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1 **(a) Title of paper:**

2 Analysis of melanoma-related microRNAs expression during the spontaneous regression of
3 cutaneous melanomas in MeLiM pigs.

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34 Dear Editor,

35 Treatment of advanced cutaneous melanoma is still challenging and new data on melanoma biology
36 are required. MicroRNAs (miRNAs) represent an emerging field in melanoma understanding. These
37 small regulators of gene expression, involved in melanoma development and progression (Da cruz *et al.*,
38 *et al.*, 2012), showed promising results as biomarkers (Nair *et al.*, 2012). As each miRNA could regulate
39 multiple protein-coding genes and conversely, one gene could be targeted by different miRNAs
40 (Bartel *et al.*, 2009), the dysregulation of miRNAs could significantly impact various pathways and
41 cells behavior. In this report, we studied the Melanoma-bearing Libechov Minipigs, a model of
42 complete spontaneous regression of invasive cutaneous melanoma (Vincent-Naulleau *et al.*, 2004), in
43 order to investigate if miRNAs could be involved in this process.

44

45 We evaluated the differential expression of 9 melanoma-related miRNAs (reviewed in Da
46 cruz *et al.*, 2012; Igoucheva and Alexeev, 2009 and Jiang *et al.*, 2012) by quantitative PCR (qPCR) in
47 MeLiM melanomas. We compared 8 tumors showing evidence of regression (clinical drying or graying
48 aspect and tumor size reduction) to 8 progressing tumors (clinical and histological characterization as
49 indicated in Vincent-Naulleau *et al.*, 2004) (Figure 1). Three miRNAs showed no significant differential
50 expression between the two conditions (let-7a, miR-34a and miR-182). Among the significant
51 differentially expressed miRNAs, only miR-92a showed a down-regulation in regressing melanoma
52 (Fold Change (FC) = 0.60, $p=0.0148$), whereas five miRNAs (miR-21, miR-221, miR-222, miR-193b and
53 let-7b) were found up-regulated with a FC ranging from 2.00 to 3.97 ($p<0.05$) (Figure 1). Interestingly,
54 in regressive swine tumors, miR-92a, let-7b and miR-193b had an expression profile opposite to the
55 profile observed during the progression of human cutaneous melanoma (Igoucheva and Alexeev,
56 2009; Schultz *et al.*, 2008; Chen *et al.*, 2010). This observation could suggest that these three miRNAs
57 could be potential actors in the regression process of cutaneous melanoma in the MeLiM model.
58 Among those, the tumor-suppressor miR-193b showed the highest up-regulation (FC = 3.97, $p<0.01$).

59

60 Thus, as miR-193b was up-regulated in regression, our hypothesis was that its known targets
61 should be concomitantly down-regulated. According to Chen *et al.* (2010), miR-193b directly and
62 indirectly down-regulated 314 mRNAs in a human melanoma cell line. In a previous study, we
63 analyzed the expression of 11,216 mRNAs during swine melanoma regression (Rambow *et al.*, 2008).
64 Out of the 314 genes of interest, 211 had a specific probe on the Porcine Genome Array used in our
65 previous study (GeneChip®, Affymetrix). We extracted the $\text{Log}_2(\text{FC})$ values of these 211 genes, to
66 build a “miR-193b-regulated” genes set and a “miR-193b-non-regulated” genes set (i.e. all other
67 genes on the micro-array, $n=11,005$). A permutation test revealed that miR-193b-regulated set is
68 enriched in down-regulated genes ($p = 1.61e-37$), as indicated by a distinct $\text{Log}_2(\text{FC})$ cumulative

69 distribution function (CDF) of the miR-193b-regulated genes set, compared to the Log₂(FC)-CDF of
70 the miR-193b-non-regulated genes set (Kolmogorov-Smirnoff $p = 2.49e-19$, figure 2). In order to
71 identify the potential function of the concomitant up-regulation of miR-193b and the regulation of its
72 down-stream genes, the 211 genes set and their respective fold change have been imported in
73 Ingenuity Pathway Analysis (IPA, Ingenuity®Systems,www.ingenuity.com) for a core analysis and
74 upstream regulators prediction. The core analysis highlighted a role for 77 of these genes in the “cell
75 cycle” pathway and the most enriched pathway identified is “DNA replication, recombination and
76 repair” (Table 1A), in accordance with our previous results. In addition, the upstream regulator
77 analysis identified 5 transcriptional regulators potentially inhibited during cutaneous melanoma
78 regression: E2F1, MYC, TBX2, E2F2 and TP63 (Table 1B). Most of these genes are described as
79 putative oncogenes or biomarkers of poor prognosis in melanoma. Among them, E2F1 (in the miR-
80 193b-regulated gene set, Log₂(FC) = -0.732) is a predicted target of miR-193b (TargetScan6.2,
81 <http://www.targetscan.org/>). Thus, we may postulate that during the early step of cutaneous
82 melanoma regression, the up-regulation of miR-193b leads to an indirect down-regulation of
83 numerous cell-cycle related genes via the modulation of E2F1. Future studies are required to explore
84 this issue.

85

86 In conclusion, we show that in our model, melanoma-related miRNAs (let-7b, miR-92a and
87 miR-193b) are expressed in an opposite direction during regression of cutaneous melanoma
88 compared to its progression in human. In addition, we showed a concomitant up-regulation of miR-
89 193b and down-regulation of its downstream genes. IPA analysis revealed that miR-193b could
90 regulate cell-cycle related genes during regression of cutaneous melanoma. The present work
91 highlight the potential implication of miRNAs in the early step of cutaneous melanoma regression in
92 swine and require future research to conclude about their potential value as therapeutic and
93 diagnosis tools.

94

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101 Physiology and Genetics, Academy of Sciences of the Czech Republic, 27721 Libečov, Czech
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103

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128

129 **Figure 1: MiRNAs expression changes during regression of swine cutaneous melanoma.** Boxplots
130 represent the relative expression of melanoma-related miRNAs in regressing melanoma (D+49,
131 n=8tumors, grey boxes) compared to progressing ones (D+8, n=8 tumors, white boxes), determined
132 by quantitative PCR. We excised one progressive and one regressive tumor on the same animal but
133 all the others, were excised on 14 different MeLiM piglets to limit impact of the animal genetic
134 background. Relative quantification was calculated by 2^{-ddCt} methods, using miR-191 as a reference
135 gene. The Normfinder analysis revealed that miR-191 (stability value 0.140) was the best normalizer
136 in our model among U6 (stability value 0.311) and miR-423 (0.173), in accordance with previous
137 reports (Peltier *et al.*, 2009; Saldanha *et al.*, 2013). Stem-loop reverse transcription has been
138 performed in triplicate for each miRNA and each cDNA product has been quantified by qPCR in
139 triplicate. Differences between groups were assessed by a Mann-Whitney U-test (Ns- no significant
140 difference, *- p<0.05, **- p<0.01).

141

142 **Figure 2: Comparison of Log2FC distribution of miR-193b-regulated and non-regulated genes in**
143 **regressing melanoma.** Log2FC cumulative frequency distribution of miR-193b targeted genes and
144 non-miR-193b targeted genes during the early phase of regression compared to progression. *Filled*

145 *line*: Log2FC cumulative frequency distribution of genes that are not regulated by miR-193b. *Dotted*
146 *line*: Log2FC cumulative frequency distribution of genes regulated by miR-193b.

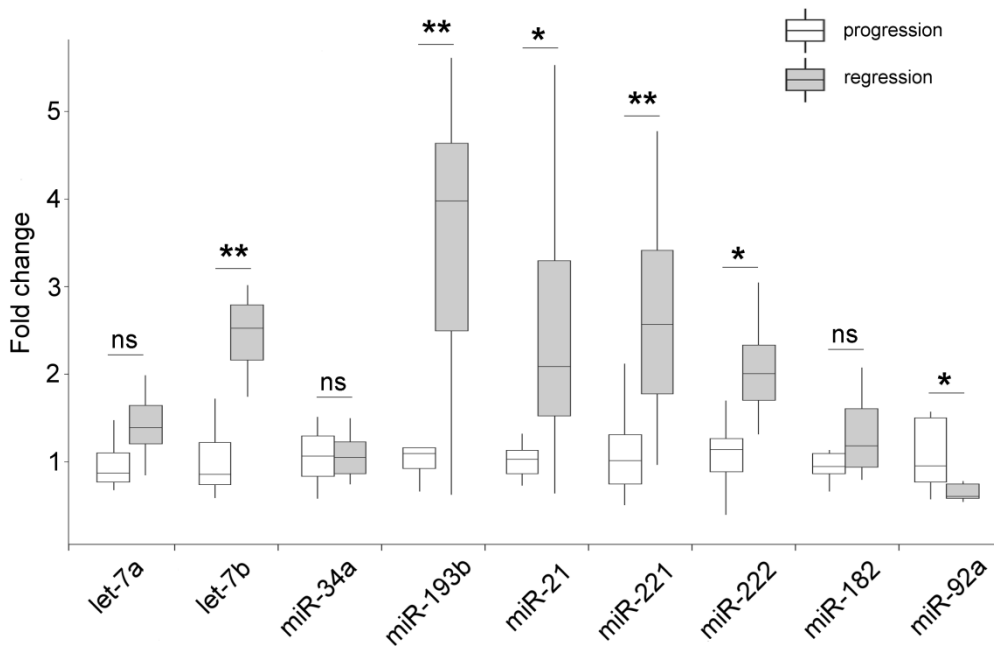
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149 **Table 1: IPA analysis results of the miR-193b-regulated genes. (A)** Molecular and cellular functions
150 associated with the 211 genes regulated by miR-193b. The top-5 functions identified are ranked
151 according to their p-value. **(B)** IPA upstream regulators prediction results. This analysis enables to
152 identify the cascade of upstream transcriptional regulators that could explain the observed gene
153 expression changes in the dataset and illuminate the biological activities occurring in the regressing
154 tumors. The top-5 upstream transcriptional regulators are presented with their predicted activation
155 or inhibition state according to the expression of their downstream genes in regression.

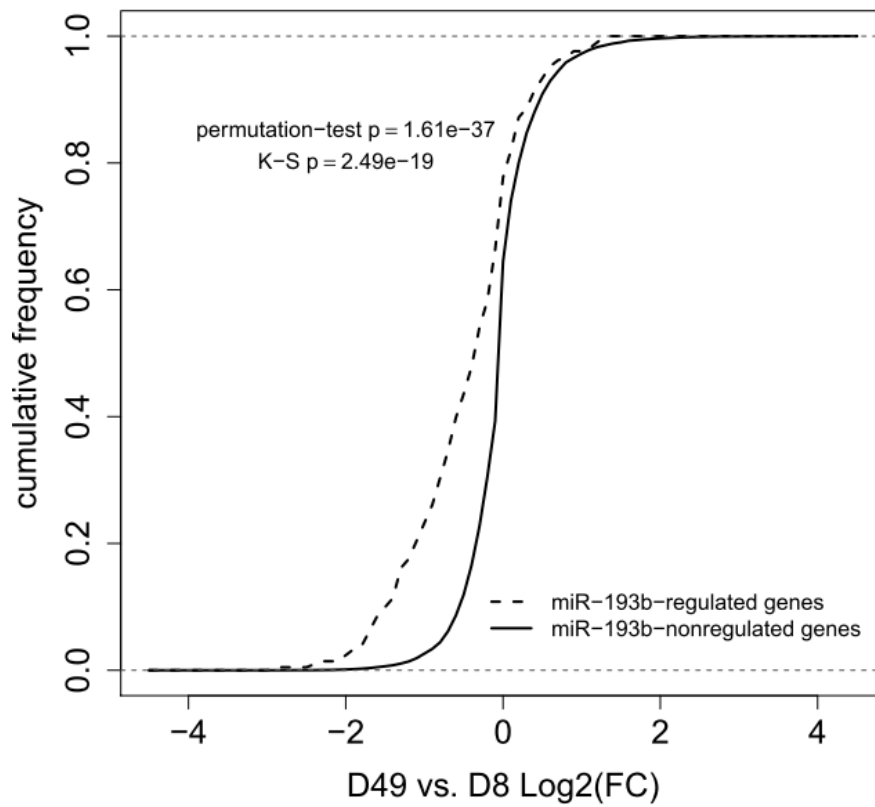
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157 Figure 1



158

159 Figure 2



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161

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163 Table I

164 **A**

Name	p-value	# Molecules
Cell Cycle	8.19E-17	77
Cellular Assembly and Organization	2.11E-11	64
DNA Replication, Recombination, and Repair	2.11E-11	80
Cellular Function and Maintenance	1.57E-09	37
Cellular Growth and Proliferation	1.89E-09	85

165

166 **B**

Upstream Regulator	Log Ratio	Predicted Activation State	Activation z-score	p-value of overlap	Target molecules in dataset
E2F1	-0,732	Inhibited	-4.477	5,08E-20	BRCA1, CCNA2, CDC25A, CDC6, CDCA4, CDK1, CHEK1, DNMT1, E2F1, ECT2, EZH2, FBXO5, FEN1, GMNN, HELLS, HMGB1, KIAA0101, MCL1, MCM4, MCM5, MCM6, MCM7, NDC80, PCNA, POLD1, RACGAP1, RRM2, STMN1, TK1, TOP2A, TYMS
TBX2		Inhibited	-4.231	6,68E-20	ASF1B, CCNA2, CDC6, CDCA5, CDK1, CDT1, CHAF1B, CHEK1, DNMT1, E2F1, ELK3, EZH2, HELLS, MCM4, MCM5, MCM6, MCM7, PKMYT1
E2F2		Inhibited	-2.236	5,57E-14	CCNA2, CDC25A, CDC6, CDCA4, CDK1, E2F1, EZH2, MCM4, MCM5, MCM6, MCM7, PCNA, RRM2
MYC		Inhibited	-4.281	6,01E-08	AIMP2, BRCA1, BUB1B, C12orf49, CCNA2, CDC20, CDC25A, CDK1, CDK6, CHEK1, DNMT1, E2F1, EZH2, KRAS, MAX, MCM5, MCM6, MCM7, PCNA, POLD1, RCC1, RRM2, SHMT1, SHMT2, SLC7A5, STMN1, TYMS, UBE2C
TP63		Inhibited	-2.236	1,58E-07	ABI2, BRCA1, CCNA2, CDK1, CDK6, DNMT1, FANCA, MAPK8, PCNA, PRNP, RAC2, RACGAP1, RCC1, SEPHS1

167