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MUSCLE WASTING IN HEAD AND NECK CARCINOMAS: INVESTIGATIONS ON THE UNDERLYING TRANSCRIPTIONAL REPROGRAMMATION

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INTRODUCTION:**Cancer cachexia:**

- Is a multifactorial syndrome often including skeletal muscle atrophy.
- Affects at least 55% of patients bearing Head and Neck Squamous Cell Carcinomas (HNSCCs)
- To our knowledge, molecular mechanisms of muscle alterations have never been investigated on clinical muscle specimens from HNSCC patients.

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*, muscle fragments collected from HNSCC patients with or without cachexia, and finally from nude mice bearing human HNSCC xenografts.

METHODS: Our study was made in three parts:

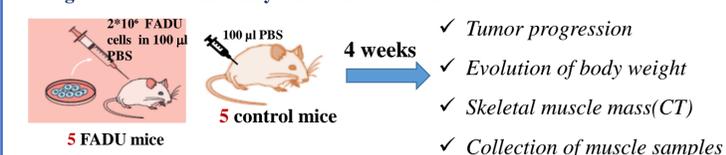
❖ *In Vitro* investigations on human myoblasts co-cultivated with HNSCC cells (FADU et SQ20B):

- Indirect co-culture system (without cell-cell contact), using Transwell inserts.
- Proliferation setting: MyoN cells were seeded in 6 well plates (5000 cells/well) and expanded using a specific "proliferation" or "growth" medium.
- Differentiation setting: MyoN cells were seeded in 6 well plates at high density (250 000 cells/well), incubated in rich medium for 24h to have cells reaching confluence and then subjected to a change of culture medium with substitution by a less rich one called "differentiation" medium. MitoTracker™ Orange (fluorescent mitochondrial dye) and Hoechst 33342 (fluorescent DNA dye), were added into the culture media : Differentiating cells were distinguished according to their mitochondrial reactivity.



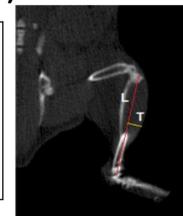
❖ *In Situ* investigations on muscles collected from nude mice xenografted with HNSCCs cells (FADU):

10 eight-week old female athymic swiss nude mice



Monitoring of body and skeletal muscle mass:

- Body weight
- Skeletal muscle mass by micro-computed tomography
 - L (Length of the tibia)
 - T (Thickness of the muscle)
 - IMM: T/L
- Gastrocnemius muscle weight



Micro-CT evaluation of the hind limb muscle mass

❖ Exploration of clinical muscle samples collected from HNSCC patients.

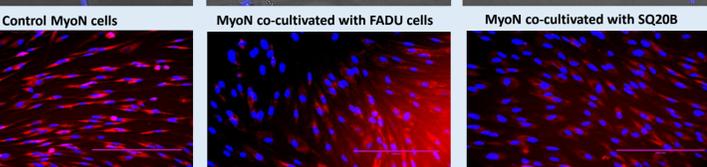
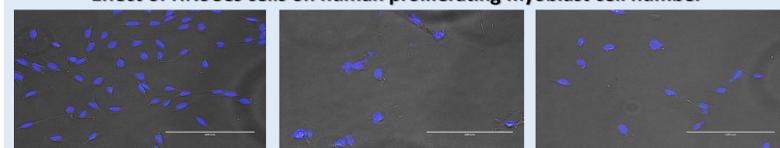
12 patients with a diagnosis of HNSCC (Department of Head and Neck surgery of Gustave Roussy Institute).
3 control skeletal muscle fragments from patients free of malignant tumors, age and sex matched.

❖ **RNA sequencing** was used to assess gene expression changes and gene function enrichment in myoblasts co-cultivated with malignant cells as well as skeletal muscle samples collected from mice xenografted with HNSCC cells (FADU) or HNSCC patients.

RESULTS

❖ Part 1 : Investigations on human myoblasts co-cultivated *in vitro* with HNSCC cells

Effect of HNSCCs cells on human proliferating myoblast cell number



Influence of HNSCC cells on human differentiating myoblasts *in vitro*

- Impact on the number and morphology of MyoN cells proportional to the amount of malignant HNSCC cells.
- Mitotracker fluorescence much weaker for cells co-cultivated with malignant cells

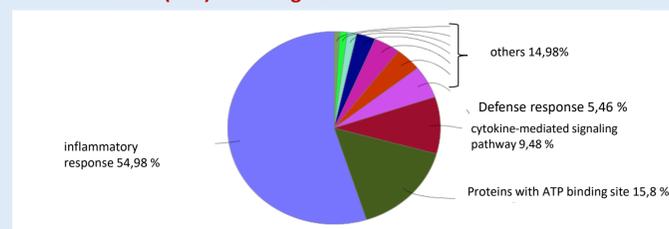
→ Significant impact of HNSCC cells on myoblast proliferation and consistent impairment of myoblast differentiation.

RESULTS:

❖ Part 2: Transcriptional alterations in human sarcopenic muscle *in vitro* and *in situ*

- Transcriptional Alteration in myoblasts (MyoN) co-cultivated with malignant cells
- Transcriptional alterations in muscles of HNSCC patients suffering from cachexia

- 58 differentially expressed genes (DGEs) ($p < 0,05$)
- 96.5% (n=56) up regulated
- 3.5% (n=2) down regulated

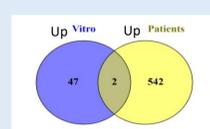


- 756 differentially expressed genes (DGEs) ($p < 0,05$)
- 71.8% (n=546) up regulated
- 28,2% (n=213) down regulated

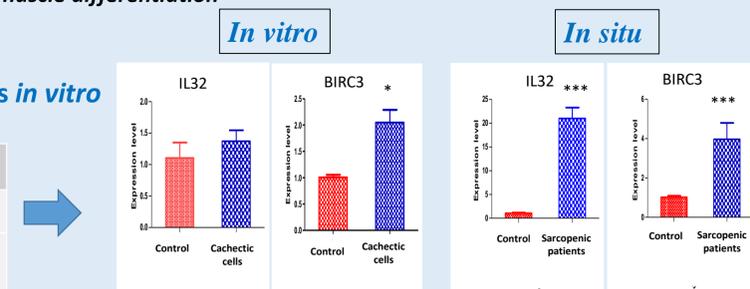
RNAseq : distinct gene profiles in skeletal muscles from cachectic patients with alterations of pathways like:

- Energy metabolism
- Immune and inflammatory response
- Mitochondrial metabolism
- Muscle development and muscle differentiation

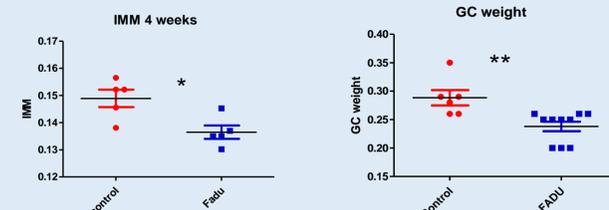
➤ Two genes are upregulated in sarcopenic muscles *in situ* as well as in co-cultivated myoblasts *in vitro*



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



❖ Part 3: Investigations of muscle wasting in nude mice xenografted with HNSCC cells



Muscle mass loss evaluated by micro CT just before sacrificing mice
Mann-Whitney test ($p < 0,05$).

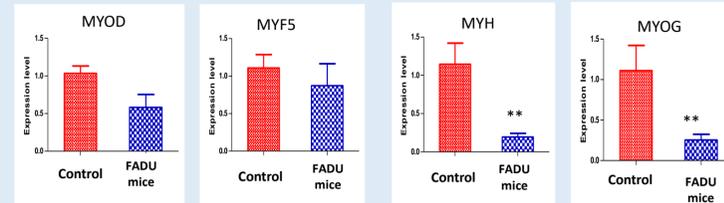
Gastrocnemius muscle weight in FADU mice compared with control mice

HNSCC cells xenograft

Capacity to induce distant muscles alterations

- Transcriptional alterations of murine MYOD, MYF5, MYH1 and MYOG in nude mice bearing or not Fadu tumors

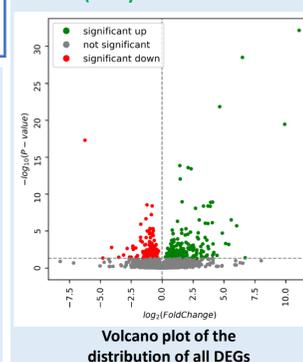
RT qPCR detection of *IL-32* and *BIRC3* transcripts upregulated in sarcopenic muscle *in situ* and myoblasts co-cultivated with malignant cells *in vitro*
Mann-Whitney test was performed to define significant difference. * $p < 0,05$ ** $p < 0,01$; *** $p < 0,001$.



RT qPCR of genes coding for factors involved in muscle growth and differentiation. RNA was extracted from gastrocnemius muscles of mice. Mann-Whitney test was performed to define significant difference. * $p < 0,05$ ** $p < 0,01$; *** $p < 0,001$.

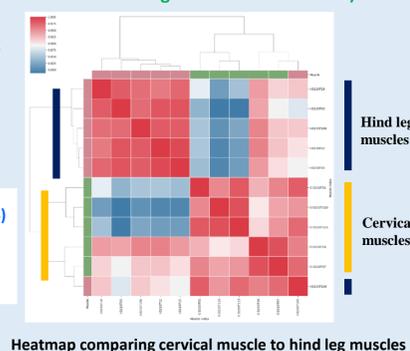
❖ Part 4: Transcriptional alterations in muscles collected from nude mice xenografted with HNSCCs cells

(Analysis of RNA extracted from 16 muscles specimen collected from 3 control mice and 3 mice xenografted with FADU cells)

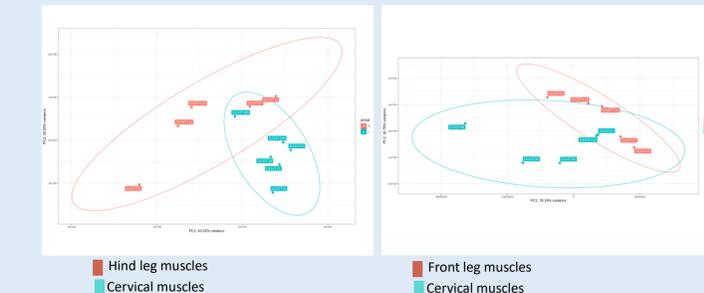


- 601 differentially expressed genes (DGEs) ($p < 0,05$)
- 58.2% (n=350) up regulated
- 41.8% (n= 251) down regulated

- Over expression of *BIRC 3* ($p < 0,05$) in muscle from tumor-bearing mice



Heatmap comparing cervical muscle to hind leg muscles



Principal component analysis (PCA) → Distinction between different muscle location

→ Major transcriptome variations are observed depending on muscle anatomic location. However, transcriptome alterations related to tumor bearing conditions are observed regardless of muscle anatomic locations, including up-regulation of *BIRC3* transcription.

CONCLUSIONS: Up-regulation of *BIRC 3* is a transcriptional modification shared by sarcopenic muscles in ENT patients and in xenografted mice as well as by myoblasts co-cultivated *in vitro* with malignant cells. Although the mechanism of *BIRC3* up-regulation in cachectic muscle cells is not yet known, this finding is likely to be important for the management of HNSCC patients in a context of promising therapeutic results obtained using Smac mimetics. Because these compounds are inhibitors of the c-IAP protein family, it will be important in the future to monitor their positive or negative effects on muscle alterations in cachectic HNSCC patients.