EFFECT OF IRISIN ON uPA/uPAR SYSTEM IN *in vitro* MODELS OF METASTATIC MELANOMA CELLS

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Introduction. Irisin is a 12kDa messenger protein as part of the fibronectin type III domain containing 5 (FNDC5) protein mainly secreted by skeletal muscles upon muscle contraction. Recently, the integrin αV/β5 has been identified as the receptor for irisin on osteocytes, Through autocrine, paracrine, and endocrine signals involving the activation of ERK cascade, irisin is involved in energy metabolism and Musculo-skeletal homeostasis and exherts pleiotropic anabolic effects also in several tissues. It has also been proven modulation effects on cellular proliferation in different tumours, even though the effect of irisin on metastatic melanoma (MM), one of the most aggressive forms of tumour, has not been described yet. We explore the direct effect of exogenous irisin treatment on the proliferation and invasion in vitro models of metastatic melanoma (Table 1) cell lines to further elucidate the role played by irisin also in melanoma cells.

Table 1. List of cell lines used in this study.

Cell line	Origin	NRAS	BRAF exon15
HBL*	Metastasis	WT/WT	WT/WT
LND1	Metastasis	WT/WT	WT/WT
Hmel1	Metastasis	WT/WT	V600K/WT
M3	Metastasis	WT/WT	V600E/V600E

* Thanks to Prof. Ghanem G., Université de Bruxelles, Belgium

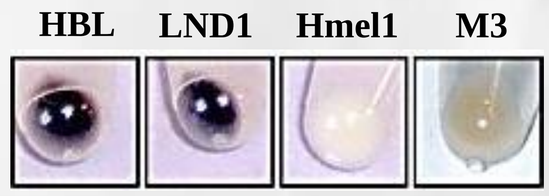
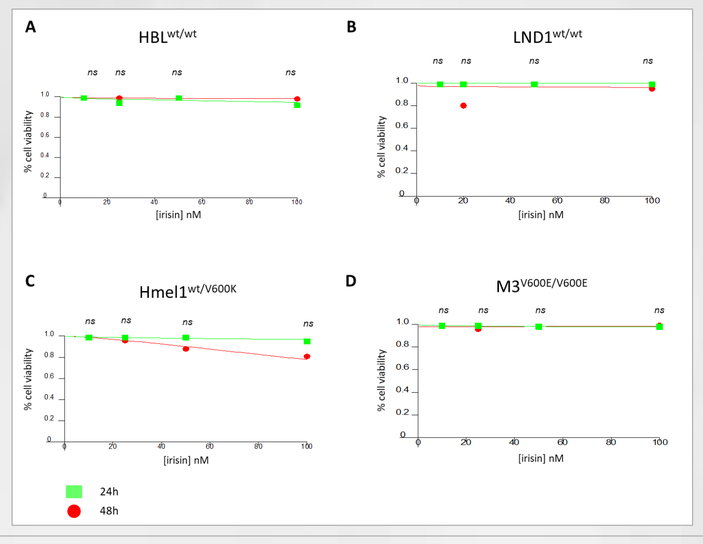


Figure 1. MTT-assay of metastatic melanoma cells treated with irisin.



We treated MM cell lines with different concentration of irisin for 24h and 48h with increasing concentration of irisin (0nM, 10nM, 20nM, 50nM, 100nM). As show in Figure 1, irisin did not impair the viability of MM cells at 24h and 48h after treatment compared to untreated cells (ns=not significant).

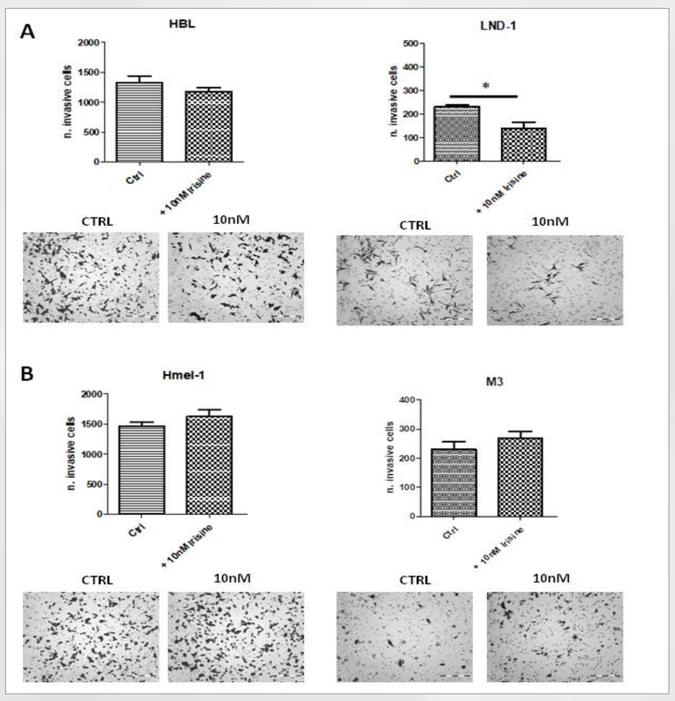
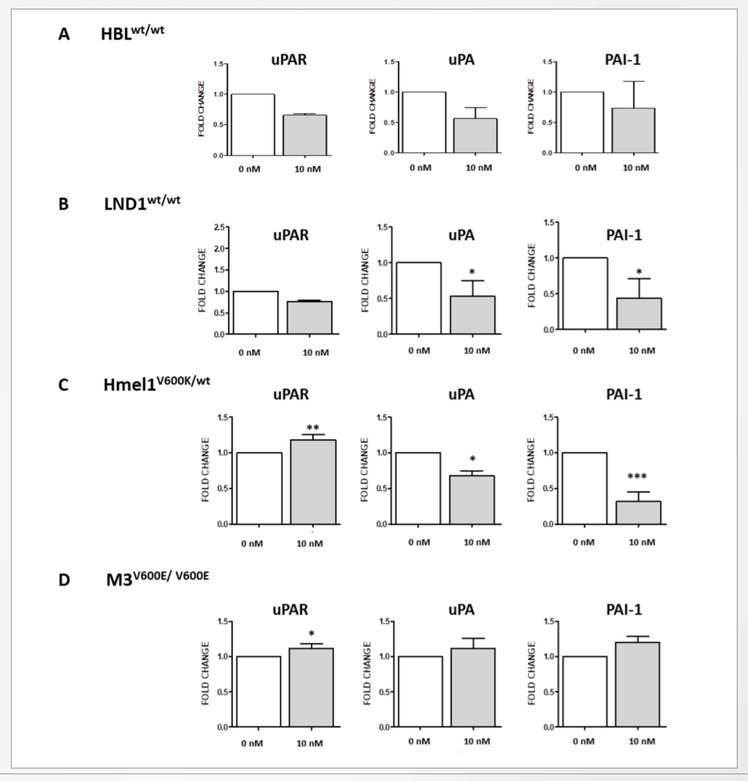


Figure 2. Chemio-Invasion assay of metastatic melanoma cells treated with irisin.

We subsequently treated MM cells with 10nM irisin, corresponding to the dose of irisin reported to exhibit biological activity in vitro, to assess whether irisin could influence cellular invasiveness across the extracellular matrix (ECM). As shown in Figure 2, irisin treatment modulated the invasive attitude of MM cells depending on BRAF: HBL invading cells was reduced while LND1 showed a significant impairment of invasive capabilities (12% and 40%, respectively, $p<0.05$), while irisin did not affect the invasion of BRAFmut cells (p=ns) Hmel-1 and M3.

Metastatic melanoma progression is mediate by activation of urokinase plasminogen activator receptor (uPAR), urokinase plasminogen activator (uPA) and its inhibitor PAI-1 as well as the gelatinase system accounting the metalloproteinases MMP-2, MMP-9 and their inhibitors TIMP-1 and TIMP-2.

Figure 3. Gene expression analysis of uPA/uPAR system.



As shown in Fig.3 (A-B), HBL showed a trend of downregulation of uPA and uPAR, while LND1 show a significant reductions of uPA and PAI-1 ($p<0.05$) upon irisin treatment compared to untreated cells, respectively. On the other hand, the BRAFmut cells Hmel1$^{V600K/wt}$ and M3$^{V600E/V600E}$ (Fig.3C-D) exhibited a clear up-regulation of uPAR following irisin treatment compared to untreated cells ($p<0.05$). In contrast, there is a decrease in the expression of uPA and PAI-1 in the Hmel1V600K/wt cell line, while no variations were observed of these genes in M3V600E/V600E cell line.

As shown in Fig.4, the bands corresponding to proteins within the fibrinolytic system, namely uPAR, uPA, and PAI-1, exhibit trends consistent with RT-PCR. Iisin treatment led to a decrease in uPAR protein expression in BRAFwt cells, with reductions of 12.10% and 30.27% in HBL and LND1, respectively. Conversely, in BRAFmut cells, irisin treatment resulted in an increase, with a rise of 4.67% in M3 and 37.53% in Hmel1. Likewise, uPA expression decreased significantly following treatment, with reductions of 42.36% and 7.11% in HBL and LND1, and decreases of 10.90% and 46.46% in Hmel1 and M3, respectively. Regarding PAI-1, irisin induced a 7.05% increase in HBL, a 28.55% reduction in LND1, and a 4.67% reduction in M3. Notably, in Hmel1, PAI-1 protein levels increased by 37.53% post-irisin treatment.

As shown in Fig.5A, irisin treatment significantly downregulated the expression of both MMPs and their inhibitors in wt MM cell lines compared to untreated cells ($p<0.001$***.). Hmel1 cell line followed the same general pattern ($p<0.01$**) as the wt cells, except of MMP-9, which expression was unchanged following treatment compared to untreated cells. On the contrary, the M3 cell line displayed a different scenario; indeed, irisin treatment increased the expression of MMP-2, MMP-9, and their inhibitors, TIMP-2 and TIMP-1, respectively ($p<0.001$***). These data were also supported by the gelatine zymography assay (Fig.5B). HBL and LND1 cell lines showed a reduction in the activation of both irisin-related MMP-2 and MMP-9, Hme1 showed no change, while M3 showed gelatinase activation following irisin treatment compared to the untreated cells.

Figure 4. Western blot analysis showing uPAR, uPA, and PAI-1 protein expression in MM cell line treated with irisin 10 nM.

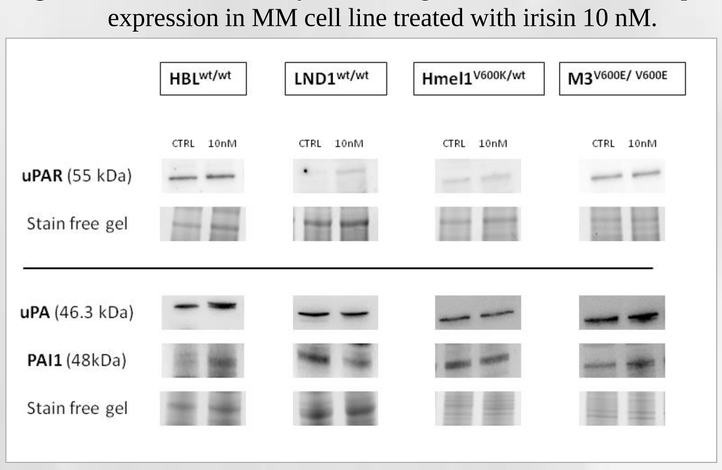
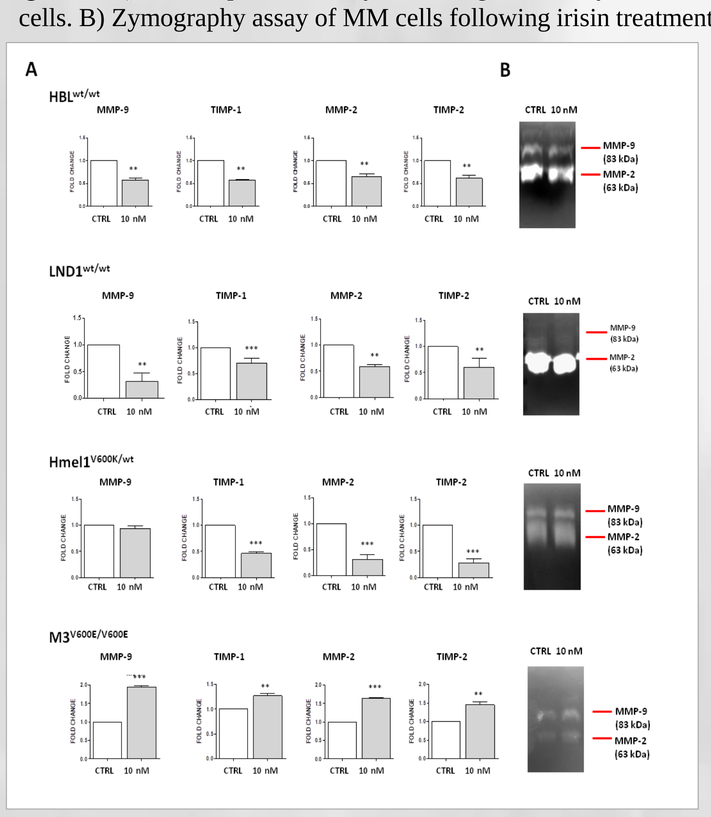


Figure 5. A) Gene expression analysis of the gelatinase system in MM cells. B) Zymography assay of MM cells following irisin treatment.



As shown in Fig.5A, HBL and LND1 cell lines showed a reduction in the activation of both irisin-related MMP-2 and MMP-9, Hme1 showed no change, while M3 showed gelatinase activation following irisin treatment compared to the untreated cells.

Conclusion. Collectively our results suggest that fibrinolytic system and MMP-2/MMP-9 gelatinases were down-regulated in BRAF wild-type melanoma cell lines in an irisin-dependent manner, while a differential expression was observed in BRAFmut cell lines.