CDKN1C mRNA upregulation, in KLK6 overexpressing cells. Expression values were normalized against expression in mock cells. B, Transfection with a miR-34A mimic resulted in inhibition of CDKN1C expression, confirming that the effect of KLK6 on CDKN1C is through inhibition by a miR-34A pathway. C, Real-time PCR showed significant MYC up-regulation, in KLK6 over-expressing compared to mock cells. D, Transfection of a miR-34A mimic resulted in a significant downregulation of MYC. All experiments were performed in triplicates.

were positively correlated with high KLK6 expressing molecular subtypes (and reduced miR-34a expression). *CDKN1C* and *MYC* show higher expression in basal-like (*CDKN1C*: 0.26 ± 1.30; *MYC*: 1.16 ± 0.10, n = 81), and lower expression in luminal B subtypes, which had lower KLK6 expression (*CDKN1C*: −0.51 ± 0.91; *MYC*: 0.24 ± 0.08, n = 131), $p < 0.0001$ (Figure 5A and B). *CDKN1B*, a validated target of mir-146a, was found to be negatively correlated to KLK6 expressing breast cancer subtypes with lower expression in basal-like subtype compared to luminal B subtypes, $p = 0.0112$ (Figure 5C). To further confirm our data, we visualized a second clinical dataset (IlluminaHiSeq, n = 1106), which demonstrated higher expression of KLK6 in normal tissue compared metastatic tissues (Figure 5D). Interestingly, expression of KLK6 was higher in basal-like and normal-like breast cancer subtypes compared to luminal B. There was reduced expression in normal tissue and basal subtypes with higher expression in luminal (ER+) and Her-2 subtypes for *GATA3*, and *CCND1*.

3.9. *Clinical correlation of KLK6 expression with miRNA gene target expression*

In order to correlate gene expression changes of KLK6 in breast cancer patients with KLK6-miRNA gene targets, a large-scale coordinate expression analysis of patients was conducted. KLK6 mRNA was positively correlated with *MYC*, *CDKN1C*, *CCNA1*, *CCNE1*, *CDK6*, and *CCNB1* expression (Figure 6A–F). KLK6 mRNA expression was negatively correlated with *CDKN1B* mRNA expression (Figure 6G); *CCND1* mRNA expression (Figure 6H); and *GATA3* mRNA expression (Figure 6I).

3.10. *Clinical correlation of KLK6 expression with miRNA gene target expression at the protein levels*

In order to correlate changes of KLK6 expression with downstream protein alterations, a co-expression analysis of patients with significantly elevated KLK6 mRNA expression were grouped in an “overexpressed” expression group (Z-

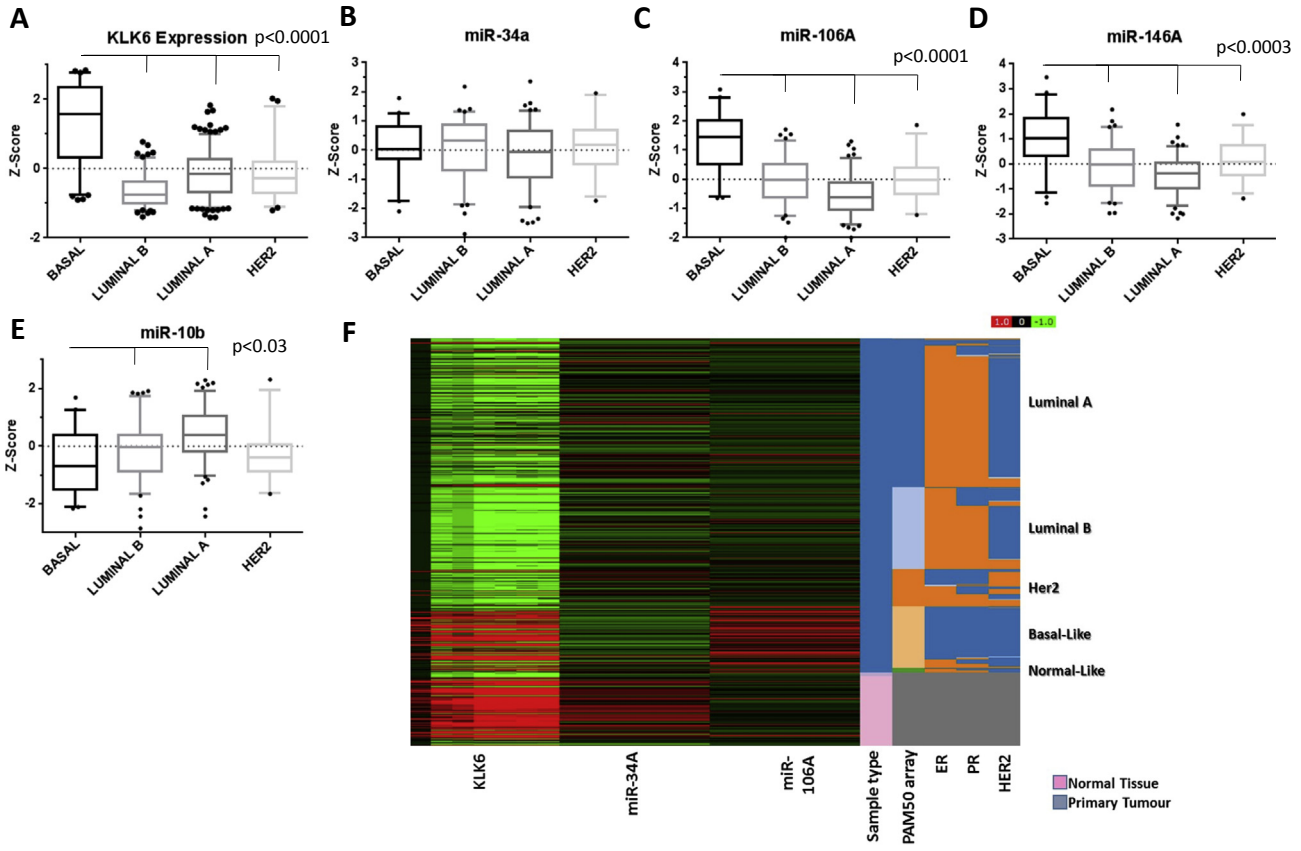

Figure 4 – Validation of the expression of KLK6 and its related miRNAs in clinical specimens. Expression levels were compared to the breast cancer molecular subtypes in the TCGA dataset. A, There was significantly increased expression of KLK6 in basal-like breast cancers (mean: 1.33) as compared with luminal B types (mean: −0.64, $p < 0.0001$), luminal A (mean: −0.15, $p < 0.0001$) and Her2 (mean: −0.15, $p < 0.0001$). B, miR-34a expression levels were lower in the basal subtype compared to luminal B, although this did not reach statistical significance. C, MiR-106A was significantly increased in basal-like (mean: 1.25) as compared to luminal B (mean: −0.04, $p < 0.0001$), luminal A (−0.55, $p < 0.0001$) and Her2 (0.005, $p < 0.0001$). D, miR-146A was significantly increased in basal-like (mean: 1.051) as compared to luminal B (mean: −0.07, $p < 0.0001$), luminal A ($p < 0.0001$) and Her2 ($p = 0.0002$). E, miR-10b was significantly reduced in basal-like (mean: −0.58) as compared to luminal B (mean: −0.16, $p < 0.03$) and to luminal A (mean: 0.40, $p < 0.0001$). F, Microarray visualization of KLK6 and its regulated miRNAs and mRNA targets in breast cancer molecular subtypes. IlluminaHiSeq data visualized using the Cancer Genomics Browser ($n = 1106$ patients). Red indicates gene expression of +1 Z-score, green −1, and black 0. Orange indicates positive and blue negative hormonal status.

score > 0.5, or expression of KLK6 mRNA more than 0.5 times the mean of all patients). Patients who had less 0.5 times the mean of KLK6 mRNA were grouped into an "underexpressed" group. The groups were then compared to RPPA protein expression of selected miRNA gene targets (Figure 7). MYC, CDK1, CCNB1, CCNE1 protein expression was significantly elevated in the overexpressed group (breast cancer patients highly expressing KLK6) (Figure 7A, B, D, and F), while CDKN1B, CCND1, and GATA3 protein expression was significantly reduced in breast cancer patients with tumors that expressed KLK6 at abnormally high levels (overexpressed group) (Figure 7C, E, and G).

3.11. *Survival analysis*

A composite gene score (KLK6-CDKN1B+MYC+CDKN1C) predicting long-term survival of breast cancer patients was generated based on our experimental findings, and following observations that in patients with metastatic tumor these genes were significantly altered regardless of breast cancer subtype. The composite survival score was equally weighted. Breast cancer patients with the lowest scores (green, n = 736) had longer long-term survival as compared to patients with a higher score (red, n = 332) (Figure 8A). Also, we tested the potential correlation of KLK6 expression on overall survival. Patients had reduced survival with over-expressed KLK6 levels (red, expression higher than 1.32, n = 352) as compared to lower (green, expression less than −1.72, n = 362) KLK6 expression levels (Figure 8B). Patients with physiological expression of KLK6 (brown; −1.72 to 1.32; n = 366) had significantly improved overall long-term survival.

3.12. *A model to predict KLK6-miRNA interactions*

Collectively, the findings of our study are depicted in Figure 9. In summary, we hypothesize that differential KLK6

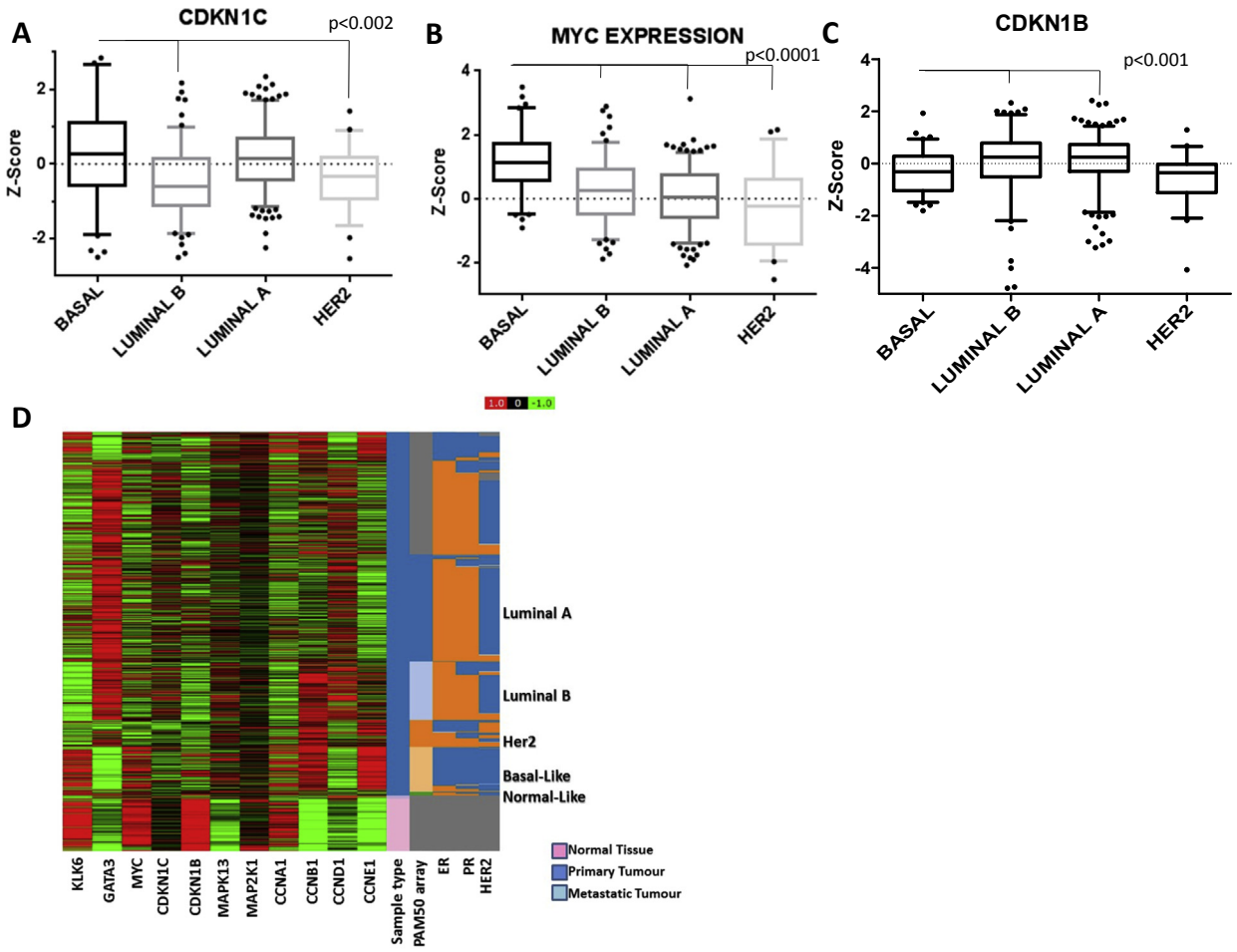

Figure 5 – *In vivo* validation of findings with clinical patient data. The mRNA expression was examined with respect to breast cancer molecular subtype in the entire TCGA clinical dataset for all subtypes. A, *CDKN1C* expression was significantly increased in basal-like (mean: 0.26 ± 1.30; n = 81) as compared to luminal B subtypes (mean: −0.51 ± 0.91; n = 131, p < 0.0001). B, MYC expression was significantly increased in basal-like (mean: 1.16 ± 0.10: n = 81) as compared with luminal B (mean: 0.24 ± 0.08; n = 131, p < 0.0001). C, CDKN1B expression was significantly reduced in basal-like (mean: −0.33 ± 0.09; n = 81) as compared to luminal B (mean: 0.07 ± 0.11; n = 131, p < 0.001). Data plotted as Box and Whiskers plot (5–95% with outliers plotted). D, Microarray visualization of KLK6 and its regulated miRNAs and mRNA targets in breast cancer molecular subtypes. Data are presented as in Figure 4, p < 0.0001. The dataset included 81 basal-like breast cancer patients, 129 luminal B breast cancers, 232 luminal A breast cancers, and 58 Her-2 breast cancers.

expression in the breast cancer molecular subtypes affects expression of a network of interacting miRNAs. These miRNA networks were enriched in MAPK, cell cycle and cell signaling targets.

4. Discussion

Dysregulation of miRNAs expression in breast cancer plays important roles in the process of carcinogenesis, and can be exploited for the stratification and identification of patients at risk in developing the disease (Mulrane et al., 2013). Our results are in line with current literature documenting miRNA involvement in breast cancer progression (Zhang et al., 2007). The data provides insights into the potential regulatory roles of KLK6 in controlling the expression of a network of miRNAs and their gene targets with putative future diagnostic and therapeutic implications.

Previously, it was known that KLK6 is highly expressed in several malignancies including breast (Wang et al., 2008; Neve et al., 2006; Yousef et al., 2004; Anisowicz et al., 1996), ovarian (Seiz et al., 2012), colon (Petraki et al., 2012; Ogawa et al., 2005), and gastric cancers (Nagahara et al., 2005). We showed that KLK6 expression in normal mammary tissue and in basal-like breast cancers is significantly elevated compared to other breast cancer subtypes. KLK6 expression negatively correlated with GATA3 expression, which correlates with a basal/myoepithelial lineage (Smalley et al., 2008). Therefore, we postulate that high KLK6 levels are important and contribute to the basal/myoepithelial lineage phenotype. In proven metastatic tumors, and molecular subtypes with a higher incidence of distant metastases (luminal B, luminal A and Her-2 types) KLK6 was found to be

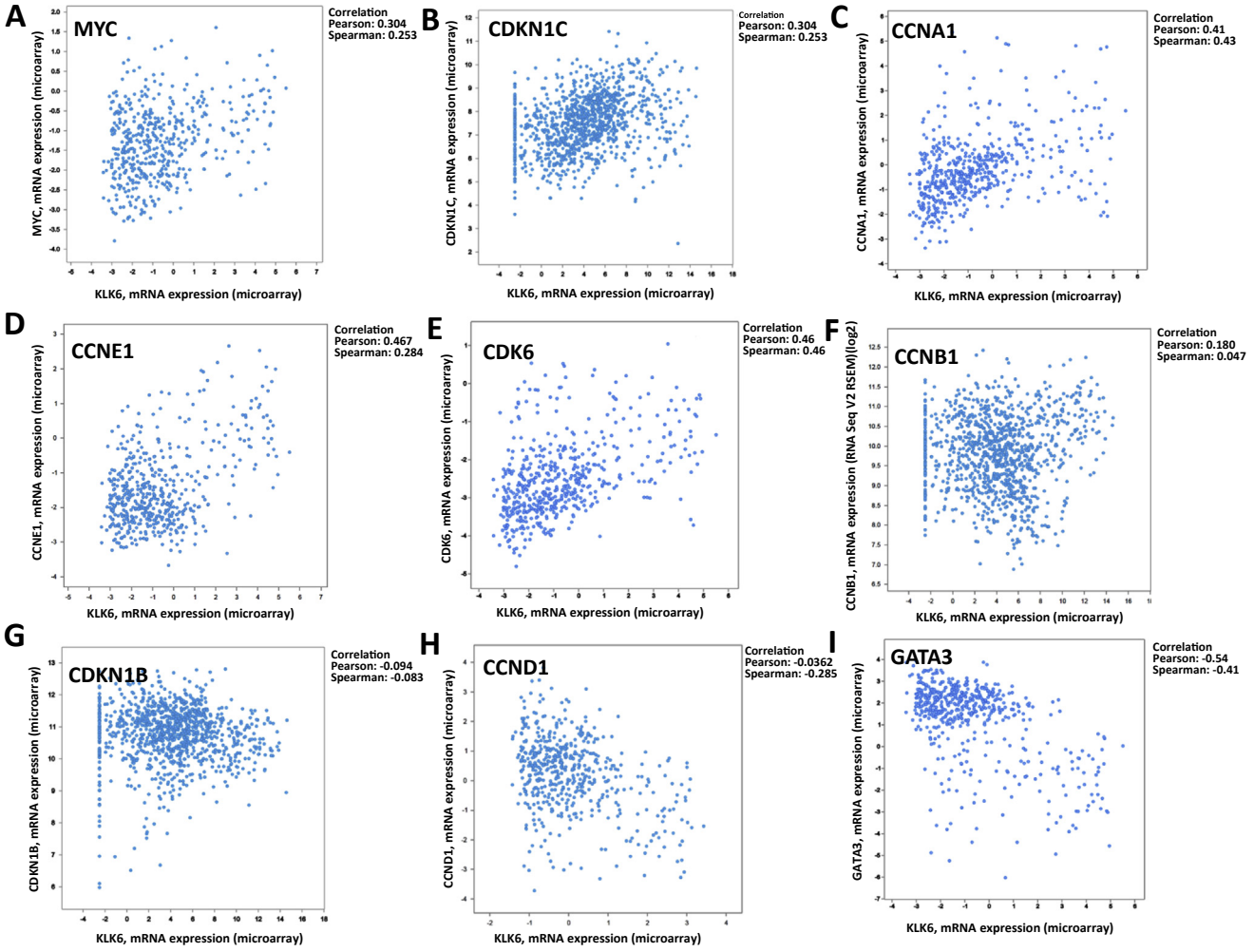

Figure 6 – Co-expressional analysis of KLK6 with miRNA gene targets. Co-expressional analysis demonstrates a positive expressional relationship between KLK6 and MYC (A), CDKN1C (B), CCNA1 (cyclin A1) (C), CCNE1 (cyclin E1) (D), CDK6 (E), and CCNB1 (cyclin B) (F). Co-expressional analysis demonstrates a negative expressional relationship between KLK6 and CDKN1B (G), CCND1 (cyclin D1) (H), and GATA3 (I) (n = 1065).

downregulated. Moreover, basal-like breast cancers are usually triple negative (ER−, PR−, HER2−), thus, our data are supported by Wang et al. which demonstrated that KLK6 protein was significantly reduced (negatively correlated) in metastatic breast cancers with positive nodal status but also significantly reduced in ER+ breast cancers (Wang et al., 2008).

In general, KLK6 has been shown to be upregulated in breast cancer, however in metastatic breast cancer it was downregulated (Pampalakis et al., 2009; Anisowicz et al., 1996). In order to study this relationship further, we performed analysis of survival data that confirmed that KLK6 expression above or below physiological levels, leads to a reduced overall survival of breast cancer patients. This may be partly explained by a balance of KLK6 serine protease activation, with downstream extracellular matrix (ECM) signaling homeostasis.

The data demonstrated that a balance between KLK6 protease activity, tumor progression and suppression exists. The KLK6 survival data, and experiments showed that reconstitution of KLK6 expression at physiological levels in MDA-MB-231 led to inhibition of tumor cell proliferation and motility, anchorage-independent growth, while remarkably abolishing their ability to form tumors in SCID mice (Pampalakis et al., 2009). However, when KLK6 was overexpressed compared to physiological levels, tumor-suppressing activity was lost (Pampalakis et al., 2009). In the present study, dysregulation of KLK6 levels, specifically overexpression, affected vital cell cycle and cell signaling.

We showed that KLK6 overexpression upregulates miR-106A and miR-146A. MiR-146A is known to inhibit migration and invasion, and to suppress NF-κB activity and reduce the metastatic potential of breast cancer cells (Bhaumik et al., 2008). MiR-106A has up to 700 gene targets and is upregulated in breast cancer patients' serum and tissue (Sinha et al., 2008; Wang et al., 2010). Interestingly, miR-106A plays an important role in regulating the cell cycle by regulating retinoblastoma (Rb) and p21/CDKN1A tumor suppressors (Zhou et al., 2010; Trimis et al., 2008). Several studies have demonstrated in a number of different cancers that miR-106A upregulation is

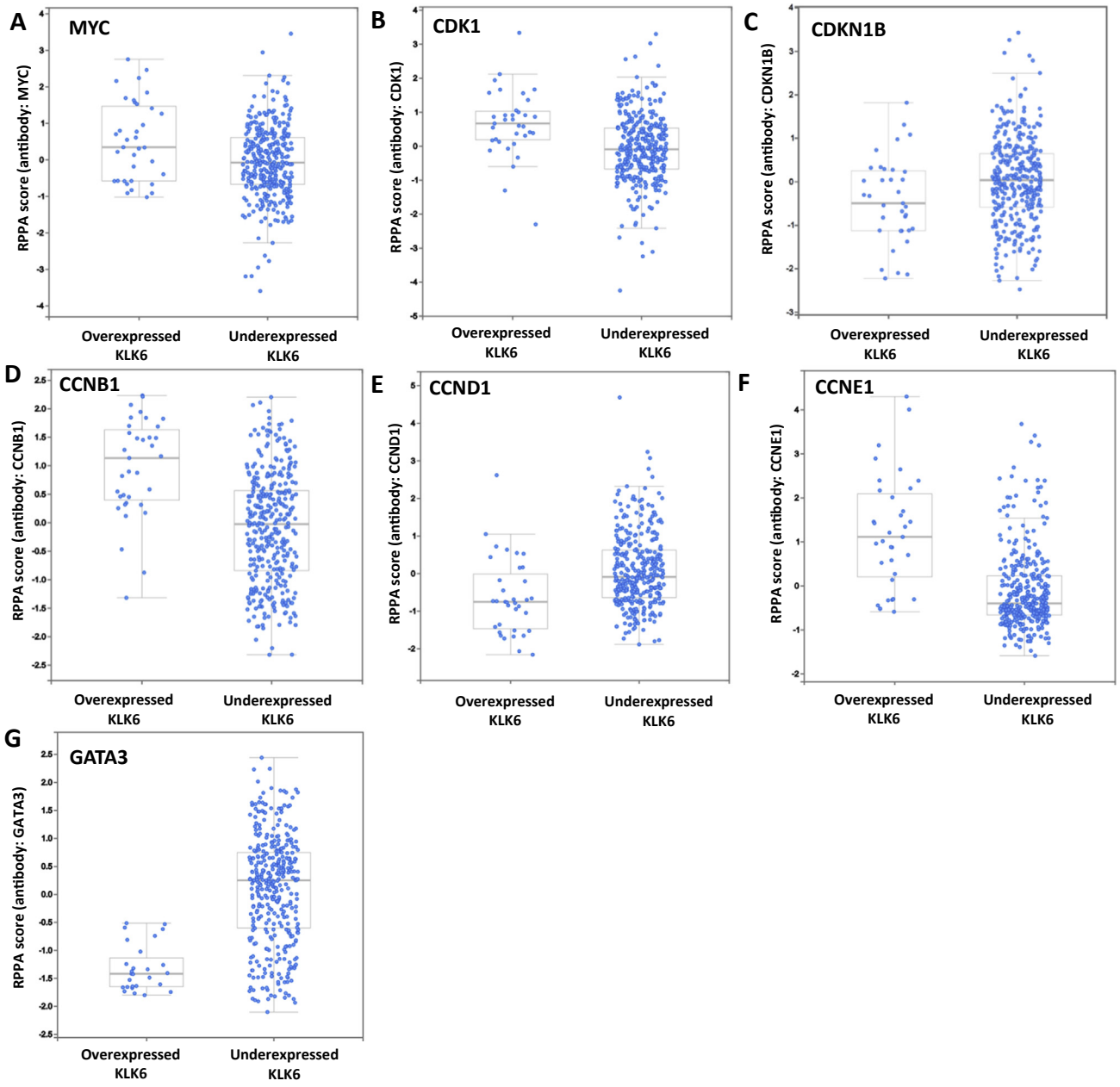

Figure 7 – RPPA protein expression analysis of KLK6-miRNA target genes. Co-expressional analysis of patients with KLK6 mRNA increased more than 0.5 times the mean KLK6 expression were grouped into an "overexpressed" KLK6 expression grouping (Z-score > +0.5; RNA Seq V2 RSEM). Patients who had KLK6 mRNA expression of less than 0.5 Z-score to the mean expression level were grouped into the "underexpressed" group. These patients were then compared to the protein (RPPA) expression of selected miRNA gene targets, from breast tumors with RPPA TCGA data, n = 409. Data plotted as Box and Whiskers Plot (0–100% with all data points plotted as blue dots per patient case). A, MYC protein expression is significantly elevated (p = 0.0025) in patients who had KLK6 mRNA expression more than 0.5 times the mean (altered cases: 0.78; unaltered cases: −0.06). B, CDK1 protein expression is significantly elevated (p = 6e-5) in patients with altered KLK6 mRNA expression greater than +0.5 Z-scores (altered cases: 0.68; unaltered cases: −0.05). C, CDKN1B protein expression is significantly reduced (p = 0.022) with altered KLK6 mRNA expression greater than +0.5 Z-scores (altered cases: −0.45; unaltered cases: 0.02). D, CCNB1 (cyclin B) protein expression is significantly elevated (p = 6e-8) with altered KLK6 mRNA expression greater than +0.5 Z-score (altered cases: 0.68; unaltered cases: 1.12). E, CCND1 (cyclin D) protein expression is significantly reduced (p = 6e-4) in patients with altered KLK6 expression greater than +0.5 Z-score (altered cases: −0.71; unaltered cases: 0.07). F, CCNE1 (cyclin E) protein expression is significantly increased (p = 6e-4) with increased KLK6 expression greater than +0.5 Z-scores (altered cases: 1.47; unaltered cases: −0.11). G, GATA3 protein expression is significantly increased (p = 5.54e-20) with elevated KLK6 expression greater than +0.5 Z-scores (altered cases: 0.10; unaltered cases: 1.32).

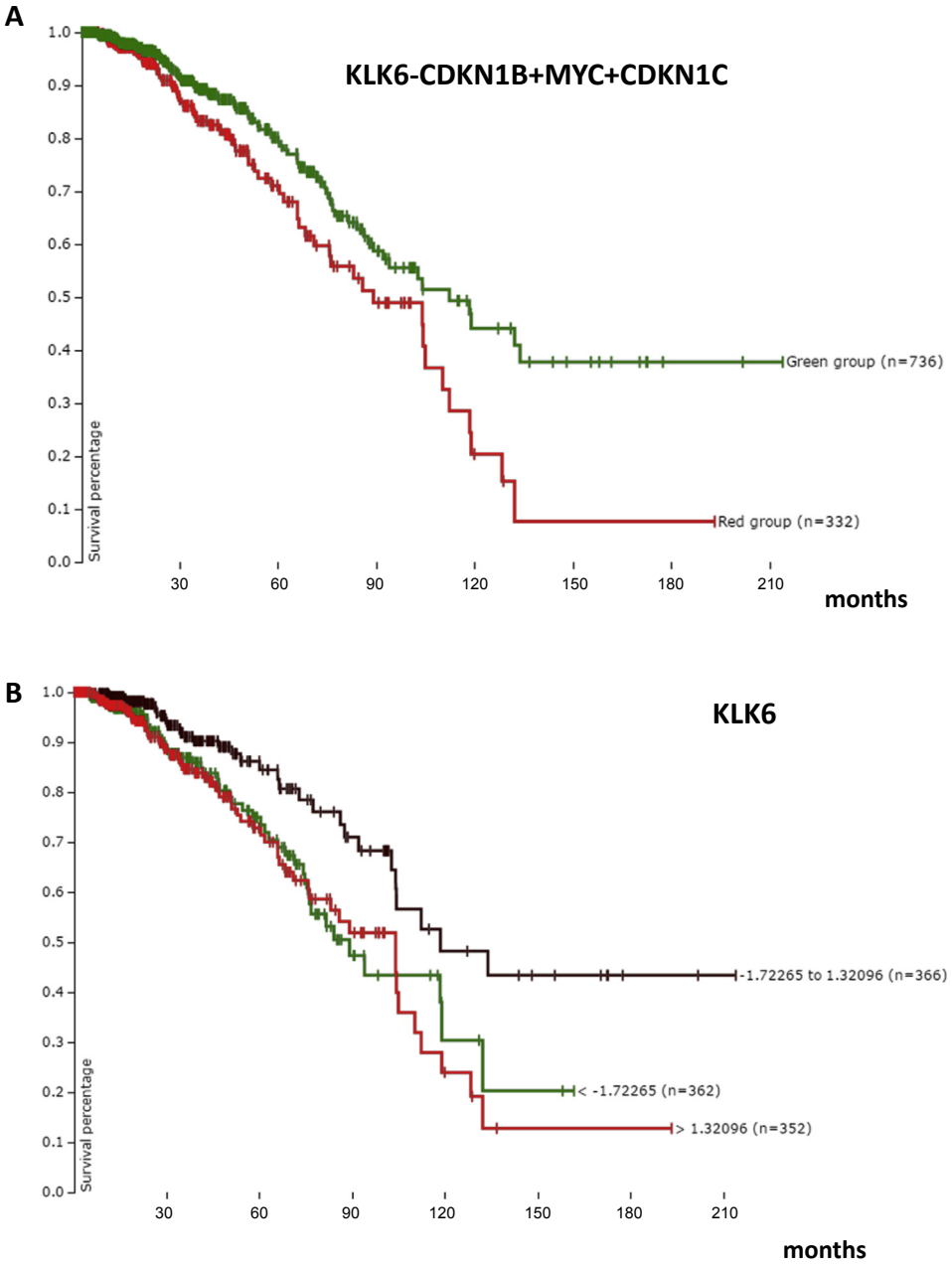

Figure 8 – Overall survival (OS) scores. A, KLK6-CDKN1B + MYC + CDKN1C Scoring Expression in Breast Cancer Patients. TCGA and IlluminaHiSeq data visualized using the Cancer Genomics Browser (n = 1106). Green indicates expression score −10.68 to 1.90; Red indicates expression score of 1.93 to 14.05. B, KLK6 scoring expression in breast cancer patients. TCGA and IlluminaHiSeq data visualized using the Cancer Genomics Browser (n = 1106). Breast cancer patients had reduced survival with over-expressed KLK6 levels (red, > 1.32, n = 352) as compared to lower (green, < −1.72, n = 362) KLK6 expression levels (Figure 8B). Patients with a physiological expression of KLK6 (brown; −1.72 to 1.32; n = 366) had much improved overall long-term survival.

accompanied by down-regulation of Rb (Zhou et al., 2010). Interestingly in non-small cell lung cancer (NSCLC), ectopic KLK6 expression dramatically enhanced cell growth with accelerated cell cycles between the G1 and S phases. This was accompanied by a marked increase in CCNE1 (cyclin E) and decrease in p21/CDKN1a (Nathalie et al., 2009). Here, in breast cancer it was demonstrated that the mechanism behind KLK6 cell cycle regulation was molecular subtype dependent, with KLK6 affecting miRNA networks involved in regulation of genes involved in cell cycle control specifically in basal-like subtypes.

KLK6 regulated miR-34a, which has been shown to be a vital regulator of cell cycle control, affecting both MYC and other cell cycle genes (Yu et al., 2010). Interestingly, KLK6 was demonstrated in NSCLC to increase MYC through an unknown mechanism (Nathalie et al., 2009) It has been shown that

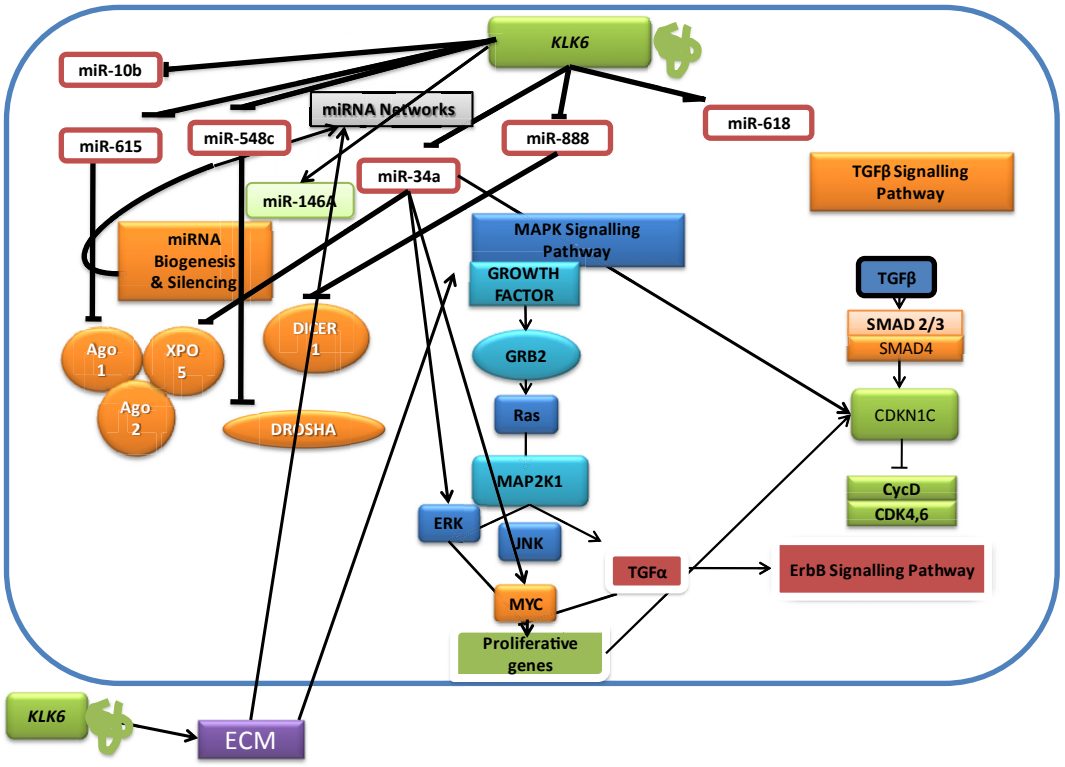

Figure 9 – A proposed model for the prediction of KLK6-miRNA interactions.

elevated miR-34a expression was associated with high proliferation rates and CCNE1 (cyclin E) (Peurala et al., 2011). Here, we demonstrated that KLK6 overexpression altered MYC mRNA expression as well in breast tissue, by regulating miR-34a, by acting on MYC through several conserved 3′UTR sites. Higher miR-34a levels have been shown to be protective against tumor progression and metastasis, as well as being associated with estrogen negative tumors (ER–), Her2-enriched tumors (Her-2+), and positive nodal status (Javeri et al., 2013). However, the exact mechanisms of these were not entirely delineated. Here, we demonstrated a significant link between hormonal status (ER, PR, HER2), KLK6 mRNA and protein expression, and miR-34a levels.

It has been demonstrated that miR-34a can affect epithelial to mesenchymal (EMT) transition of cancers (Hahn et al., 2013; Siemens et al., 2011), making it an important regulator of tumor metastatic potential and ultimately patient long-term survival. It was shown that miR-34a is downregulated in breast cancer, and it is also detected in breast cancer serum and other cancers (Eichelser et al., 2013; Luo et al., 2013; Nugent et al., 2012). One mechanism of miRNA down-regulation by cancers, including breast cancer, was shown to be promoter methylation (Lodygin et al., 2008; Vogt et al., 2011). Another mechanism accounting for tumor downregulation of miR-34a includes NF-κB transcriptional regulation, known to be dysregulated in breast cancers with important roles in EMT (Li et al., 2012; Huber et al., 2004; Nakshatri et al., 1997).

It is known that miR-34a is regulated by p53 and, in fact, itself regulates p53 to affect cell transformation (Hahn et al., 2013). ECM signals regulate not only matrix interactions, but also cell survival and apoptosis through pathways including p53 (Hermeking, 2012; Ilic et al., 1998). Here, we also demonstrated that differential KLK6 expression in breast cancers affects miRNA networks and their downstream ECM targets. Cellular and ECM matrix interactions have been known to regulate downstream cellular pathways including miRNA networks, cell survival and apoptosis through pathways such as p53 (Hermeking, 2012; Ilic et al., 1998). KLK6 protease activity may also lead to miRNA network alterations through its proteolytic actions on the ECM, generating downstream signaling cascades affecting methylation, transcription and miRNA processing.

In conclusion, we demonstrate that differential KLK6 expression in the breast cancer molecular subtypes affects expression of a network of interacting miRNAs. These networks were enriched in MAPK, cell cycle and cell signaling molecule targets. Reduction of KLK6 mRNA through promoter methylation as found in many metastatic breast cancers, or reduction in KLK6 protein levels would lead to loss of anti-metastamir miRNA expression and increased metastatic potential. On the other hand, very high levels of KLK6 would lead to cell cycle progression and MAPK signaling molecule activation. KLK6 affects mRNA expression of ECM molecules and, as a serine protease, it is known to regulate ECM proteins and cell adhesion directly through proteolysis (Ghosh et al., 2004). Thus, these pathways merge to have an amplified downstream impact on cell cycle progression, MAPK signaling, ECM regulation, breast cancer phenotype, survival and metastatic potential.

Conflict of interest

The authors declare that they do not have a conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.molonc.2016.03.008.

REFERENCES

Anisowicz, A., Sotiropoulou, G., Stenman, G., Mok, S.C., Sager, R., 1996. A novel protease homolog differentially expressed in breast and ovarian cancer. Mol. Med. 2, 624–636.

Bayés, A., Tsetsenis, T., Ventura, S., Vendrell, J., Aviles, F.X., Sotiropoulou, G., 2004. Human kallikrein 6 activity is regulated *via* an autoproteolytic mechanism of activation/inactivation. Biol. Chem. 385, 517–524.

Bhaumik, D., Scott, G.K., Schokrpur, S., Patil, C.K., Campisi, J., Benz, C.C., 2008. Expression of microRNA-146 suppresses NF-kappaB activity with reduction of metastatic potential in breast cancer cells. Oncogene 27, 5643–5647.

Cancer Genome Atlas Network, 2012. Comprehensive molecular portraits of human breast tumours. Nature 490, 61–70.

Chow, T.F., Crow, M., Earle, T., El-Said, H., Diamandis, E.P., Yousef, G.M., 2008. Kallikreins as microRNA targets: an *in silico* and experimental-based analysis. Biol. Chem. 389, 731–738.

Creighton, C.J., 2012. The molecular profile of luminal B breast cancer. Biologics 6, 289–297.

Diamandis, E.P., Yousef, G.M., 2002. Human tissue kallikreins: a family of new cancer biomarkers. Clin. Chem. 48, 1198–1205.

Eichelser, C., Flesch-Janys, D., Chang-Claude, J., Pantel, K., Schwarzenback, H., 2013. Deregulated serum concentrations of circulating cell-free microRNAs miR-17, miR-34a, miR-155, and miR-373 in human breast cancer development and progression. Clin. Chem. 59, 1489–1496.

Engstrom, M.J., Opdahl, S., Hagen, A.I., Romundstad, P.R., Akslen, L.A., Haugen, O.A., Vatten, L.J., Bofin, A.M., 2013. Molecular subtypes, histopathological grade and survival in a historic cohort of breast cancer patients. Breast Cancer Res. Treat. 140, 463–473.

Ghosh, M.C., Grass, L., Soosaipillai, A., Sotiropoulou, G., Diamandis, E.P., 2004. Human kallikrein 6 degrades extracellular matrix proteins and may enhance the metastatic potential of tumour cells. Tumour Biol. 25, 193–199.

Hahn, S., Jackstadt, R., Siemens, H., Hunten, S., Hermeking, H., 2013. SNAIL and miR-34a feed-forward regulation of ZNF281/ZBP99 promotes epithelial-mesenchymal transition. EMBO J. 32, 3079–3095.

Hermeking, H., 2012. MicroRNAs in the p53 network: micromanagement of tumour suppression. Nat. Rev. Cancer 12, 613–626.

Huber, M.A., Beug, H., Wirth, T., 2004. Epithelial-mesenchymal transition: NF-kappaB takes center stage. Cell Cycle 3, 1477–1480.

Ilic, D., Almeida, E.A., Schlaepfer, D.D., Dazin, P., Aizawa, S., Damsky, C.H., 1998. Extracellular matrix survival signals transduced by focal adhesion kinase suppress p53-mediated apoptosis. J. Cell. Biol. 143, 547–560.

Javeri, A., Ghaffarpour, M., Taha, M.F., Houshmand, M., 2013. Downregulation of miR-34a in breast tumors is not associated with either p53 mutations or promoter hypermethylation while it correlates with metastasis. Med. Oncol. 30, 413.

Jemal, A., Bray, F., Center, M.M., Ferlay, J., Ward, E., Forman, D., 2011. Global cancer statistics. CA Cancer J. Clin. 61, 69–90.

Li, J., Wang, K., Chen, X., Meng, H., Song, M., Wang, Y., Xu, X., Bai, Y., 2012. Transcriptional activation of microRNA-34a by NF-kappa B in human esophageal cancer cells. BMC Mol. Biol. 13, 4.

Lodygin, D., Tarasov, V., Epanchintsev, A., Berking, C., Knyazeva, T., Korner, H., Knyazev, P., Diebold, J., Hermeking, H., 2008. Inactivation of miR-34a by aberrant CpG methylation in multiple types of cancer. Cell Cycle 7, 2591–2600.

Luo, D., Wilson, J.M., Harvel, N., Liu, J., Pei, L., Huang, S., Hawthorn, L., Shi, H., 2013. A systematic evaluation of miRNA:mRNA interactions involved in the migration and invasion of breast cancer cells. J. Transl. Med. 11, 57.

Mulrane, L., McGee, S.F., Gallagher, W.M., O'Connor, D.P., 2013. miRNA dysregulation in breast cancer. Cancer Res. 73, 6554–6562.

Nagahara, H., Mimori, K., Utsunomiya, T., Barnard, G.F., Ohira, M., Hirakawa, K., Mori, M., 2005. Clinicopathologic and biological significance of kallikrein 6 overexpression in human gastric cancer. Clin. Cancer Res. 11, 6800–6806.

Nakshatri, H., Bhat-Nakshari, P., Martin, D.A., Goulet Jr., R.J., Sledge Jr., G.W., 1997. Constitutive activation of NF-kappaB during progression of breast cancer to hormone-independent growth. Mol. Cell. Biol. 17, 3629–3639.

Nathalie, H.V., Chris, P., Serge, G., Catherine, C., Benjamin, B., Claire, B., Christelle, P., Briollais, L., Pascale, R., Marie-Lise, J., Yves, C., 2009. High kallikrein-related peptidase 6 in non-small cell lung cancer cells: an indicator of tumour proliferation and poor prognosis. J. Cell. Mol. Med. 13, 4014–4022.

Neve, R.M., Chin, K., Fridlyand, J., Yeh, J., Baehner, F.L., Fevr, T., Clark, L., Bayani, N., Coppe, J.P., Tong, F., Speed, T., Spellman, P.T., DeVries, S., Lapuk, A., Wang, N.J., Kuo, W.L., Stilwell, J.L., Pinkel, D., Albertson, D.G., Waldman, F.M., McCormick, F., Dickson, R.B., Johnson, M.D., Lippman, M., Ethier, S., Gazdar, A., Gray, J.W., 2006. A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes. Cancer Cell 10, 515–527.

Ni, X., Zhang, W., Huang, K.C., Wang, Y., Ng, S.K., Mok, S.C., Berkowitz, R.S., Ng, S.W., 2004. Characterisation of human kallikrein 6/protease M expression in ovarian cancer. Br. J. Cancer 91, 725–731.

Nugent, M., Miller, N., Kerin, M.J., 2012. Circulating miR-34a levels are reduced in colorectal cancer. J. Surg. Oncol. 106, 947–952.

Ogawa, K., Utsunomiya, T., Mimori, K., Tanaka, F., Inoue, H., Nagahara, H., Murayama, S., Mori, M., 2005. Clinical significance of human kallikrein gene 6 messenger RNA expression in colorectal cancer. Clin. Cancer Res. 11, 2889–2893.

Pampalakis, G., Sotiropoulou, G., 2006. Multiple mechanisms underlie the aberrant expression of the human kallikrein 6 gene in breast cancer. Biol. Chem. 387, 773–782.

Pampalakis, G., Prosnikli, E., Agalioti, T., Vlahou, A., Zoumpourlis, V., Sotiropoulou, G., 2009. A tumor-protective role for human kallikrein-related peptidase 6 in breast cancer mediated by inhibition of epithelial-to-mesenchymal transition. Cancer Res. 69, 3779–3787.

Papadopoulos, G.L., Alexiou, P., Maragkakis, M., Reczko, M., Hatzigeorgious, A.G., 2009. DIANA-mirPath: integrating human and mouse microRNAs in pathways. Bioinformatics 25, 1991–1993.
Pasic, M.D., Samaan, S., Yousef, G.M., 2013. Genomic medicine: new frontiers and new challenges. Clin. Chem. 59, 158–167.
Pasic, M.D., Olkhov, E., Bapat, B., Yousef, G.M., 2012. Epigenetic regulation of kallikrein-related peptidases: there is a whole new world out there. Biol. Chem. 393, 319–330.
Peter, M.E., 2010. Targeting of mRNAs by multiple miRNAs: the next step. Oncogene 29, 2161–2164.
Petraki, C., Dubinski, W., Scorilas, A., Saleh, C., Pasic, M.D., Komborozos, V., Khalil, B., Gabril, M.Y., Streutker, C., Diamandis, E.P., Yousef, G.M., 2012. Evaluation and prognostic significance of human tissue kallikrein-related peptidase 6 (KLK6) in colorectal cancer. Pathol. Res. Pract. 208, 104–108.
Peurala, H., Greco, D., Heikkinen, T., Kaur, S., Bartkova, J., Jamshidi, M., Aittomaki, K., Heikkila, P., Bartek, J., Blomqvist, C., Butzow, R., Nevanlinna, H., 2011. MiR-34a expression has an effect for lower risk of metastasis and associates with expression patterns predicting clinical outcome in breast cancer. PLoS One 6, e26122.
Qin, W., Zhang, K., Kliethermes, B., Ruhlen, R.L., Browne, E.P., Arcaro, K.F., Sauter, E.R., 2012. Differential expression of cancer associated proteins in breast milk based on age at first full term pregnancy. BMC Cancer 12, 100.
Reis-Filho, J.S., Pusztai, L., 2011. Gene expression profiling in breast cancer: classification, prognostication, and prediction. Lancet 378, 1812–1823.
Seiz, L., Dorn, J., Kotzsch, M., Walch, A., Grebenchtchikov, N.I., Gkazepis, A., Schmalfeldt, B., Kiechle, M., Bayani, J., Diamandis, E.P., Langer, R., Sweep, F.C., Schmitt, M., Magdolen, V., 2012. Stromal cell-associated expression of kallikrein-related peptidase 6 (KLK6) indicates poor prognosis of ovarian cancer patients. Biol. Chem. 393, 391–401.
Sidiropoulos, K.G., White, N.M., Bui, A., Ding, Q., Boulos, P., Pampalakis, G., Khella, H., Samuel, J.N., Sotiropoulou, G., Yousef, G.M., 2014. Kallikrein-related peptidase 5 induces miRNA-mediated anti-oncogenic pathways in breast cancer. Oncoscience 1, 709–724.
Siemens, H., Jackstadt, R., Hunten, S., Kaller, M., Menssen, A., Gotz, U., Hermeking, H., 2011. miR-34 and SNAIL form a double-negative feedback loop to regulate epithelial-mesenchymal transitions. Cell Cycle 10, 4256–4271.
Sinha, A.U., Kaimal, V., Chen, J., Jegga, A.G., 2008. Dissecting microregulation of a master regulatory network. BMC Genomics 9, 88.
Smalley, M.J., Reis-Filho, J.S., Ashworth, A., 2008. BRCA2 and stem cells: tumour typecasting. Nat. Cell Biol. 10, 377–379.
Sotiropoulou, G., Rogakos, V., Tsetsenis, T., Pampalakis, G., Zafiropoulos, N., Simillides, G., Yiotakis, A., Diamandis, E.P., 2003. Emerging interest in the kallikrein gene family for understanding and diagnosing cancer. Oncol. Rep. 13, 381–391.
Sotiropoulou, G., Pampalakis, G., Diamandis, E.P., 2009a. Functional roles of human kallikrein-related peptidases. J. Biol. Chem. 284, 32989–32994.
Sotiropoulou, G., Pampalakis, G., Lianidou, E., Mourelatos, Z., 2009b. Emerging roles of microRNAs as molecular switches in the integrated circuit of the cancer cell. RNA 15, 1443–1461.
Sotiropoulou, G., Pampalakis, G., 2012. Targeting the kallikrein-related peptidases for drug development. Trends Pharmacol. Sci. 33, 623–634.
Trimis, G., Chatzistamou, I., Politi, K., Kiaris, H., Papavassiliou, A.G., 2008. Expression of p21waf1/Cip1 in stromal fibroblasts of primary breast tumors. Hum. Mol. Genet. 17, 3596–3600.
Voduc, K.D., Cheang, M.C., Tyldesley, S., Gelmon, K., Nielsen, T.O., Kennecke, H., 2010. Breast cancer subtypes and the risk of local and regional relapse. J. Clin. Oncol. 28, 1684–1691.
Vogt, M., Munding, J., Gruner, M., Liffers, S.T., Verdoodt, B., Hauk, J., Steinstrasser, L., Tannapfel, A., Hermeking, H., 2011. Frequent concomitant inactivation of miR-34a and miR-34b/c by CpG methylation in colorectal, pancreatic, mammary, ovarian, urothelial, and renal cell carcinomas and soft tissue sarcomas. Virchows Arch. 458, 313–322.
Wang, S.M., Mao, J., Li, B., Wu, W., Tang, L.L., 2008. [Expression of KLK6 protein and mRNA in primary breast cancer and its clinical significance]. Xi Bao Yu Fen Zi Mian Yi Xue Za Zhi 24, 1087–1089.
Wang, F., Zheng, Z., Guo, J., Ding, X., 2010. Correlation and quantitation of microRNA aberrant expression in tissues and sera from patients with breast tumor. Gynecol. Oncol. 119, 586–593.
White, N.M., Bui, A., Mejia-Guerrero, S., Chao, J., Soosaipillai, A., Youssef, Y., Mankaruos, M., Honey, R.J., Stewart, R., Pace, K.T., Sugar, L., Diamandis, E.P., Dore, J., Yousef, G.M., 2010a. Dysregulation of kallikrein-related peptidases in renal cell carcinoma: potential targets of miRNAs. Biol. Chem. 391, 411–423.
White, N.M., Chow, T.F., Mejia-Guerrero, S., Diamandis, M., Rofael, Y., Faragalla, H., Mankaruous, M., Gabril, M., Girgis, A., Yousef, G.M., 2010b. Three dysregulated miRNAs control kallikrein 10 expression and cell proliferation in ovarian cancer. Br. J. Cancer 102, 1244–1253.
White, N.M., Youssef, Y.M., Fendler, A., Stephan, C., Jung, K., Yousef, G.M., 2012. The miRNA-kallikrein axis of interaction: a new dimension in the pathogenesis of prostate cancer. Biol. Chem. 393, 379–389.
Yousef, G.M., Obiezu, C.V., Luo, L.Y., Magklara, A., Borgono, C.A., Kishi, T., Memari, N., Michael, I.P., Sidiropoulos, M., Kurlender, L., Economopoulou, K., Kapadia, C., Komatsu, N., Petraki, C., Elliott, M., Scorilas, A., Katsaros, D., Levesque, M.A., Diamandis, E.P., 2005. Human tissue kallikreins: from gene structure to function and clinical applications. Adv. Clin. Chem. 39, 11–79.
Yousef, G.M., Diamandis, E.P., 2002. Expanded human tissue kallikrein family–a novel panel of cancer biomarkers. Tumour Biol. 23, 185–192.
Yousef, G.M., Yacoub, G.M., Polymeris, M.E., Popalis, C., Soosaipillai, A., Diamandis, E.P., 2004. Kallikrein gene downregulation in breast cancer. Br. J. Cancer 90, 167–172.
Yu, Z., Baserga, R., Chen, L., Wang, C., Lisanti, M.P., Pestell, R.G., 2010. microRNA, cell cycle, and human breast cancer. Am. J. Pathol. 176, 1058–1064.
Zhang, B., Pan, X., Cobb, G.P., Anderson, T.A., 2007. microRNAs as oncogenes and tumor suppressors. Dev. Biol. 302, 1–12.
Zhou, H., Guo, J.M., Lou, Y.R., Zhang, X.J., Zhong, F.D., Jiang, Z., Cheng, J., Xiao, B.X., 2010. Detection of circulating tumor cells in peripheral blood from patients with gastric cancer using microRNA as a marker. J. Mol. Med. (Berl) 88, 709–717.
Zhu, J., Sanborn, J.Z., Benz, S., Szeto, C., Hsu, F., Kuhn, R.M., Karolchink, D., Archie, J., Lenburg, M.E., Esserman, L.J., Kent, W.J., Haussler, D., Wang, T., 2009. The UCSC Cancer Genomics Browser. Nat. Methods 6, 239–240.