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Overexpression of IL-32 and Birc-3 transcripts in sarcopenic muscles from patients bearing Head and Neck carcinomas

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INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) are generally associated with a reduction in skeletal muscle mass called sarcopenia, which is a major medical concern. One difficulty is our profound ignorance of the molecular alterations occurring *in situ* in skeletal muscles. Another difficulty is the virtual absence of *in vitro* models. The aim of this study was to perform transcriptome profiling by bulk RNAseq on human immortalized myoblasts co-cultivated with malignant HNSCC cells *in vitro* and on muscle fragments collected from HNSCC patients with or without sarcopenia.

METHODS

➤ *In Vitro* investigations on human myoblasts stimulated by HNSCC cells:

- Indirect co-culture of myoblasts (the target immortalized myoblasts were MyoN and AB1190; Mamchaoui et al., Skeletal Muscle, 2011, 1:34) and head and neck carcinoma cells: HNSCC cell lines were FaDu and Detroit; Cellosaurus accession numbers: CVCL_1218 and CVCL_1171, using Transwell 6-well plates (4µm) with internal cups. Carcinoma cells were seeded in the upper compartment and myoblasts in the lower compartment. Control condition: myoblasts were seeded in the absence of cells in the upper compartment. In some cases, co-cultivation was replaced by stimulation with clarified conditioned media. Crude conditioned media were obtained from 48h culture of malignant cells at 75% confluence in 75 cm² flasks with 15 ml of culture medium and 5% fetal calf serum. Clarification was achieved by ultracentrifugation at 36000 rpm, 4°C for 2 hours (Optima XPN-80 Ultracentrifuge). Myoblasts were treated with culture medium containing 1/4th of clarified conditioned medium.
- Assessment of morphological changes: empowered by AI (CellProfiler) as a first simple read-out of global cellular alterations; two parameters - major axis length and perimeter of cell - were selected to represent morphological changes.
- RT-qPCR was done using a StepOnePlus apparatus (Applied Biosystems). The abundance of transcripts was calculated using the 2^{-ΔCT} method with PPIA (peptidylprolyl isomerase A), RPLPO (ribosomal protein lateral stalk subunit P0) and ACE1 (angiotensin-converting enzyme 1) mRNAs as calibrators.

➤ Exploration of clinical muscle samples collected from HNSCC patients:

- **Collection of muscle fragments.** Muscle fragments were obtained from 12 HNSCC patients undergoing surgery at Gustave Roussy without previous oncological treatment (in the frame of the Magnolia protocol ; NCT 04842162). Most of these fragments were from the sternocleidomastoid muscle. Control muscle fragments (quadriceps or fascia lata tensor) were from patients free of malignant tumors and muscle diseases ("Institut de Myologie", hôpital Salpêtrière, Paris).
- **Assessment of global skeletal muscle mass.** Skeletal muscle index (SMI) was calculated using medical imaging. Paravertebral muscle area was measured on CT scan section at the level of C3. It was normalized to body surface area. The Cutoff values for sarcopenia was 52.4 cm²/m² for males and 38.5 cm²/m² for females.

RESULTS

➤ 1. Proliferating myoblasts stimulated by malignant cells undergo morphological changes, mainly a greater elongation.

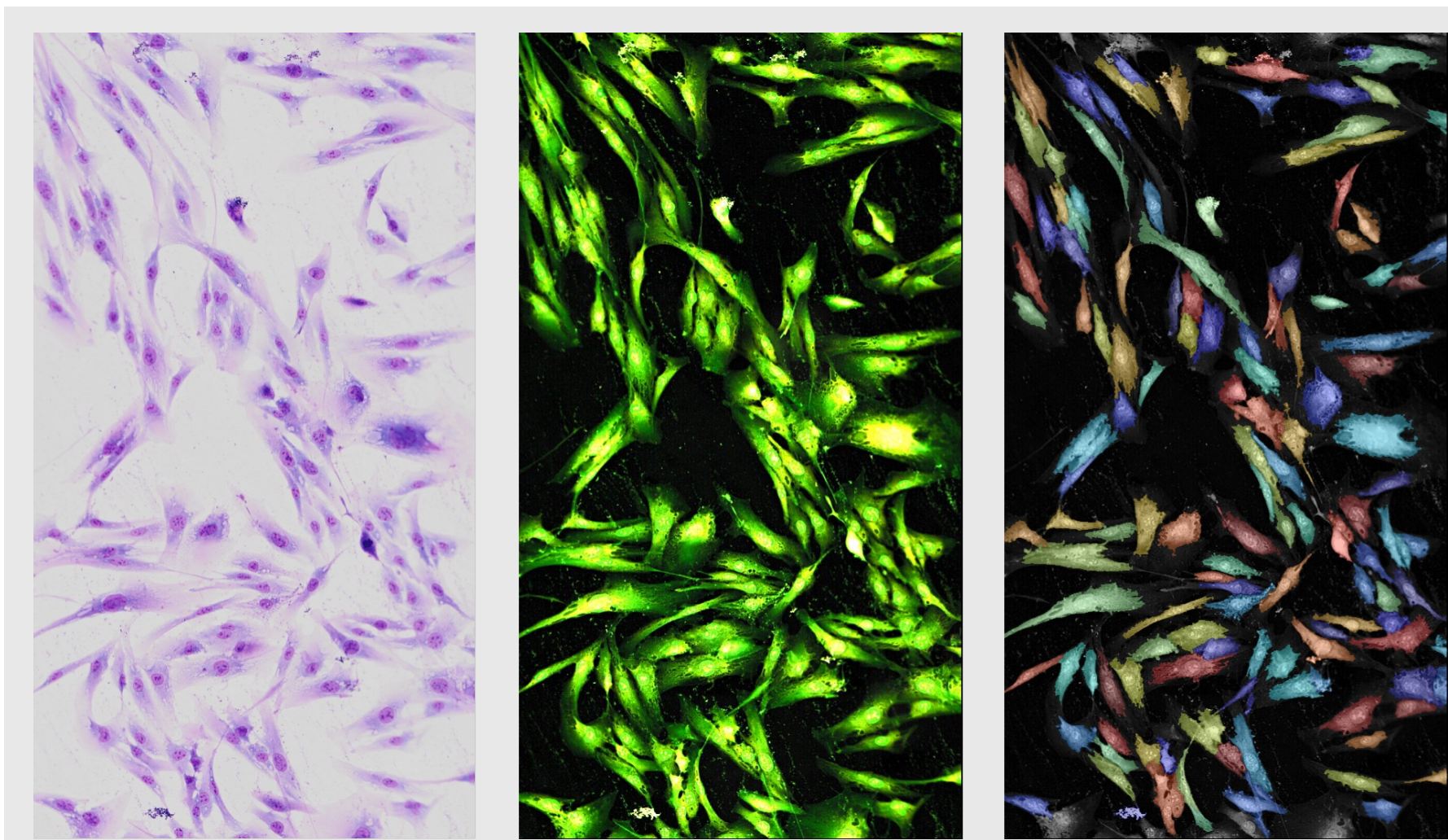


Fig 1a. The outlines of Giemsa stained AB1190 myoblasts (stimulated by conditioned medium) are identified and marked by CellProfiler "AI software".

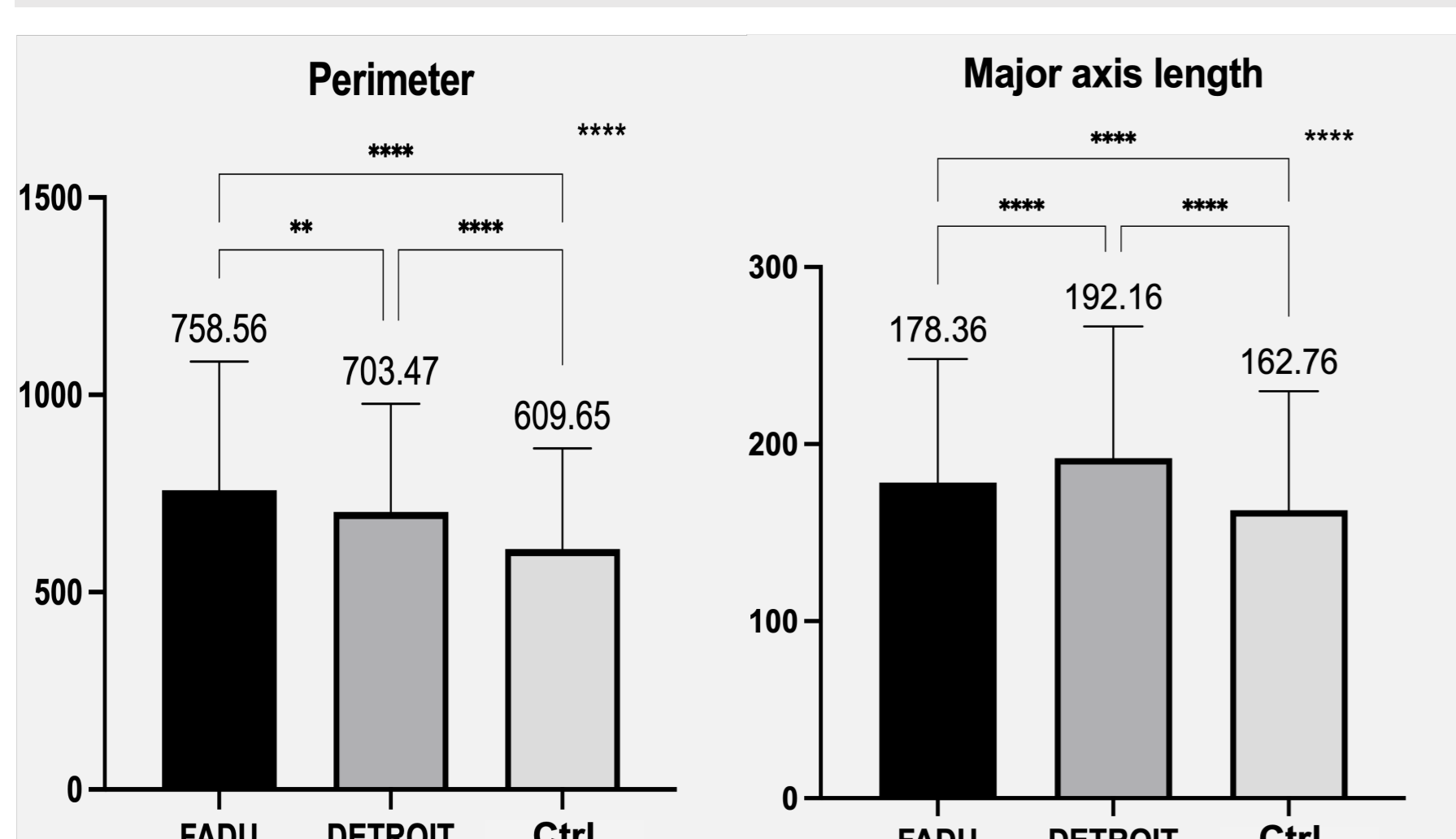


Fig 1b. AB1190 myoblasts co-cultured with FaDu or Detroit conditioned medium exhibit increased perimeters and major axis lengths (unit is in pixels; *p<0.05, **p<0.01, ***p<0.001).

➤ 2. Transcriptional analysis of muscle cells exposed or not exposed to the influence of malignant HNSCC cells *in vitro* or in the patients:

Comparative bulk RNAseq of 1) proliferating Myo-N cells exposed (n=3) or not exposed (n=1) to FaDu cells (co-cultures using the Transwell system); 2) proliferating AB1190 cells exposed (n=4) or not exposed (n=4) to clarified FaDu conditioned media for 48h (controls exposed to mock conditioned medium); 3) muscle fragments from HNSCC patients (n=12) and control donors without known cancer or muscle disease (n=3). Among the mRNAs up-regulated in these 3 experimental settings were IL-32 and BIRC3 mRNAs.

Gene ID	Description	Functions	log2FC, p<0.05 <i>In vitro</i>	log2FC, p<0.05 Patients
IL32	Interleukin 32	regulates human myogenesis, insulin sensitivity and ATP levels in muscle cells	1.465	4.197
BIRC3	Baculoviral IAP repeat containing 3 (also called c-IAP2)	Inhibits apoptosis, modulates inflammatory signaling and immunity, mitogenic kinase signaling and cell proliferation	2.244	2.148

Fig 2. RNAseq: Two genes (IL32 and BIRC3) are upregulated in co-cultivated myoblasts *in vitro* as well as in muscle fragments from HNSCC patients *in situ*.

➤ 3. RT-qPCR detection of IL-32 and BIRC3 mRNAs in AB1190 myoblasts co-cultured with HNSCC cells

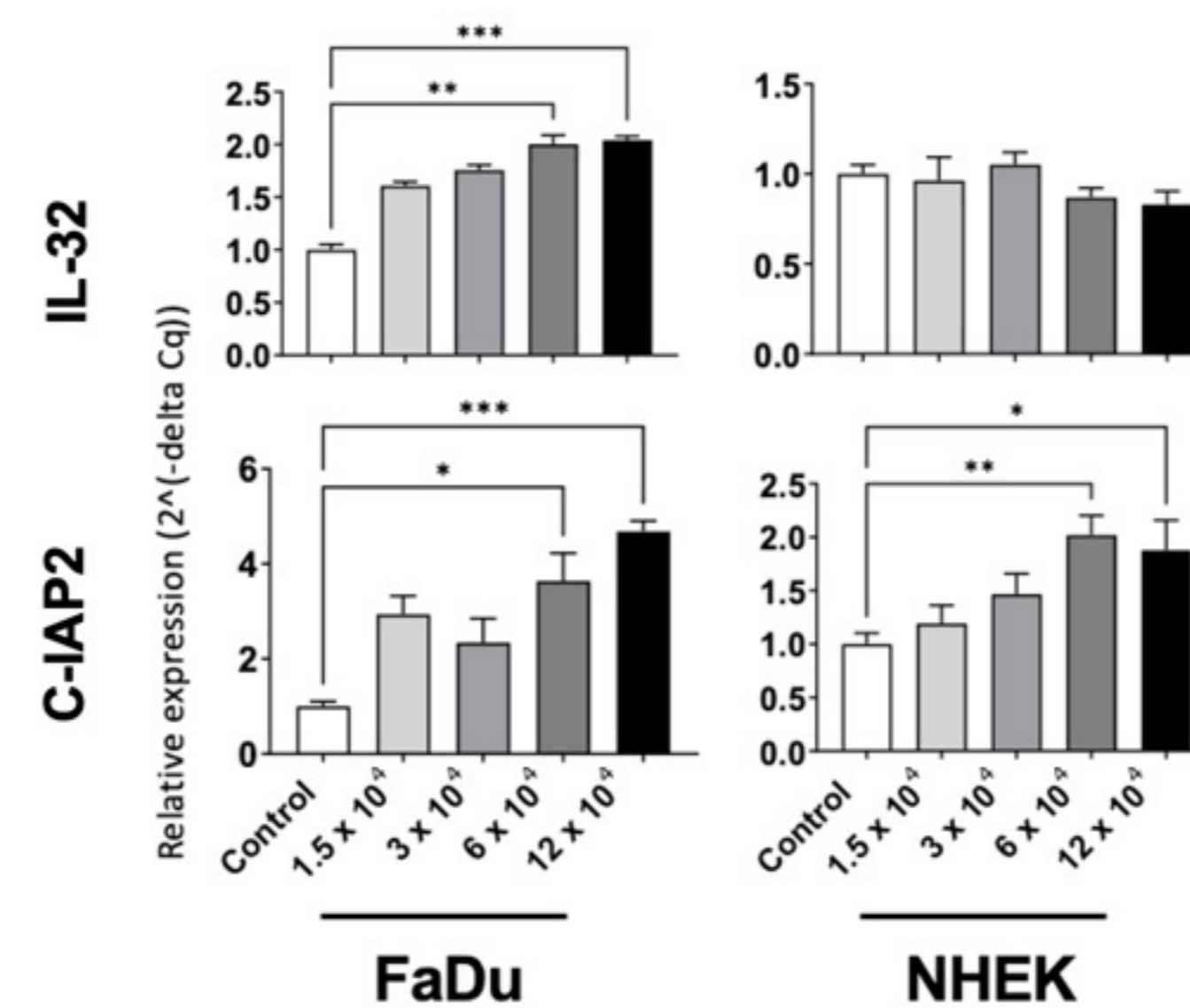


Fig 3. RT-qPCR: Contactless influence of HNSCC cells on the expression of IL32 and C-IAP2 (BIRC3) in immortalized human myoblasts (AB1190). For the control condition, we made co-cultures with Normal Epidermal Keratinocytes (NHEK) instead of malignant cells.

➤ 4. Greater abundance of IL-32 and BIRC3 mRNAs in muscle fragments from patients bearing HNSCC

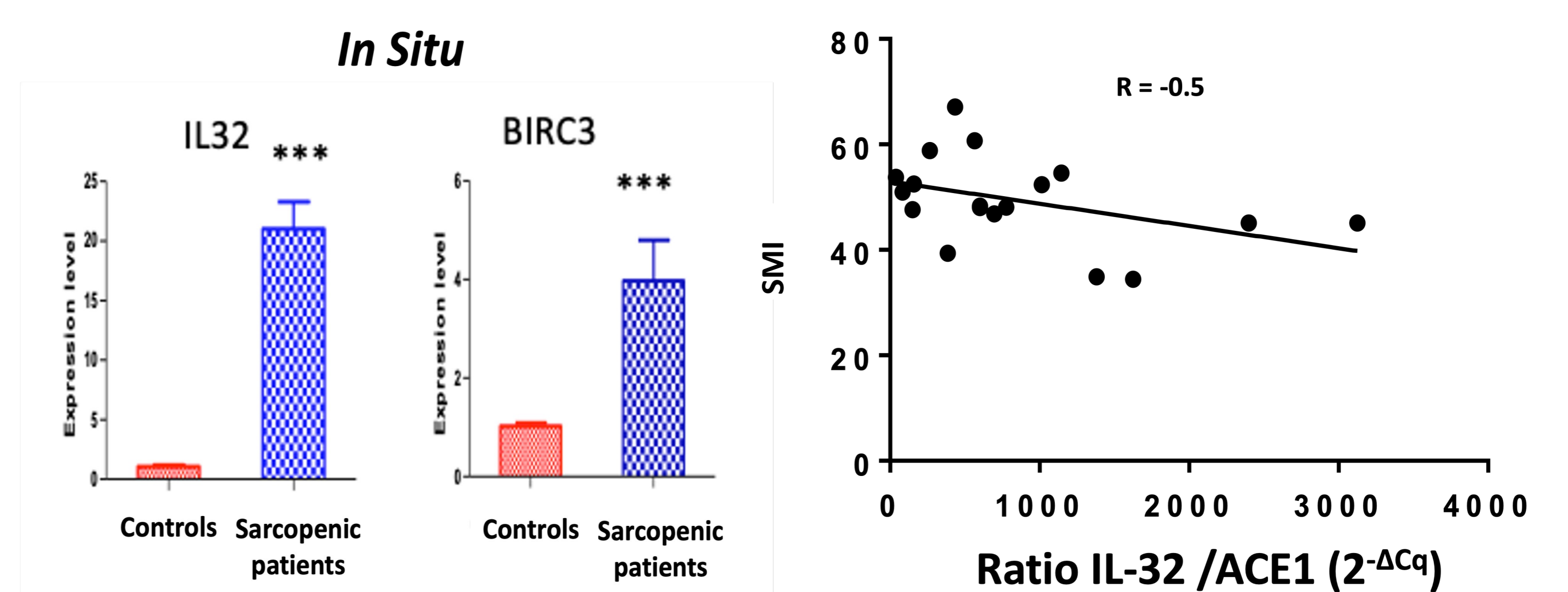


Fig 4a. RT-qPCR: Detection of IL-32 and BIRC3 transcripts upregulated in myoblasts co-cultivated with malignant cells *in vitro*.

Fig 4b. RNAseq: A negative correlation between IL-32 transcripts in muscle fragments from HNSCC patients and SMI.

CONCLUSION

IL32 and Birc3 mRNAs emerge as candidate biomarkers of muscle alterations in HNSCC patients. Our co-cultivation system may provide a basis for *in vitro* modelization of cancer-related sarcopenia.

PERSPECTIVES

In forthcoming research, exploring larger series of clinical specimens and incorporating the detection of IL-32 and BIRC3 proteins, alongside a range of other mRNAs and proteins, could offer valuable insights into the reciprocal interaction between muscle cells and HNSCC cells.