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# Déconditionnement et régénération du muscle strié squelettique : rôle du niveau d'activité contractile sur le développement d'infiltrations graisseuses

Allan Pagano

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# THÈSE

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**Docteur**

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**Sciences du Mouvement Humain**

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Spécialité :  
**Sciences du mouvement humain**

Présentée par **Allan PAGANO**

**Déconditionnement et régénération du  
muscle strié squelettique : rôle du niveau  
d'activité contractile sur le développement  
d'infiltrations graisseuses**

Soutenue le **25 Novembre 2016** devant le jury composé de

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# TABLE DES MATIERES

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LISTE DES ABREVIATIONS.....	1
LISTE DES FIGURES.....	3
LISTE DES PUBLICATIONS.....	5
<b>Publications indexées ISI</b> .....	<b>5</b>
<b>Articles en cours de soumission</b> .....	<b>5</b>
AVANT-PROPOS.....	6
ETAT DE LA LITTERATURE.....	8
<b><u>CHAPITRE 1 : LA PLASTICITE DU MUSCLE STRIE SQUELETTIQUE</u></b> .....	<b>9</b>
<b>1.1. Généralités</b> .....	<b>9</b>
<b>1.2. Le déconditionnement musculaire</b> .....	<b>9</b>
1.2.1. Définition et facteurs d'apparition.....	9
1.2.2. Caractéristiques du déconditionnement.....	10
<b>1.3. Les modèles expérimentaux du déconditionnement musculaire</b> .....	<b>12</b>
1.3.1. Modèles de microgravité réelle.....	13
1.3.2. Modèles humains de microgravité simulée et d'hypoactivité.....	14
1.3.3. Modèles animaux de microgravité simulée et d'hypoactivité.....	17
1.3.4. Modèles cellulaires.....	18
<b>1.4. Perturbation de la balance protéique : mécanismes cellulaires et moléculaires</b> .....	<b>19</b>
1.4.1. Une augmentation de l'activité des systèmes protéolytiques.....	19
1.4.2 Les facteurs de transcription FoxO et NFκB : des régulateurs essentiels des systèmes protéolytiques.....	24

1.4.3 Une diminution de la synthèse protéique .....	26
1.4.4. Le déconditionnement musculaire : une perte de masse rapide .	28
<b>CHAPITRE 2 : LA REGENERATION MUSCULAIRE .....</b>	<b>32</b>
<b>2.1. Généralités.....</b>	<b>32</b>
<b>2.2. Modèles expérimentaux.....</b>	<b>33</b>
2.2.1. Modèles humains .....	33
2.2.2. Modèles animaux .....	36
<b>2.3. Les cellules satellites.....</b>	<b>38</b>
2.3.1. Un rôle majeur dans la régénération.....	38
2.3.2. Les régulateurs de l'activité des cellules satellites.....	40
2.3.3. Une population hétérogène .....	46
<b>2.4. Autres cellules souches dans l'environnement musculaire.....</b>	<b>48</b>
2.4.1. Les fibro-adipogenic progenitors : FAPs.....	49
2.4.2. PW1 <sup>+</sup> /Pax7 <sup>-</sup> interstitial cells : PICs .....	51
2.4.3. Les péricytes.....	51
2.4.4. Les myo-endothelial progenitors: MECs.....	52
<b>2.5. Rôle clé du système immunitaire.....</b>	<b>52</b>
2.5.1. Les éosinophiles et neutrophiles.....	53
2.5.2. Les macrophages.....	54
2.5.3. Les regulatory T cells : Treg .....	55
<b>2.6. Suppléments alimentaires et optimisation du processus de régénération.....</b>	<b>57</b>
<b>CHAPITRE 3 : LES INFILTRATIONS GRAISSEUSES.....</b>	<b>59</b>
<b>3.1. Définition et localisation.....</b>	<b>59</b>
<b>3.2. Altération de la fonction musculaire .....</b>	<b>60</b>
<b>3.3. Les modèles expérimentaux .....</b>	<b>62</b>
3.3.1. Modèles sans régénération musculaire.....	63
3.3.2. Modèles avec régénération musculaire.....	65

<b>3.4. L'origine des IMAT .....</b>	<b>67</b>
3.4.1. L'implication majeure des FAPs .....	67
3.4.2. PDGFR $\alpha$ : un récepteur clé .....	67
3.4.3. Du progéniteur à l'adipocyte mature .....	69
<b>3.5. IMAT et fibrose : signe d'une régénération musculaire perturbée .....</b>	<b>71</b>
3.5.2. Perturbation de la régénération musculaire avec l'avancée en âge .....	71
3.5.3. Altération du recrutement et de la fonction des macrophages.....	75
3.5.4. Exemple de la dystrophie de Duchenne .....	76
3.5.5. Le modèle glycérol : pourquoi davantage d'IMAT ? .....	77
<b>3.6. L'activité physique comme principale mesure pour lutter contre l'apparition d'IMAT .....</b>	<b>78</b>
<b>3.7. Interactions FAPs et cellules satellites/immunitaires .....</b>	<b>81</b>
3.7.1. Un système complexe pour une régénération efficace .....	81
3.7.2. Effets d'une modulation de l'activité contractile ? .....	85
<b>OBJECTIFS DE LA THESE .....</b>	<b>87</b>
<b>RESULTATS ET DISCUSSION .....</b>	<b>89</b>
<b>ETUDE 1 : LA REGENERATION MUSCULAIRE ET L'ACCUMULATION D'IMAT EST MODULEE PAR LES CONTRAINTES MECANIQUES .....</b>	<b>90</b>
<b>Contexte de l'étude 1 .....</b>	<b>91</b>
<b>Discussion de l'étude 1 .....</b>	<b>109</b>
<b>ETUDE 2 : LA PERTURBATION DE L'AXE TNF<math>\alpha</math>/TGF-<math>\beta</math>1 DURANT LA REGENERATION MUSCULAIRE FAVORISE L'APPARITION D'IMAT .....</b>	<b>112</b>
<b>Contexte de l'étude 2 .....</b>	<b>113</b>
<b>Discussion de l'étude 2 .....</b>	<b>139</b>



<b>ETUDE 3 : 3 JOURS DE REDUCTION D'ACTIVITE CHEZ L'HOMME INDUIT LE</b>	
<b>DEVELOPEMENT D'IMAT.....</b>	<b>142</b>
<b>Contexte de l'étude 3 .....</b>	<b>143</b>
<b>Discussion de l'étude 3 .....</b>	<b>168</b>
<b>DISCUSSION GENERALE ET PERSPECTIVES.....</b>	<b>171</b>
<b>TRAVAUX SUPPLEMENTAIRES.....</b>	<b>182</b>
<b>Muscle wasting and aging: Experimental models, fatty infiltrations, and prevention.....</b>	<b>183</b>
<b>Autophagy and Protein Turnover Signaling in Slow-Twitch Muscle during Exercise .....</b>	<b>216</b>
<b>The role of AMP-activated protein kinase in the coordination of skeletal muscle turnover and energy homeostasis .....</b>	<b>229</b>
<b>REFERENCES.....</b>	<b>242</b>

# LISTE DES ABREVIATIONS

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<b>4EBP1</b> : eIF4E binding protein 1	<b>FGF</b> : Fibroblast growth factor
<b>Akt</b> : Protein kinase B (PKB)	<b>Fstn</b> : Follistatin
<b>AMP/ADP/ATP</b> : Adenosine Mono/Di/Tri-Phosphate	<b>HDAC</b> : Histone Deacetylase
<b>AMPK</b> : AMP-activated protein kinase	<b>HU</b> : Hindlimb unloading
<b>Atg</b> : Autophagy related Gene	<b>IFN<math>\gamma</math></b> : Interferon $\gamma$
<b>BNIP3</b> : BCL2/adenovirus E1B 19 kDa interacting protein 3	<b>IGF</b> : Insulin-like growth factor
<b>C/EBPs</b> : CCAAT-enhancer-binding proteins	<b>IGFR</b> : Insulin-like growth factor receptor
<b>Caspases</b> : Cysteine-dependent aspartate-directed proteases	<b>Il</b> : Interleukin
<b>CCL2</b> : Chemokine ligand 2	<b>Il-r</b> : Interleukin receptor
<b>CCR2</b> : C-C chemokine receptor type 2	<b>IMAT</b> : InterMuscular Adipose Tissue
<b>CD</b> : Cluster of differentiation	<b>IRM</b> : Imagerie par résonance magnétique
<b>CHOP10</b> : C/EBP homologous protein-10	<b>KO</b> : Knock-out
<b>CSA</b> : Surface de section	<b>MAFbx</b> : Muscle atrophy F-box ou Atrogin1
<b>CTX</b> : Cardiotoxine	<b>MAPK</b> : Mitogen-activated protein kinases
<b>DI</b> : Dry immersion	<b>Mdx</b> : X-linked muscular dystrophy
<b>DMD</b> : Dystrophie musculaire de Duchenne	<b>MECs</b> : Myo-endothelial progenitors
<b>E3 ligase</b> : Ubiquitin protein enzyme	<b>MRF4</b> : Muscle-specific regulatory factor 4
<b>EDL</b> : <i>Extensor digitorum longus</i>	<b>MRFs</b> : Myogenic regulatory factors
<b>eIF3f</b> : Eukaryote initiation factor 3f	<b>mRNA</b> : ARN messagers
<b>FAPs</b> : Fibro/adipogenic progenitors	<b>MSC</b> : Cellule souche mésenchymateuse
<b>FOXO</b> : Forkhead box class O	<b>Mstn</b> : Myostatin
	<b>mTORC1/2</b> : Mammalian target of rapamycin complex 1/2

**mTOR** : Mammalian target of rapamycin

**MuRF1** : Muscle ring finger 1

**MyHC** : Myosin Heavy Chain

**Myf5** : Myogenic factor 5

**MyHC** : Myosin Heavy Chain

**MyoD** : Myogenic differentiation 1

**NF- $\kappa$ B** : Nuclear Factor-kappa B

**NG2** : Neuron glia antigen 2

**NO** : Nitric Oxide

**Notch** : Neurogenic locus notch homolog protein

**NTX** : Notexine

**Pax7** : Paired box protein Pax-7

**PBS** : Phosphate buffer saline

**PDGF** : Platelet-derived growth factor

**PDGFR** : Platelet-derived growth factor receptor

**PI3K** : Phosphoinositide 3 kinase

**PICs** : PW1<sup>+</sup>/Pax7<sup>-</sup> interstitial cells

**PPAR** : Peroxysome proliferator-activated receptor

**PW1** : Zinc finger transcription factor  
PW1

**S6K1** : p70 S6 kinase 1

**SC** : Cellule satellite

**Ser** : Sérine

**STAT3** : Signal transducer and activator of transcription 3

**SUP** : Système ubiquitine-protéasome

**Thr** : Thréonine

**TGF** : Transforming Growth Factor

**TNF $\alpha$**  : Tumor necrosis factor  $\alpha$

**Treg** : Regulatory T cells

**Tyr** : Tyrosine

**Ub** : Ubiquitine

**ULK1** : unc-51 like autophagy activating kinase

**ULLS** : Unilateral limb suspension

**VL** : *Vastus lateralis*

**Wnt** : Wingless-type MMTV integration site

# LISTE DES FIGURES

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<b>Figure 1.</b> Les modèles expérimentaux du déconditionnement musculaire .....	13
<b>Figure 2.</b> Modèle expérimental humain de suspension unilatérale du membre inférieur (ULLS) .....	15
<b>Figure 3.</b> Modèle expérimental humain d'immersion sèche (Dry Immersion).....	16
<b>Figure 4.</b> Le système autophagique-lysosomal .....	21
<b>Figure 5.</b> Le système ubiquitine-protéasome .....	24
<b>Figure 6.</b> Variation de la balance protéique en condition de réduction d'activité.....	26
<b>Figure 7.</b> PI3K/Akt/mTOR : principale voie de synthèse protéique .....	28
<b>Figure 8.</b> Principaux mécanismes impliqués dans la régulation de la masse musculaire .....	30
<b>Figure 9.</b> Les mécanismes d'apoptose et de nécrose.....	33
<b>Figure 10.</b> La dystrophine et les différentes protéines de structure du tissu musculaire.....	34
<b>Figure 11.</b> Histopathologie musculaire liée à la DMD chez l'homme .....	35
<b>Figure 12.</b> Localisation des cellules satellites dans le tissu musculaire .....	39
<b>Figure 13.</b> Processus de différenciation d'une cellule satellite : expression de Pax7 et des MRFs.....	40
<b>Figure 14.</b> Schéma représentatif des différents facteurs influençant les cellules satellites .....	46
<b>Figure 15.</b> Les différentes divisions possibles pour une cellule satellite .....	46
<b>Figure 16.</b> Les différentes populations de cellules souches résidentes au sein du tissu musculaire .....	49
<b>Figure 17.</b> Localisation des FAPs au sein du tissu musculaire .....	50
<b>Figure 18.</b> L'implication majeure du système immunitaire dans la régénération musculaire .....	56

<b>Figure 19.</b> Illustration de la localisation des IMAT au sein de l'environnement musculaire .	60
<b>Figure 20.</b> Corrélations entre contenu en IMAT et avancée en âge/sensibilité à l'insuline....	61
<b>Figure 21.</b> Sévère accumulation d'IMAT dans les modèles murins de blessures tendineuses de la coiffe des rotateurs.....	64
<b>Figure 22.</b> Posture d'un patient atteint de camptocormie et histopathologie du muscle strié squelettique.....	65
<b>Figure 23.</b> Corrélation positive entre l'expression de PDGFR $\alpha$ et le degré de sévérité de la DMD.....	66
<b>Figure 24.</b> PPAR $\gamma$ et les C/EBPs en tant qu'acteurs majeurs de l'adipogenèse.....	69
<b>Figure 25.</b> Modifications de l'environnement musculaire lors de l'avancée en âge et son impact sur la régénération musculaire.....	73
<b>Figure 26.</b> Quantification par IRM des infiltrations graisseuses chez des jeunes hommes atteints de la DMD .....	76
<b>Figure 27.</b> Cinétique du développement des IMAT par le modèle glycérol de régénération .	77
<b>Figure 28.</b> L'entraînement régulier préserve l'apparition du déconditionnement musculaire lié à l'avancée en âge .....	81
<b>Figure 29.</b> Illustration des différentes interactions entre cellules immunitaires, endothéliales, satellites et FAPs lors de la régénération musculaire .....	82
<b>Figure 30.</b> Mécanismes d'apoptose et de survie des FAPs après blessure musculaire .....	84
<b>Figure 31.</b> Illustration des différents inhibiteurs de la voie du TGF- $\beta$ 1 .....	180

# LISTE DES PUBLICATIONS

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## **Publications indexées ISI**

- Allan F. Pagano, Thomas Brioché, Guillaume Py, Angèle Chopard (2016). Muscle wasting and aging: experimental models, fatty infiltrations, and prevention. **Molecular Aspects of Medicine** (IF=10,2).

- Allan F. Pagano, Rémi Demangel, Thomas Brioché, Élodie Jublanc, Christelle Bertrand-Gaday, Robin Candau, Claude A. Dechesne, Christian Dani, Anne Bonnieu, Guillaume Py, Angèle Chopard (2015). Muscle regeneration with intermuscular adipose tissue accumulation is modulated by the level of mechanical constraints. **PLOS ONE** (IF=3.2).

- Allan F. Pagano, Guillaume Py, Henri Bernardi, Robin B. Candau, Anthony M.J. Sanchez (2014). Autophagy and Protein Turnover Signaling in Slow-Twitch Muscle during Exercise. **Medicine and Science in Sports and Exercise**. (IF=4.5)

- Anthony M.J. Sanchez, Robin Candau, Alfredo Csibi, Allan F. Pagano, Audrey Raibon, Henri Bernardi (2012). The role of AMP-activated protein kinase in the coordination of skeletal muscle turnover and energy homeostasis. **American Journal of Physiology - Cell physiology**. (IF=3.5)

## **Articles en cours de soumission**

- Allan F. Pagano, Thomas Brioché, Coralie Arc-Chagnaud, Angèle Chopard, Guillaume Py. TNF $\alpha$ /TGF- $\beta$ 1 axis disruption during skeletal muscle regeneration promotes fatty infiltrations development. En soumission. **eLIFE** (IF=7)

- Allan F. Pagano, Thomas Brioché, Coralie Arc-Chagnaud, Rémi Demangel, Angèle Chopard, Guillaume Py. 3 days of human skeletal muscle disuse promote fatty infiltrations development. En soumission. **Frontiers in Physiology** (IF=3.9)

# AVANT-PROPOS

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Le muscle strié squelettique est un tissu fascinant qui permet d'assurer les fonctions essentielles à notre existence : se mouvoir, maintenir sa posture, se nourrir, communiquer ou tout simplement respirer. Les muscles, qui représentent environ 40% de notre poids de corps, sont capables de faire varier leur masse et leurs propriétés intrinsèques de manière rapide en réponse aux différents niveaux de stimuli. Les nombreuses situations physiologiques ou pathologiques, impliquant une diminution des sollicitations fonctionnelles des muscles, engendrent un déconditionnement musculaire. C'est le cas, par exemple, lors d'un alitement prolongé du sportif à la suite d'une blessure, lors d'un séjour en environnement de microgravité, ou encore lors de maladies chroniques telles que le cancer, le diabète de type 2, la broncho-pneumopathie obstructive, ou tout simplement dans le contexte d'un style de vie sédentaire. Ce déconditionnement se caractérise par une perte de masse et de force musculaire, une augmentation de la fatigabilité, le développement d'une insulino-résistance mais également par l'apparition d'infiltrations graisseuses. Dans de nombreux cas, la littérature scientifique a mis en évidence que le pourcentage de perte de force musculaire excède largement le pourcentage d'atrophie musculaire, soulignant ainsi le rôle d'autres facteurs dans la perte de force. Les infiltrations graisseuses, bien qu'encore peu étudiées, sont une caractéristique importante du déconditionnement musculaire qui contribue en partie à expliquer cette disproportion. Les infiltrations graisseuses ont tout d'abord été étudiées dans un contexte de vieillissement et d'obésité, tout en étant corrélées avec une diminution de la fonction musculaire et l'apparition d'une insulino-résistance. Des études ont ensuite mis en lumière le développement d'infiltrations graisseuses associé avec une régénération musculaire altérée. Ainsi, ces études ont également permis de mettre en évidence une population de cellules souches résidant au sein de l'environnement musculaire et étant à l'origine de ces infiltrations graisseuses : les fibro-adipogenic progenitors (FAPs). Des études récentes ont ensuite démontré que ces FAPs étaient influencés par de nombreux facteurs issus du tissu musculaire lui-même, des cellules satellites ou encore des cellules du système immunitaire/inflammatoire, pouvant ainsi moduler l'apparition des infiltrations graisseuses. Ces nombreux facteurs sont, pour la plupart, modulés par le niveau d'activité physique et, par conséquent, une modulation du niveau de l'activité contractile pourrait ainsi moduler le devenir des FAPs au sein du tissu musculaire.

L'objectif de ce travail de thèse a donc été d'étudier le rôle du niveau d'activité contractile sur l'apparition d'infiltrations graisseuses au sein du tissu musculaire, aussi bien dans un contexte de régénération perturbée, que dans un contexte de déconditionnement musculaire dénué de régénération. Ainsi, mon travail de thèse est composé de deux grands axes afin d'étudier le développement d'infiltrations graisseuses. Un premier axe traite de la modulation de l'activité contractile au travers d'un modèle de régénération permettant l'apparition d'infiltrations graisseuses (études 1 et 2) ; un deuxième axe concerne le développement précoce des infiltrations graisseuses dans un modèle de déconditionnement musculaire simulant les effets de la microgravité chez l'homme (étude 3).

A la suite d'une revue de la littérature, les résultats de ces 3 études sont synthétisés et discutés (les publications relatives aux trois études sont insérées dans les chapitres respectifs). Trois autres publications, sans lien direct avec les travaux de thèse, sont également présentées et placées en annexe.



# ETAT DE LA LITTERATURE

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# **CHAPITRE 1 : LA PLASTICITE DU MUSCLE STRIE SQUELETTIQUE**

## **1.1. Généralités**

Le tissu musculaire strié squelettique représente environ 40% de notre masse corporelle, et joue un rôle primordial dans le maintien postural et la production de mouvements. C'est un tissu qui présente une capacité d'adaptation exceptionnelle, que l'on définit comme étant sa plasticité. Il peut en effet s'adapter pour répondre aux différents niveaux de sollicitations qui lui sont imposés. Les muscles sont capables de faire varier non seulement leur masse mais aussi leurs caractéristiques fonctionnelles, le tout grâce à de vastes modifications structurales et métaboliques, afin de s'adapter aux différentes contraintes mécaniques et environnementales rencontrées. Cette plasticité repose en grande partie sur la balance entre processus anaboliques et cataboliques, responsables de l'homéostasie musculaire, concourant ainsi à un équilibre et une régulation fine de notre masse musculaire. Ces mécanismes de protéosynthèse et de protéolyse sont régulés par des voies de signalisation, elles-mêmes influencées par une multitude de facteurs incluant le stress mécanique, l'activité contractile, la disponibilité en nutriments ou encore de nombreuses hormones (Egan & Zierath, 2013, Goldspink, 1999, Sandri, 2008, Schiaffino et al., 2013). Outre l'aspect structural de la plasticité musculaire fortement développé dans cet état de la littérature, la réorganisation structurale du tissu musculaire squelettique s'opère également en concert avec des adaptations métaboliques toutes aussi importantes.

## **1.2. Le déconditionnement musculaire**

### **1.2.1. Définition et facteurs d'apparition**

A l'inverse de l'hypertrophie musculaire liée à une augmentation des sollicitations fonctionnelles, comme par exemple liée à un entraînement en résistance, le déconditionnement musculaire peut se définir comme une adaptation négative des processus physiologiques en réponse à un environnement défavorable, incluant une diminution des sollicitations fonctionnelles (Baldwin et al., 2013, Bodine, 2013, Chopard et al., 2009). Ce déconditionnement musculaire peut être décrit comme primaire, conséquence directe d'un environnement défavorable, ou comme secondaire s'il est indirectement lié à une pathologie. Le déconditionnement primaire est donc fortement lié aux conditions d'inactivité musculaire rencontrées lors de l'alitement prolongé (séjours hospitaliers ou grossesse pathologique par exemple), lors de l'immobilisation d'une ou plusieurs articulations à la suite d'une blessure,

lors d'une exposition à un environnement en microgravité, ou tout simplement en conséquence d'un style de vie sédentaire. Par ailleurs, le simple fait d'avancer en âge est également accompagné d'un déconditionnement musculaire considéré comme un syndrome gériatrique appelé sarcopénie. Le déconditionnement musculaire secondaire est quand à lui associé à des maladies chroniques telles que le cancer, le diabète de type 2, la broncho-pneumopathie obstructive ou encore de nombreuses myopathies (Brioche et al., 2016).

### 1.2.2. Caractéristiques du déconditionnement

Le déconditionnement musculaire provoqué par une diminution des contraintes mécaniques se caractérise, de manière non exhaustive, par une diminution de masse et de force musculaire, un shift phénotypique, une augmentation de la fatigabilité, une résistance à l'insuline et un développement d'infiltrations graisseuses (**Fig.1**).

Depuis de nombreuses années, un bon nombre de publications ont démontré une perte de masse musculaire rapide et importante dans différentes situations de déconditionnement musculaire chez l'animal et chez l'homme (Baldwin et al., 2013, Brioche et al., 2016, Cros et al., 1999, Hanson et al., 2013, Kawashima et al., 2004, Manini et al., 2007). L'étude de Manini et al. (2007) a par exemple montré, chez l'homme, une diminution d'environ 7% du volume musculaire (mollet et cuisse) après 4 semaines d'inactivité et des valeurs similaires ont été retrouvées au niveau des adducteurs après 20 jours d'alitement (Kawashima et al., 2004). Une réduction de 15 et 10% du volume musculaire du soléaire et des gastrocnémiens respectivement a également été observée chez des astronautes à la suite de 6 mois de microgravité à bord de l'ISS (Trappe et al., 2009). L'étude de Cros et al. (1999) a par ailleurs démontré, chez l'animal, que seulement 2 jours d'hypoactivité (modèle de suspension du train arrière chez le rat) entraînait une diminution de la masse du soléaire de 20%, pour ensuite atteindre une diminution de 40% après 20 jours de suspension.

La force générée par un muscle est, par définition, corrélée à sa surface de section. Ainsi, de manière logique, la force musculaire est bien évidemment diminuée lors du déconditionnement musculaire. Toutefois, il est classiquement reporté, dans les situations d'hypoactivité chronique ou de microgravité, que la perte de force excède largement le degré d'atrophie musculaire. Dans ce contexte, d'autres facteurs, tels que les infiltrations graisseuses, les propriétés intrinsèques de la machinerie contractile ou encore les facteurs nerveux contribueraient ainsi à la perte de force accrue observée. Par exemple l'étude longitudinale d'une durée de 5 ans de Delmonico et al. (2009) a démontré, dans le contexte de

l'avancée en âge, une diminution de force 2 à 5 fois supérieure à celle de la masse musculaire. Cette étude a, par ailleurs, souligné l'importance de l'apparition d'infiltrations graisseuses. La revue de littérature de Di Prampero & Narici (2003) souligne également une diminution de la force maximale développée par différents groupes musculaires à la suite de vols spatiaux allant jusqu'à 25%, ces valeurs de perte de force musculaire atteignant des valeurs bien plus élevées que le niveau de perte de masse, soulignant ainsi l'importance d'autres facteurs influençant la perte de force.

Concernant le shift phénotypique musculaire, on distingue au sein d'un même muscle des fibres lentes riches en chaînes lourdes de myosine (MyHC) de type I qui développent des forces plus faibles que des fibres rapides riches en MyHC de type II. Il existe aussi plusieurs isoformes des MyHC de type II que l'on distingue en fonction de leur vitesse de contraction, par ordre croissant : IIa, IIx et IIb chez le rongeur, l'isoforme IIb n'existant pas chez l'homme (Egan & Zierath, 2013). Il est clairement établi dans la littérature que les muscles les plus touchés par le déconditionnement musculaire lié à l'hypoactivité sont les muscles posturaux, lents et donc constitués majoritairement de fibres de type I (Baldwin et al., 2013). Dans des conditions d'inactivité musculaire, ces muscles vont perdre leur fonction spécifique de maintien postural et vont être la cible privilégiée de la dégradation des chaînes lourdes de myosine. Ainsi, l'étude de Baldwin et al. (1990) portant sur un vol spatial d'environ 12 jours chez le rat a montré que les MyHCI représentaient l'isoforme la plus dégradée. Hormis dans quelques cas comme celui de la sarcopénie pour lequel le shift phénotypique est inversé (Brioche et al., 2016), les différentes situations de déconditionnement musculaire résultent généralement d'une diminution de la quantité en MyHCI au profit des MyHCII (Baldwin et al., 2013). Ce shift phénotypique, en plus d'être conditionné par une situation globale d'atrophie musculaire, a pour conséquence d'induire une fatigabilité accrue du muscle concerné. En effet, les MyHC de type II ont un régime de contraction plus rapide mais sont d'avantage fatigables en comparaison aux MyHCI.

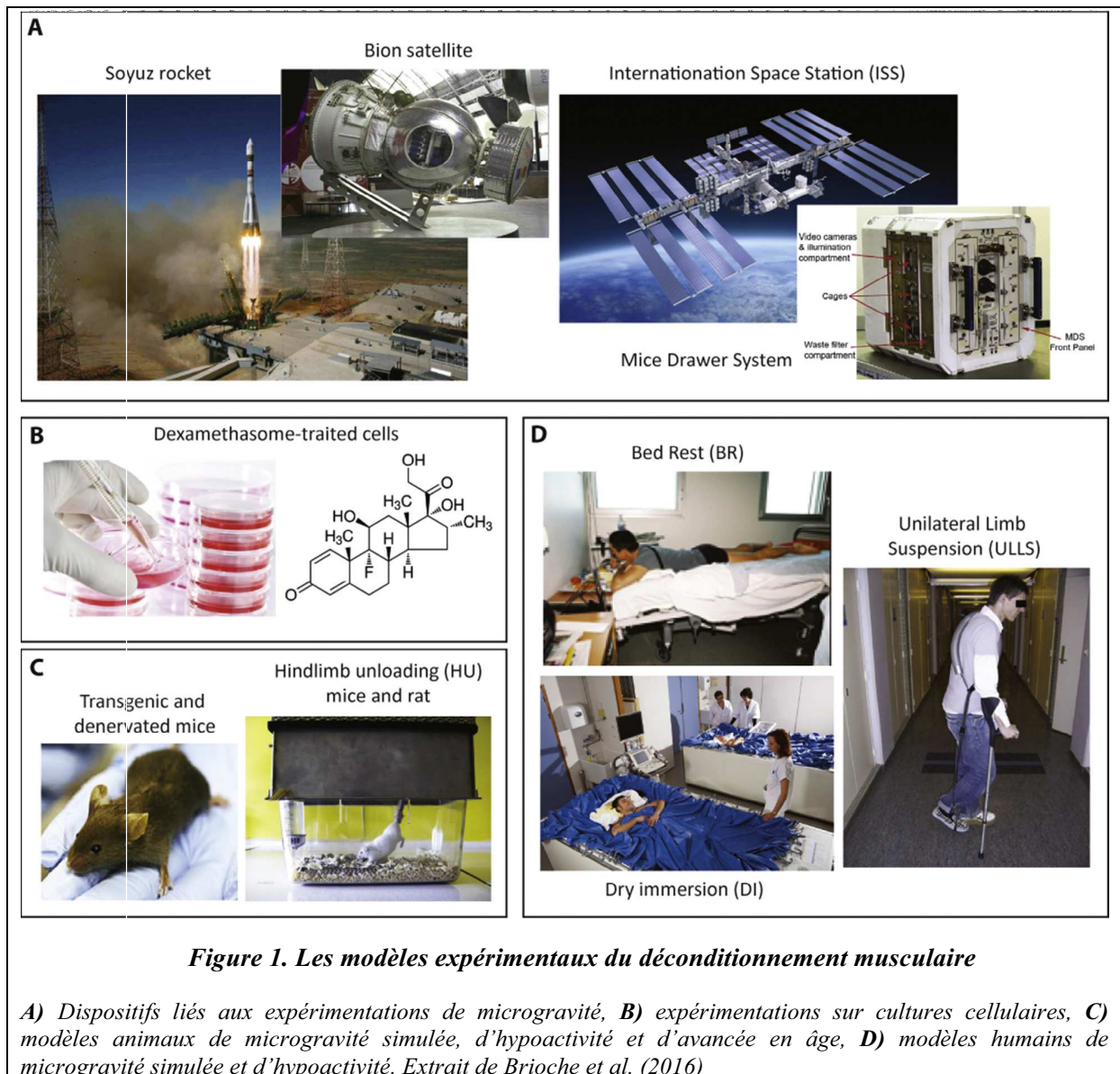
Si, comme précédemment évoqué, la littérature a déjà souligné que la perte de force musculaire excède assez largement la perte de masse engendrée par différentes situations de déconditionnement musculaire (Berg et al., 1997, Delmonico et al., 2009, Di Prampero & Narici, 2003, Jubrias et al., 1997), l'aspect de l'apparition d'infiltrations graisseuses entre les fibres musculaires (IMAT pour « *Inter-Muscular Adipose Tissue* ») est un facteur important pouvant influencer la perte de force. Le développement et l'apparition d'IMAT, bien que de nombreuses fois observé, est encore peu étudié et apparaît comme un facteur important du

déconditionnement musculaire (Addison et al., 2014, Brioche et al., 2016). En effet, même si les IMAT semblent être une caractéristique du muscle sain chez l'homme, leur développement et accumulation est fortement corrélé aux différentes situations de déconditionnement musculaire mais également de perturbation de la régénération (Marcus et al., 2010, Sciorati et al., 2015, Uezumi et al., 2014b). Cette partie concernant les IMAT sera traitée de manière complète et spécifique dans le chapitre 3 de cet état de la littérature.

Si les mécanismes cellulaires et moléculaires entraînant le développement et l'accumulation des IMAT sont encore incomplètement décrits, ceux concernant la perte de masse musculaire ont été largement étudiés ces dernières décennies. La littérature a ainsi pu, grâce à de nombreux modèles expérimentaux, démontrer le rôle majeur de l'augmentation de l'activité des différents systèmes protéolytiques, mais également une diminution de ceux régissant la synthèse protéique, dans la perte de masse musculaire.

### **1.3. Les modèles expérimentaux du déconditionnement musculaire**

Dans les dernières décennies, la communauté scientifique a validé de nombreux modèles expérimentaux cellulaires, animaux et humains afin d'étudier et décortiquer les mécanismes du déconditionnement musculaire. De nombreux modèles, en relation avec l'environnement spatial, ont largement contribué aux avancées scientifiques et ont fait avancer de manière importante la recherche autour de l'appareil musculo-squelettique (Fitts et al., 2000, Trappe et al., 2009) (**Fig.1**).



### 1.3.1. Modèles de microgravité réelle

Ces expérimentations en condition de microgravité réelle incluent majoritairement des études concernant les rongeurs et les humains. Par exemple, des programmes basés sur les sciences la vie comme Spacelab ou Spacehab ont inclus de nombreuses expérimentations impliquant des astronautes, mais également des animaux et des insectes (Fitts et al., 2010; Miu et al., 1990; Roffino et al., 1998). Des expériences conduites grâce au programme BION, d'abord soviétique puis russe, représentent une série de missions de satellites scientifiques lancés par une fusée Soyuz depuis le cosmodrome de Baïkonour, dans le nord de la Russie, et placé sur une orbite à 575km d'altitude. Après 12 missions réalisées entre 1966 et 1996 incluant des collaborations internationales, y compris avec les Etats-Unis pendant la guerre

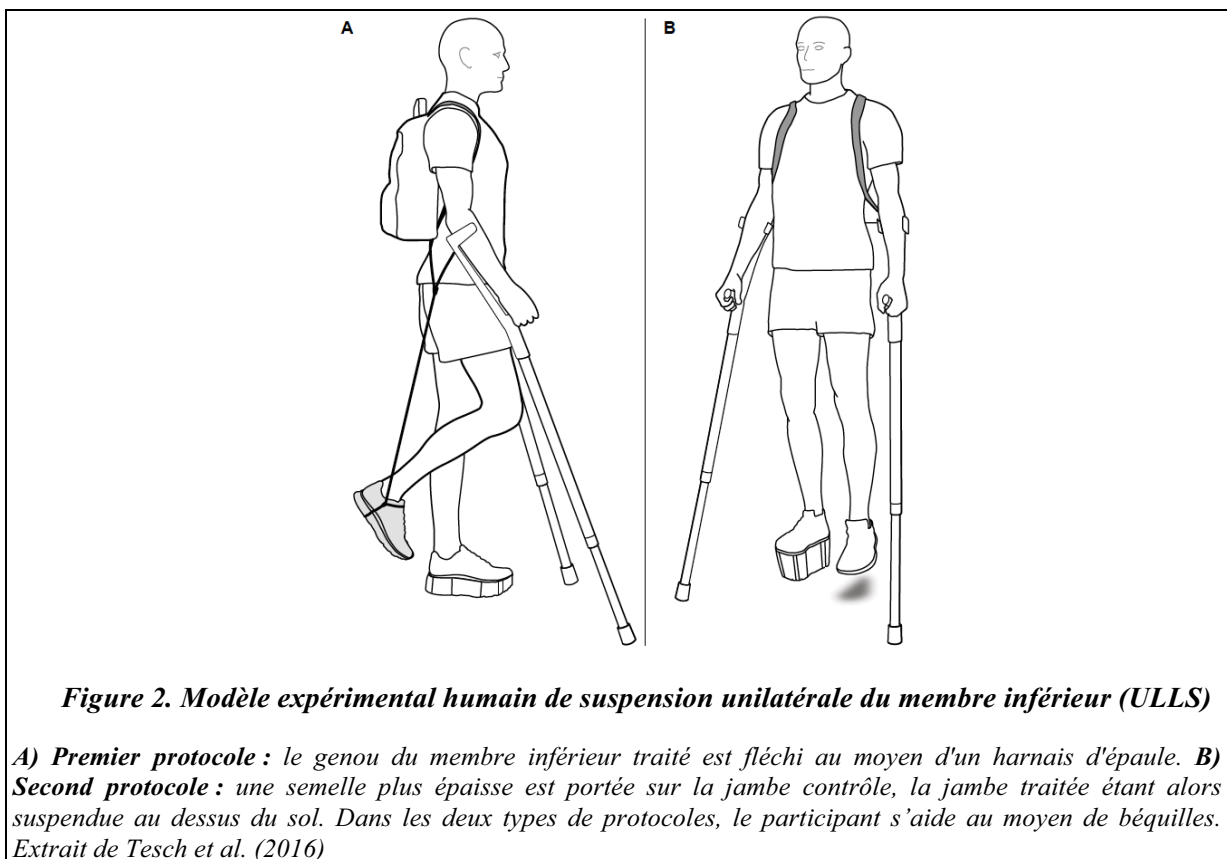
froide, le programme est réactivé par la Russie en 2005 et rebaptise le programme BION-M. La première mission, BION-M1, réalisée en avril/mai 2013 a consisté en un vol spatial de 30 jours et a emporté des souris, des gerbilles, des escargots ou encore des poissons. Ce programme a d'ores et déjà engendré de nombreuses publications scientifiques, notamment des résultats obtenus chez la souris, examinant les effets d'un séjour spatial prolongé sur les différentes fonctions physiologiques (Berg-Johansen et al., 2016, Gritsyna et al., 2015, Islamov et al., 2014, Naumenko et al., 2015, Novoselova et al., 2015, Ogneva et al., 2015, Ogneva et al., 2014, Popova et al., 2015, Proshchina et al., 2015, Sofronova et al., 2015, Tsybko et al., 2015, Ulanova et al., 2015).

L'actuelle station spatiale internationale (ISS), nous donne également l'opportunité de réaliser des expériences de microgravité réelle. Cette station, qui vole à 400km du sol avec une vitesse de 28.800km/h, ne met que 90min pour faire un cycle complet autour de la Terre. Ainsi, les astronautes vivant et travaillant à bord de l'ISS sont sujets à pas moins de 16 levers et couchers de soleil par jour ! L'ISS permet donc, encore aujourd'hui, de fournir l'opportunité de travailler sur les effets à long terme de l'environnement de microgravité et de nombreuses études ont déjà démontré la considérable variabilité dans les proportions de perte de masse musculaire et de perte fonctionnelle parmi les astronautes (Fitts et al., 2000, Trappe et al., 2009). Le système « Mice Drawer » à bord de l'ISS permet également d'accueillir des souris et a donc donné l'opportunité de travailler sur les conséquences de 91 jours de microgravité réelle (Cancedda et al., 2012, Sandona et al., 2012).

### 1.3.2. Modèles humains de microgravité simulée et d'hypoactivité

De nombreux modèles expérimentaux menés chez l'homme sain, et cette fois sur la terre ferme, permettent d'induire un déconditionnement musculaire. Ils sont pour la plupart dédiés à étudier les mécanismes impliqués dans les différentes situations impliquant l'inactivité musculaire mais sont également utilisés afin d'identifier et d'évaluer différentes contre-mesures afin de lutter contre ces effets néfastes. Les études conduites chez l'homme sain se sont principalement centrées sur l'utilisation du modèle de suspension unilatérale de la jambe (ULLS pour *Uni-Lateral Limb Suspension*) (Hackney & Ploutz-Snyder, 2012), de l'alitement prolongé (*bed rest*) (Pavy-Le Traon et al., 2007) ou encore d'immersion sèche (DI pour *Dry Immersion*) (Navasiolava et al., 2011) et ont permis aux chercheurs de distinguer les effets liés à l'inactivité plus que ceux liés aux comorbidités lors du déconditionnement musculaire.

Le protocole expérimental ULLS est un protocole permettant de mettre une des deux jambes en suspension : dans un premier protocole la jambe traitée est fléchie et suspendue au-dessus du sol au moyen d'un harnais d'épaule (Berg et al., 1991, Horstman et al., 2012), dans un second protocole, une semelle plus épaisse est portée sur la jambe contrôle, la jambe traitée étant alors suspendue au-dessus du sol (Tesch et al., 2016) (**Fig.2**). Ce modèle a donc été utilisé depuis 1991 afin d'induire une inactivité musculaire sur une seule jambe (Hackney & Ploutz-Snyder, 2012). De plus, le fait de n'induire une atrophie musculaire que sur une seule des deux jambes permet également d'être au plus proche des aspects cliniques rencontrés lors d'une immobilisation à la suite d'une fracture osseuse par exemple. ULLS cause donc logiquement un déconditionnement musculaire qui, par définition, est associé de manière non exhaustive à une perte de masse musculaire (environ 0.44% de perte par jour pour le vaste latéral), à une diminution de force mais également à l'apparition d'infiltrations graisseuses (Hackney & Ploutz-Snyder, 2012).

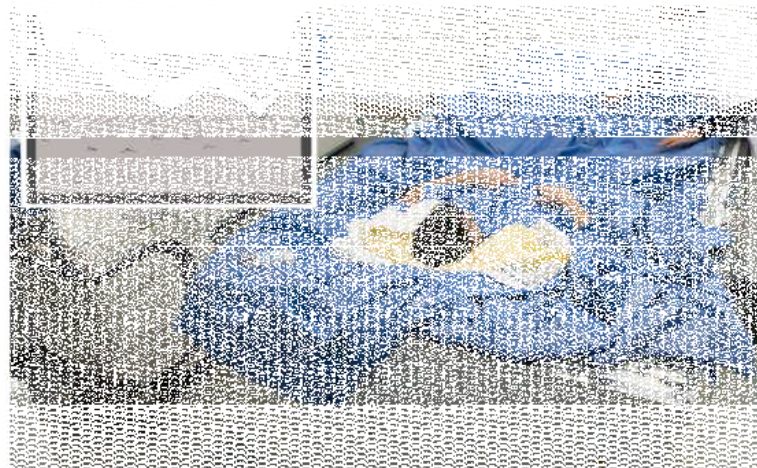


Le modèle de bed rest, et plus précisément le « head-down tilt bed rest », permet d'exposer le sujet à un alitement prolongé sur un lit orienté de manière à mettre le sujet à  $-6^{\circ}$  degrés la tête en bas. Ce modèle est reconnu pour être un modèle valide afin de mimer les effets physiologiques de la microgravité et permet également d'appliquer et de tester de manière



simple différentes contre-mesures. Les expérimentations issues de bed rest (tête en bas ou non) ont déjà permis de générer, et génèrent encore, un grand nombre de données sur les effets de l'inactivité. De nombreuses études ont par exemple collectivement observé une atteinte plus prononcée des muscles extenseurs du genou et de la cheville (Alkner & Tesch, 2004, Arentson-Lantz et al., 2016, Dirks et al., 2016, LeBlanc et al., 1992, Rittweger et al., 2005).

Dans les années 1970, à l'heure où le développement des programmes spatiaux était en plein essor, les soviétiques proposèrent le modèle « dry immersion » (DI) comme un moyen de simuler au sol les effets de la microgravité. Ce modèle expérimental, décrit et validé pour la première fois par des spécialistes en biologie et médecine spatiales de Moscou (Shulzhenko et al., 1976), consiste à immerger un sujet dans de l'eau thermiquement neutre, et recouverte d'un tissu élastique étanche. Le sujet est alors immergé, et repose librement dans l'eau, tout en restant sec (**Fig.3**).



*Figure 3. Modèle expérimental humain d'immersion sèche (Dry Immersion)*

*Adapté de Navasiolava et al. (2011)*

L'avantage considérable de cette technique, par rapport à celle du traditionnel bed rest, est représenté par l'absence de structure de support sous le corps des sujets. Ainsi l'absence de support mécanique crée alors un état plus proche de la situation retrouvée lors d'un environnement de microgravité réelle (Navasiolava et al., 2011). DI est un modèle de microgravité simulée largement utilisé en Russie, mais reste bien moins connu partout ailleurs, et plus particulièrement dans les régions occidentales pour lesquelles le modèle bed rest est encore privilégié. Le modèle de DI a la caractéristique de placer les sujets en situation d'hypokinésie et d'hypodynamie, réduisant ainsi le stress mécanique au niveau des muscles et

des os, ce qui induit donc une diminution rapide du tonus et de la tension musculaire jusqu'à des niveaux inférieurs à ceux observés lors d'expérimentations de bed rest. La chute du tonus des muscles posturaux apparaît dans les premières heures d'immersion, avec des valeurs atteignant -40% à -50%. Pendant cette même période, il a également été rapporté une diminution de l'activité électromyographique des muscles extenseurs du genou (Navasiolava et al., 2011). Une réduction de la surface de section des fibres du vaste latéral a également été rapportée après 3 jours (-5 à -9%) et 7 jours (-15 à -18%) d'immersion sèche (Shenkman et al., 2004a), soulignant ainsi la précocité des mécanismes d'atrophie des fibres musculaires.

### 1.3.3. Modèles animaux de microgravité simulée et d'hypoactivité

Les rongeurs, principalement la souris et le rat, sont les animaux les plus utilisés dans l'étude du déconditionnement musculaire. Parmi les différents modèles d'atrophie musculaire utilisés, les plus pertinents et les plus utilisés restent les modèles de dénervation et d'hindlimb unloading (HU).

La dénervation est une technique consistant à enlever, par chirurgie, une partie du nerf sciatique. Le muscle perd alors sa capacité à se contracter, puisqu'il n'y a plus aucune stimulation nerveuse. Cette situation entraîne de rapides et drastiques effets délétères sur le tissu musculaire incluant une forte dégradation protéique (Furuno et al., 1990). Comme chez l'homme, et dans différents modèles de déconditionnement musculaire, la recherche souligne une perte de force disproportionnée à celle de la masse musculaire. Par exemple, l'étude menée par Carlson et al. (1996) a montré une perte de masse du muscle *extensor digitorum longus* (EDL) de 75% couplée avec une perte de force maximale isométrique, mesurée avec un système de contractilité *ex vivo*, de 99% après 4 mois de dénervation.

Le modèle HU, développé par la NASA (National Aeronautics and Space Administration) dans le milieu des années 1970, a depuis été largement validé comme étant un modèle simulant les effets de la microgravité (Baldwin et al., 2013, Morey-Holton & Globus, 2002). Plusieurs centaines d'articles scientifiques, relatifs à l'utilisation de ce modèle de suspension, ont été publiés. Un paramètre crucial de ce modèle réside tout simplement dans la cage et le matériel de suspension en lui-même. En effet, le matériel utilisé doit pouvoir permettre à l'animal de bouger librement dans sa cage tout en ayant les pattes postérieures dans le vide grâce à une suspension assurée par un harnais au niveau du bassin ou une attache à la base de la queue (Brioche et al., 2016, Morey-Holton & Globus, 2002). Des études ayant utilisé une suspension par le bassin ou par la base de la queue ont donné des résultats équivalents

(Chowdhury et al., 2013). Par exemple, une perte de masse d'environ 40% du soléaire a été collectivement observée après 14 jours de suspension (Chowdhury et al., 2013, Cros et al., 1999, Mueller et al., 2005, Picquet & Falempin, 2003).

#### 1.3.4. Modèles cellulaires

Afin d'étudier plus précisément les mécanismes cellulaires et moléculaires impliqués dans la perte de masse musculaire, de nombreux modèles en culture cellulaire ont été développés. Trois modèles expérimentaux apparaissent comme majeurs dans l'étude de l'atrophie musculaire en culture et seront brièvement présentés : le modèle mimant le jeûne (« *starvation* »), le traitement aux glucocorticoïdes (GCs) et l'utilisation de cytokines inflammatoires.

L'un des plus simples, et des plus utilisé, reste tout simplement le fait de priver les cultures de cellules d'un milieu riche en nutriments (appelé « *starvation* »). De ce fait, en remplaçant le milieu de culture par du PBS (phosphate-buffered saline), l'étude de Sandri et al. (2004) a pu mettre en évidence le rôle majeur joué par les facteurs de transcription FoxO dans l'atrophie musculaire. Un simple traitement allant de quelques heures, jusqu'à 2 jours, au PBS suffit donc pour entraîner une atrophie rapide de myotubes de différentes lignées (C2C12, L6 ou culture primaire de cellules satellites) (Desgeorges et al., 2014, Stevenson et al., 2005).

Les GCs sont également connus comme des médiateurs clés de la protéolyse musculaire (Britto et al., 2014, Schakman et al., 2013) et de nombreuses études ont pu observer des mécanismes similaires entre des modèles de déconditionnement *in vivo* et des expérimentation *in vitro* utilisant de la dexaméthasone (Klaude et al., 2007, Thissen, 2005, Thompson et al., 1999, Tiao et al., 1997, Wang et al., 1998).

Différentes cytokines pro-inflammatoires ont également été utilisées dans différentes études. En effet, lorsqu'il est ajouté au milieu de culture, tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) est connu pour induire une atrophie musculaire *in vitro* en induisant une augmentation des processus apoptotiques et protéolytiques (Li et al., 1998, Magee et al., 2008, Mirza et al., 2014, Moylan et al., 2008). Un cocktail pro-inflammatoire composé de TNF- $\alpha$  et d'interferon  $\gamma$  (INF- $\gamma$ ) a aussi été utilisé dans la littérature pour stimuler l'atrophie musculaire dans des cultures de lignées C2C12 (Dehoux et al., 2007, Kimura et al., 2014).

## **1.4. Perturbation de la balance protéique : mécanismes cellulaires et moléculaires**

### **1.4.1. Une augmentation de l'activité des systèmes protéolytiques**

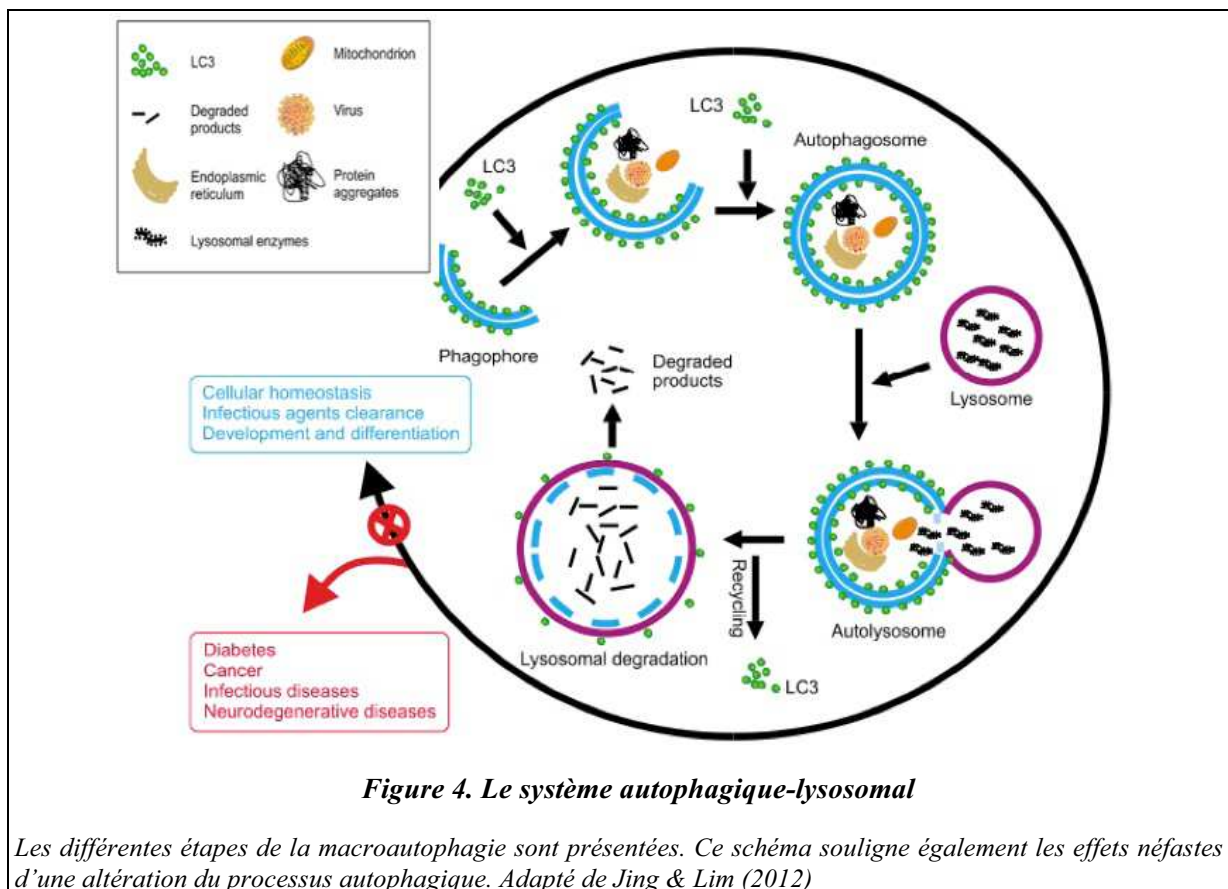
La perte de masse musculaire repose en grande partie sur une perturbation de la balance entre processus anaboliques et cataboliques. En plus d'une synthèse protéique diminuée, de nombreux processus de protéolyse musculaire se mettent en place rapidement dans un environnement défavorable. La littérature scientifique a déjà démontré qu'à la suite d'une inactivité musculaire par exemple, des changements significatifs dans les mécanismes de dégradation apparaissent dès les deux premiers jours d'inactivité (Cros et al., 1999). Le volume de la fibre musculaire étant occupé à 80% par des protéines (Hoppeler, 1986), les mécanismes de dégradation des protéines (protéolyse) ont une responsabilité très importante dans les mécanismes menant à l'atrophie musculaire. En effet, dans différentes situations impliquant une perte de masse musculaire, de nombreuses études (et revues de littérature) ont déjà décrit le rôle de l'augmentation de l'activité des différents systèmes protéolytiques. (Bodine, 2001, Bonaldo & Sandri, 2013, Gomes et al., 2012, Sandri, 2013, Sandri et al., 2004). A l'inverse, dans un muscle sain et de manière physiologique, la protéolyse est le processus cellulaire permettant la dégradation des protéines (altérées ou en fin de vie) en peptides puis en acides aminés réutilisables pour l'organisme. Ces acides aminés sont nécessaires à la synthèse de nouvelles protéines par exemple. La littérature scientifique a mis en évidence 3 voies majeures impliquées dans la protéolyse : le système des calpaïnes, l'autophagie et le système ubiquitine protéasome (SUP).

Concernant les calpaïnes, ce système calcium-dépendant est responsable d'une digestion partielle de protéines cibles. Les calpaïnes, composées de 14 membres dont les plus représentés sont les calpaïnes 1 et 2 et 3 dans le tissu musculaire, ont donc une activité majoritairement dépendante de la concentration intracellulaire de calcium mais également de leur inhibiteur endogène la calpastatine. De nombreux substrats des calpaïnes ont été identifiés à ce jour : les myosines, les troponines, la tropomyosine, la titine, la dystrophine, la desmine (Huang & Zhu, 2016, Murphy, 2010). Le système des calpaïnes est donc intimement lié à la dégradation partielle des protéines liées au sarcomère et au cytosquelette. De nombreuses études ont déjà montré une augmentation anormale de l'activité des calpaïnes dans des conditions de perte de masse musculaire incluant les traitements aux glucocorticoïdes ou la dénervation (Fareed et al., 2006, Matsumoto et al., 2014, Nelson et al.,

2012). De plus, deux études réalisées *in vitro* ont montré qu'une inhibition de la calpaïne 2 permettait de limiter les flux de dégradation (Huang & Forsberg, 1998, Purintrapiban et al., 2003). Dans un contexte de perte de masse musculaire liée à une septicémie chez l'animal, deux études réalisées *in vivo* ont également démontré un intérêt de bloquer l'augmentation de la concentration en calcium, et ainsi de bloquer la réponse des calpaïnes, pour limiter l'atrophie musculaire (Fischer et al., 2001, Williams et al., 1999). Par ailleurs, différentes études ont mis en évidence le rôle des calpaïnes dans la transition phénotypique vers un phénotype lent ainsi que dans les adaptations liées à l'exercice induisant des dommages musculaires (Belcastro, 1993, Belcastro et al., 1998, Raastad et al., 2010, Sultan et al., 2001, Sultan et al., 2000). Dans ce contexte, les calpaïnes seraient directement impliquées dans le démantèlement du cytosquelette par digestion partielle des protéines structurales.

Le système autophagique lysosomal est un processus catabolique qui émerge comme étant un système majeur dans le contrôle de la masse musculaire ainsi que dans la survie de la cellule. Suite à un stress environnemental ou un stress cellulaire (manque de nutriments ou hypoxie par exemple), l'autophagie se présente comme une réponse de survie car elle permet de recycler des protéines ou organelles usagées (mitochondries endommagées, agrégats protéiques ou pathogènes) et de générer des substrats métaboliques nécessaires au maintien de l'activité cellulaire basale (Sandri, 2010). Il existe différents types d'autophagies mais nous ne détaillerons ici que la macroautophagie plus communément appelée « autophagie » qui représente le système autophagique principal au sein du tissu musculaire (**Fig.4**). L'autophagie est donc la seule voie qui puisse dégrader massivement des macromolécules et des organites, elle représente la voie de dégradation alternative à celle du système ubiquitine-protéasome (Klionsky & Emr, 2000). L'autophagie possède un niveau basal d'activité permettant d'assurer une homéostasie cellulaire, notamment par le renouvellement du pool d'acides aminés et par l'élimination de macromolécules et de structures cellulaires endommagées (Klionsky & Emr, 2000, Sandri, 2010). L'autophagie est un système qui implique deux types de vésicules, l'autophagosome qui capture dans un premier temps les substrats, et le lysosome, qui dans une étape ultérieure, fusionne avec l'autophagosome et le dégrade avec ses constituants (Levine & Klionsky, 2004). Afin de dégrader les substrats, les lysosomes contiennent des hydrolases qui fonctionnent à pH acide (4 à 5.5) et catalysent l'hydrolyse de toutes les molécules (protéines, glucides, lipides et acides nucléiques) (Roberts et al., 2003). On peut compter parmi les hydrolases, des protéases, des phosphatases, des glycosidases et des lipases (Bechet et al., 2005). Par ailleurs, une quinzaine de protéines Atgs

(Autophagy related genes) sont nécessaires à la biogenèse de l'autophagosome. Ces Atgs, recrutées dans le cytoplasme, s'agrègent les uns aux autres, s'associent de façon transitoire à la membrane pré-autophagosomale et à celle de l'autophagosome et sont nécessaires pour l'induction de l'autophagie. Plus précisément, le complexe Ulk1-Atg13-FIP200 joue un rôle clé dans l'initiation de l'autophagie. Il peut être phosphorylé par mTORC1, menant à son inhibition, et par AMPK, menant à son activation (Jung et al., 2010). Une fois le complexe actif, Ulk1 phosphoryle Beclin1 sur sa ser-14, permettant l'activation de Vps34 et ainsi l'induction de la formation de l'autophagosome (Russell et al., 2013). La formation de l'autophagosome implique ensuite l'action de nombreuses Atgs, et notamment de LC3 (microtubule-associated protein light chain 3) nécessaire à l'élongation et la formation d'un autophagosome mature. Pro-LC3 est d'abord clivée par Atg4 pour former l'isoforme LC3-I, qui, une fois conjuguée au phosphatidylethanolamine de la membrane autophagosomale par l'action de Atg7, devient LC3-II (Nakatogawa et al., 2007). Enfin, la protéine p62 est couramment employée en tant que marqueur de l'activité lysosomale puisqu'elle est dégradée au même titre que l'autophagosome et ses composants.



**Figure 4. Le système autophagique-lysosomal**

Les différentes étapes de la macroautophagie sont présentées. Ce schéma souligne également les effets néfastes d'une altération du processus autophagique. Adapté de Jing & Lim (2012)

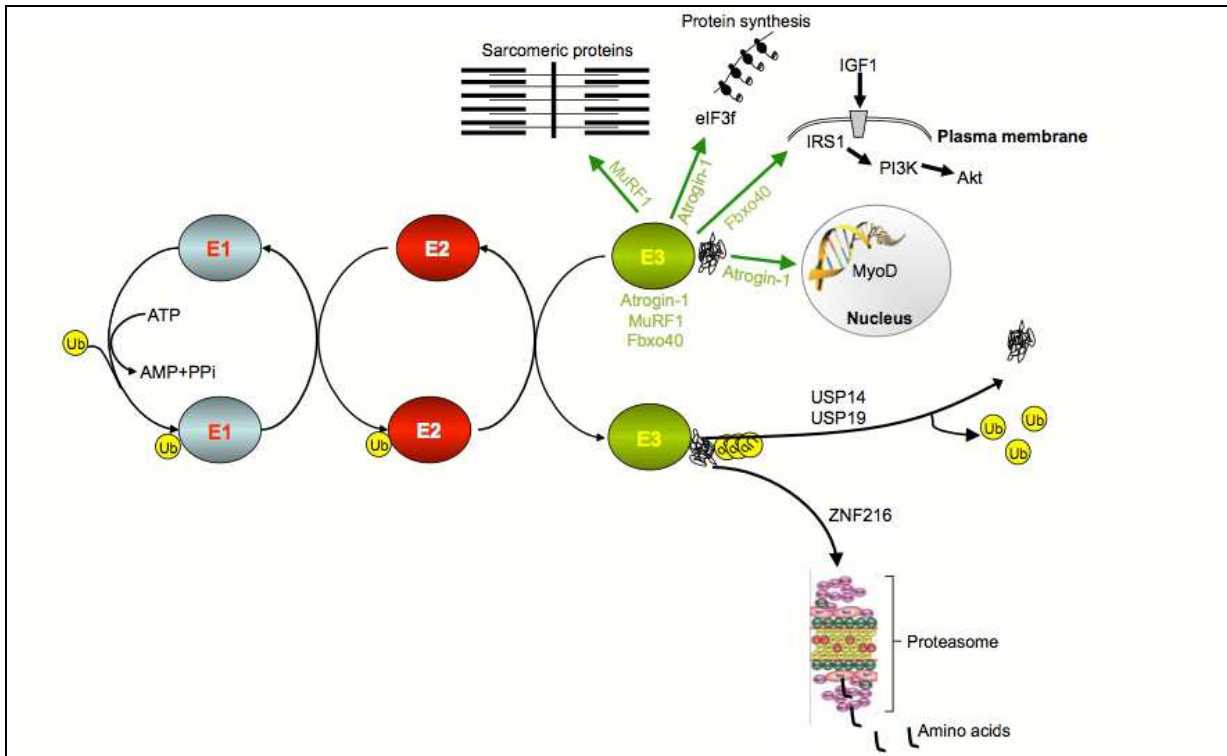
Le processus autophagique apparaît comme primordial au sein du tissu musculaire (Masiero et al., 2009, Masiero & Sandri, 2010). En effet, ces auteurs ont réalisé des expérimentations sur des souris KO Atg7, un gène nécessaire au déroulement du processus autophagique. Les résultats de ce groupe montrent que ces souris présentent une accumulation de protéines endommagées, de radicaux libres, une détérioration des structures cellulaires internes et une activation de l'apoptose (mort cellulaire programmée). Les auteurs montrent clairement que l'absence du processus autophagique au sein du tissu musculaire mène à l'atrophie du muscle squelettique. Cependant, une hyperactivité du processus autophagique conduit également à une atrophie ainsi qu'à des pathologies musculaires (Kaneda et al., 2003, Mammucari et al., 2007, Sandri, 2010, Zhao et al., 2007). L'étude de Mammucari et al. (2007) a par exemple démontré, chez l'animal, qu'une augmentation du processus autophagique était retrouvée dans des situations de privation alimentaire et de dénervation. Une augmentation de l'expression de différents marqueurs liés à l'autophagie, comme Beclin-1 ou LC3-II/LC3-I, a été également retrouvée chez l'homme après 21 jours de bed rest (Brocca et al., 2012).

Le système ubiquitine-protéasome (SUP) joue un rôle majeur dans de nombreux processus cellulaires. C'est la voie principale, non-lysosomale, de dégradation protéique intracellulaire chez les eucaryotes. Ce système est un processus ATP dépendant impliquant une cascade de réactions enzymatiques conduisant *in fine* un substrat au protéasome dans lequel il sera dégradé (Geng et al., 2012, Goldberg, 2003, Lecker et al., 2006) (**Fig.5**). Tout d'abord, l'ubiquitine possède un rôle majeur dans ce système. Cette petite protéine de 9kDa est capable de se fixer sur les résidus lysine des protéines. Les protéines substrats poly-ubiquitinées sont alors dirigées vers le protéasome pour y être dégradées. La poly-ubiquitination d'un substrat nécessite plusieurs enzymes clés. La première enzyme (E1 ou ubiquitin activating enzyme) permet, de manière ATP dépendante, d'activer les ubiquitines. La deuxième (E2 ou ubiquitin conjugating enzyme) permet la liaison des ubiquitines avec le substrat en catalysant une fixation covalente sur les protéines cibles. Enfin, la dernière enzyme (E3 ou ubiquitin protein ligase) est responsable de la sélection du substrat grâce à des sites de liaisons spécifiques. Pour finir, il est à noter que les «deubiquitinating enzymes» (DUbs) catalysent la réaction inverse et permettent alors de désubiquitiner les protéines (Geng et al., 2012). Le protéasome musculaire 26S est une protéase multi-catalytique, nucléaire et cytosolique. Ce complexe protéolytique de 2500kDa est formé par l'association du protéasome 20S, responsable de la dégradation du substrat, et du complexe régulateur 19S contrôlant l'entrée du substrat à

dégrader (Goldberg, 2003). A l'entrée du protéasome, le substrat est désubiquitiné (permettant le recyclage des ubiquitines) et ensuite dégradé en peptides. Le SUP semble être le système le plus impliqué dans l'atrophie musculaire dans différentes situations de déconditionnement. Ainsi de nombreuses études ont montré que l'utilisation d'inhibiteurs spécifiques du SUP (comme le MG132 par exemple) permettait de limiter l'atrophie induite par différentes situations chez l'animal (Caron et al., 2011, Jamart et al., 2011, Tawa et al., 1997, Zhang et al., 2013). Par exemple, l'étude de Jamart et al. (2011) a montré une diminution de 50% de la perte de masse musculaire engendrée par 6 jours de HU chez la souris.

Une découverte majeure a été l'identification de deux E3 ligases clés, MAFbx et MuRF1 (muscle atrophy F-box protein et muscle ring finger protein 1 respectivement) dans l'atrophie musculaire (Bodine, 2001, Gomes et al., 2001). L'augmentation de l'expression de ces deux E3 ligases, baptisées « atrogenes », est associée à de nombreuses situations de déconditionnement musculaire primaire ou secondaire (Bodine & Baehr, 2014a). L'E3 ligase MuRF1 étant impliquée dans la dégradation des protéines de structure et du métabolisme glucidique, en ciblant notamment les chaînes lourdes et légères de myosine ou la pyruvate déshydrogénase par exemple. MAFbx est quant à elle plus impliquée dans la diminution de la synthèse protéique via la dégradation de la protéine eIF3f, essentielle à l'initiation de la traduction, mais également via la dégradation de MyoD, un facteur de transcription myogénique essentiel (Bodine & Baehr, 2014b, Csibi et al., 2009, Lagirand-Cantaloube et al., 2008). Cependant, outre la découverte majeure des atrogenes, de nombreuses autres E3 ligases ont été par la suite découvertes et ayant un rôle majeur dans les mécanismes d'atrophie musculaire (Sandri, 2013, Schiaffino et al., 2013). Ainsi, TRIM32, CHIP, TRAF6, FBx040, Nedd4, TRIM62, Cbl-b, Mdm2, MUSA1 ou encore SMART sont toutes des E3 ligases impliquées dans l'amyotrophie mais ne seront pas détaillées ici.





**Figure 5. Le système ubiquitine-protéasome**

*Représentation schématique des différentes étapes du système ubiquitine-protéasome permettant de dégrader des protéines reconnues de manière spécifique par des E3 ligases. Les cibles de MuRF1, MAFbx (ou atrogin-1) et Fbxo40 sont présentées. Extrait de Bonaldo & Sandri (2013)*

#### 1.4.2 Les facteurs de transcription FoxO et NFκB : des régulateurs essentiels des systèmes protéolytiques

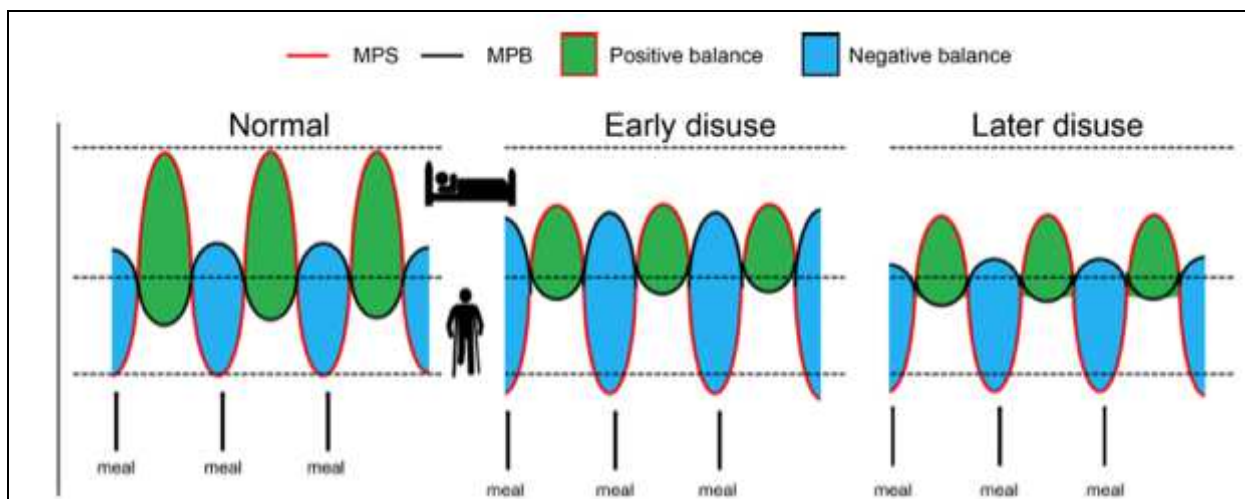
Les facteurs de transcription de la famille Forkhead box class O (FoxO) sont au nombre de 4 (FoxO1, 3, 4 et 6) et sont tous exprimés dans le tissu musculaire. Cependant, seuls FoxO1 et FoxO3 ont été précisément étudiés au sein du muscle. Même si les FoxO's sont impliqués dans la régulation du métabolisme musculaire, ils possèdent un rôle majeur dans la régulation des mécanismes protéolytiques. FoxO1 et FoxO3 sont particulièrement impliqués dans l'augmentation de l'activité du SUP et de l'autophagie (Sanchez et al., 2014, Sandri, 2010). Ces facteurs de transcription sont soumis à de nombreuses modifications post-traductionnelles, régulant leur translocation dans le noyau et ainsi leur activité. Par exemple, Akt phosphoryle et exclut du noyau FoxO3, via la protéine 14-3-3, en le phosphorylant sur les Thr32, la Ser253 et la Ser315 (Brunet et al., 1999). Alors que d'autres kinases ou déacétylases, jouent le rôle inverse et activent les FoxO's, par exemple l'AMPK et SIRT1 (Canto et al., 2009, Canto et al., 2010, Nakashima & Yakabe, 2007, Sanchez et al., 2012b). Une augmentation de la transcription de MAFbx et MuRF1 a été démontrée à la suite d'une augmentation de l'activité transcriptionnelle de FoxO3 et FoxO1, mais également une

augmentation de l'expression de nombreux Atgs impliqués dans l'autophagie (Mammucari et al., 2007, Sandri et al., 2004, Zhao et al., 2007). Collectivement, les données de la littérature semblent démontrer un rôle prépondérant de FoxO1 dans l'augmentation du SUP, alors que FoxO3 posséderait également un rôle primordial dans l'autophagie (Sanchez et al., 2013). Ainsi, deux études ont montré qu'une surexpression de FoxO1 et FoxO3 *in vivo* induisait une diminution de la masse musculaire, associée à avec une augmentation de l'expression de MAFbx et MuRF1 (Kamei, 2004, Sandri et al., 2004). Inversement, une inhibition de l'activité de ces deux facteurs de transcription permet ainsi de résister à l'atrophie musculaire (Reed et al., 2012, Sandri et al., 2004), soulignant une fois de plus le rôle primordial de ces facteurs de transcription de les mécanismes d'atrophie musculaire.

La voie NF- $\kappa$ B semble également être une composante essentielle des mécanismes d'atrophie musculaire. Ce facteur de transcription, stocké sous forme inactive dans le cytoplasme, est finement contrôlé par son inhibiteur I $\kappa$ B. De nombreux stimuli mènent à une activation de cette voie, les mécanismes inflammatoires via TNF- $\alpha$  ou IL-1 $\beta$ , les espèces oxygénées réactives (ROS) ou encore tout simplement l'exercice parmi d'autres. Principalement activé par la dégradation de son inhibiteur, NF- $\kappa$ B peut alors jouer son rôle de facteur de transcription et est connu pour être impliqué dans de nombreux processus cellulaires, dans de nombreux tissus. Les cibles de NF- $\kappa$ B sont très nombreuses et variées, et peuvent même être comptées par centaines (Jackman et al., 2013, Peterson et al., 2011). Concernant le tissu musculaire, une activation chronique de NF- $\kappa$ B a été reportée dans de nombreuses études relatives à l'atrophie musculaire (Acharyya et al., 2005, Hunter & Kandarian, 2004, Hunter et al., 2002, Li et al., 2008, Mourkioti et al., 2006, Penner et al., 2001). L'étude de Cai et al. (2004) a cependant été la première, grâce à des jeux d'activations et d'inhibitions, à mettre en avant le rôle primordial du facteur de transcription NF- $\kappa$ B dans l'atrophie musculaire. Ces auteurs ont notamment démontré qu'une surexpression de NF- $\kappa$ B causait une perte de masse musculaire, alors que son inhibition dans des situations de dénervation ou de cachexie limitait l'amyotrophie induite par ces situations. D'autres études ont ensuite également démontré le rôle protecteur, sur la perte de masse musculaire, de inhibition de NF- $\kappa$ B dans des situations d'inactivité prolongée (Hunter & Kandarian, 2004) et de nouveau dans une situation de dénervation (Mourkioti et al., 2006). Ces différentes études ont donc mis en avant le rôle majeur de NF- $\kappa$ B dans l'atrophie musculaire et l'étude de Cai et al. (2004) a par ailleurs mis en évidence que NF- $\kappa$ B favorise l'atrophie par une augmentation de l'expression de MuRF1.

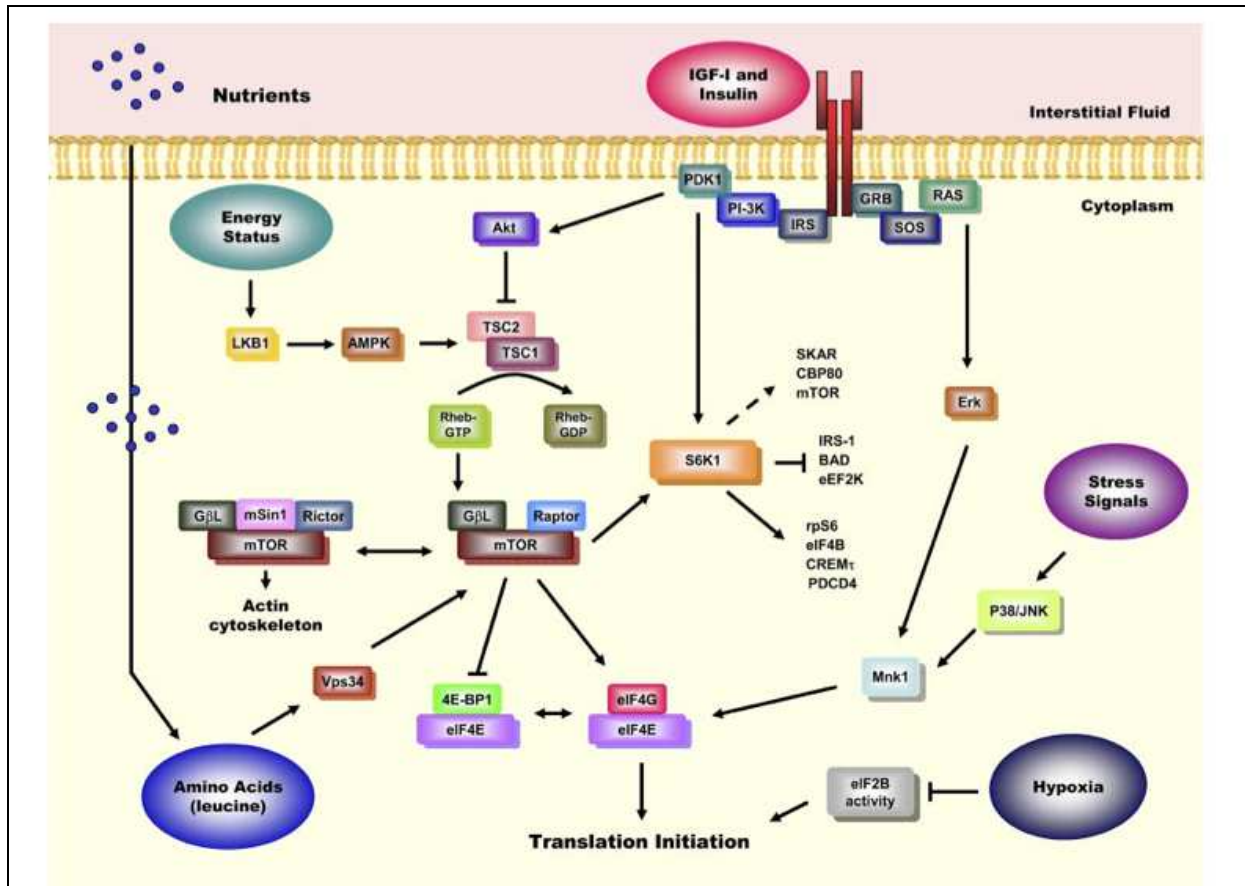
### 1.4.3 Une diminution de la synthèse protéique

Si l'augmentation de l'activité des systèmes protéolytiques est nécessaire pour induire une atrophie musculaire, la littérature scientifique a également montré que la diminution des processus de synthèse protéique l'est tout autant. La revue très récente de Atherton et al. (2016) souligne le rôle majeur de la diminution des flux de synthèse lors du déconditionnement musculaire (associé ou non à une pathologie). En effet, les flux de synthèse, post-prise alimentaire, sont par exemple largement diminués après immobilisation d'une articulation (Glover et al., 2008). Les auteurs ont également retrouvé le même résultat à la suite d'une supplémentation en acides aminés. L'étude de Stein et al. (1999) a également démontré une diminution de la synthèse protéique chez des astronautes sur des longues durées de vols spatiaux (supérieures à 3 mois). Une diminution des flux de synthèse musculaire après 14 jours de bed rest a également été observée dans la littérature (Ferrando et al., 1996). Chez l'animal, des études ont montré que seulement 3 jours d'hypoactivité suffisaient pour engendrer une diminution des flux de synthèse (Gomes et al., 2012). Dans ce modèle de déconditionnement (HU) les flux de synthèse continuent de diminuer pendant 7 jours puis restent réprimés. Une perte de masse musculaire est donc simultanément liée à une augmentation des processus cataboliques et une diminution des processus anaboliques, et de manière prédominante pour les muscles posturaux (Atherton et al., 2016) (**Fig.6**).



Ce schéma présente les hypothèses émises concernant la variation des flux de synthèse et de dégradation protéiques dans différentes conditions d'activité physique. Lors d'une situation d'hypoactivité de moins de 10 jours (« early disuse ») ou de plus de 10 jours (« later disuse »), les flux de synthèse (MPS) sont largement diminués et engendrent une balance protéique négative. Les flux de dégradation (MPB) protéique sont également augmentés dans les 10 premiers jours mais reviennent à des valeurs basales après 10 jours d'hypoactivité, soulignant le rôle joué par la diminution de la synthèse dans les processus de perte de masse dans des situations chroniques de réduction d'activité. Extrait de Atherton et al. (2016).

Au niveau cellulaire, la voie PI3k/Akt/mTOR constitue la voie majeure de la synthèse des protéines musculaires (Miyazaki & Esser, 2009, Mounier et al., 2011) (**Fig.7**). De nombreux facteurs conduisent à l'activation de la protéine kinase PI3k comme l'insuline ou encore l'IGF-1 (Rommel et al., 2001). PI3k permet ensuite d'augmenter l'activité de la Protéine Kinase B, également dénommée Akt. Cette kinase joue un rôle inhibiteur sur le complexe suppresseur de tumeur TSC1/TSC2 en phosphorylant TSC1 sur les Ser-939/981 et la Thr-1462 (Manning & Cantley, 2003). TSC2 est alors incapable d'exercer le rôle inhibiteur qu'il exerce normalement sur la protéine Rheb, un activateur de mTOR, conduisant ainsi à son activation. A l'inverse, lorsque la voie Akt n'est pas activée, TSC1/TSC2 inhibe l'activité de mTOR par l'intermédiaire de Rheb (Miyazaki & Esser, 2009). mTOR (mammalian Target of Rapamycin) est une Serine/Thréonine kinase très conservée dans l'évolution des espèces. Elle exerce un rôle essentiel au sein de la cascade signalétique PI3k/Akt/mTOR. La protéine mTOR représente l'unité catalytique présente dans 2 complexes distincts, mTORC1 et mTORC2. Le complexe mTORC1 reste à ce jour le plus étudié des deux, c'est un complexe régulant de nombreuses voies de signalisation. En effet, il permet non seulement une activation de la synthèse protéique et de la croissance cellulaire mais aussi une augmentation de l'activité du métabolisme lipidique (Csibi et al., 2010, Wang & Proud, 2010, Wullschlegel et al., 2006, Zoncu et al., 2010). Ce complexe multi-protéique joue également un rôle inhibiteur sur l'autophagie (Jung et al., 2010) et activateur de l'angiogenèse (Dormond-Meuwly et al., 2011). En se liant au facteur d'initiation à la traduction eIF3f, mTORC1 sera en mesure de phosphoryler ses substrats 4E-BP1 et S6K1 pour initier la traduction protéique. eIF3f joue ici un rôle d'échafaudage et accueille mTORC1, S6K1, 4E-BP1 et eIF4E (Csibi et al., 2010, Holz et al., 2005). Après phosphorylation, S6K1 se dissocie de eIF3f et active la traduction ribosomale en phosphorylant la protéine ribosomale S6. S6K1 est également responsable d'un rétrocontrôle positif en activant mTORC1 par phosphorylation de ses résidus Thr-2446 et Ser-2448 (Chiang & Abraham, 2005, Holz & Blenis, 2005). La phosphorylation de 4E-BP1 par mTORC1 favorise à son tour l'initiation de la traduction en le dissociant de eIF4E (Fingar et al., 2002).



**Figure 7. PI3K/Akt/mTOR : principale voie de synthèse protéique**

*Ce schéma présente les acteurs principaux de la voie PI3K/Akt/mTOR régulant l'initiation de la translation des protéines. De nombreux stress/stimuli permettent de moduler cette voie tels que la disponibilité en nutriments, l'hormone de croissance IGF-1 ou encore l'hypoxie. Extrait de Lang et al. (2007)*

Cette voie PI3K/Akt/mTOR, reconnue comme essentielle pour maintenir une balance protéique positive, est donc largement perturbée lors de perte de masse musculaire. Les nombreux acteurs qui influencent cette voie de signalisation sont autant de marqueurs utilisés par les études scientifiques voulant évaluer l'impact d'une diminution de cette voie sur le déconditionnement musculaire. Chez l'animal, l'étude de Bodine et al. (2001) a par exemple démontré que 14 jours de hindlimb unloading diminuaient l'activité de nombreux marqueurs de cette voie incluant Akt ou S6K1 par exemple. Les mêmes auteurs ont ensuite activé de manière constitutive Akt et observé une résistance à l'atrophie induite par le modèle de dénervation. De plus, l'étude de Hornberger et al. (2001) a observé une diminution des niveaux d'expression protéique de S6K1 et eIF2- $\alpha$  après 14 jours de HU et de dénervation.

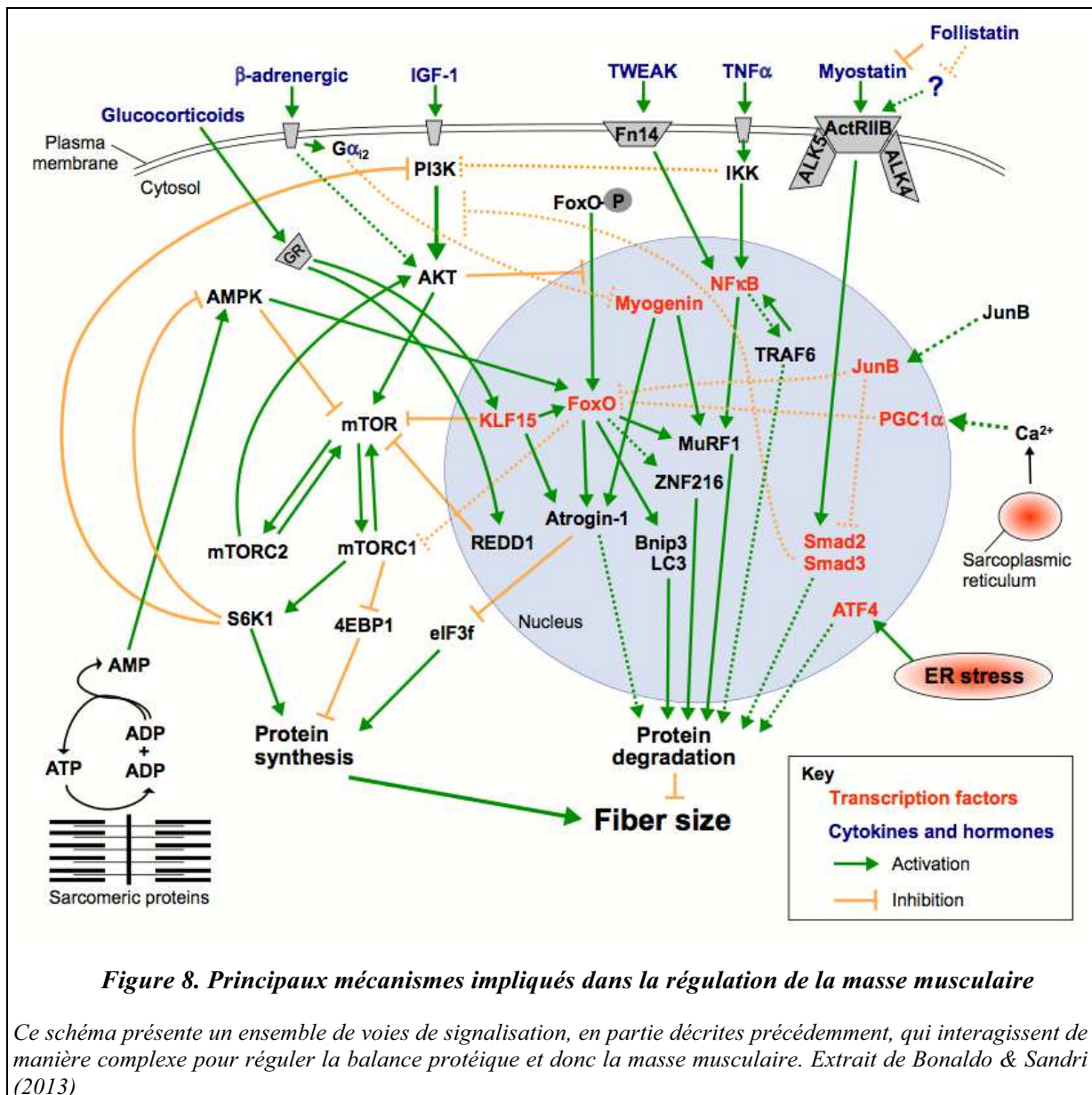
#### 1.4.4. Le déconditionnement musculaire : une perte de masse rapide

Depuis de nombreuses décennies il est donc clairement établi que la perte de masse musculaire est intimement liée à une perturbation de la balance protéique (**Fig.8**). Cependant

la majeure partie des études concernant le déconditionnement musculaire a étudié la perte de masse et ses mécanismes avec des protocoles de longues durées alors que la plus grande perte de masse musculaire s'opère dans les premiers jours d'une situation défavorable (Akima et al., 1997, Cros et al., 1999, Edgerton et al., 1995, Hanson et al., 2013, Kawashima et al., 2004). En effet, l'étude de Kawashima et al. (2004) a observé une baisse significative d'environ 5% de la surface de section (CSA) de plusieurs groupes musculaires dès 10 jours de bed rest. Une forte diminution de la CSA des fibres de type I (-16%) et des fibres de type II (-36%) du *vastus lateralis* a également été observée à la suite de 11 jours de vol spatial (Edgerton et al., 1995). Enfin, grâce au modèle de dry immersion, des études ont souligné que seulement 3 jours et 7 jours de microgravité simulée par ce modèle permettaient de diminuer la CSA du *vastus lateralis* de 5-9% et 15-18% respectivement. Par ailleurs, des études utilisant des modèles animaux ont également pu déterminer l'impact des différents modèles de déconditionnement musculaire sur de très courtes périodes. L'étude de Cros et al. (1999) a par exemple montré que seulement 2 jours de HU chez le rat diminuaient de 20% la masse du soléaire. Chez la souris, moins sensible aux altérations provoquées par le HU, 7 jours de suspension ont aussi engendré une diminution de la CSA des fibres de type I et IIa du soléaire (Hanson et al., 2013). Enfin, chez le lapin, une diminution de la CSA de 17% a été observée après 6 jours d'immobilisation de la cheville (Smith et al., 2000). Ainsi ces nombreuses études menées chez l'homme ou chez l'animal ont souligné que la perte de masse musculaire engendrée par des situations de déconditionnement musculaire, liées à l'hypoactivité, est très précoce et apparaît dès les premiers jours.

La perte de masse étant liée aux mécanismes régissant la balance protéique, il semble alors logique que les mécanismes de dégradation et de synthèse protéique soient perturbés également dès les premiers jours. A ma connaissance, peu d'études ont identifié ces mécanismes dans des temps aussi courts que dans les études précédemment exposées. Cependant, chez l'homme, l'étude récente de Wall et al. (2016) a identifié une résistance anabolique postprandiale après 5 jours d'immobilisation du genou. L'étude de Dirks et al. (2014) a également montré, chez des personnes âgées en bonne santé, qu'une immobilisation du genou de 5 jours augmentait l'expression des mRNA (ARN messagers) codant pour MAFbx et MuRF1. Sur un autre protocole d'inactivité (ULLS) d'une durée de 3 jours, Gustafsson et al. (2010) ont montré, sur le soléaire, une augmentation de l'induction génique de MuRF1 et MAFbx. Chez l'animal, seulement 3 jours d'hypoactivité suffisaient pour engendrer une diminution des flux de synthèse (Gomes et al., 2012) et l'étude de Hornberger

et al. (2001) a souligné une diminution de la phosphorylation de S6K1 après seulement 12h de dénervation ou de HU. Par ailleurs, une augmentation de l'expression des mRNA codant pour MAFbx et MuRF1 a été reportée dès 2 jours d'immobilisation (Ryder et al., 2015) ou de HU (Hanson et al., 2013). De plus, cette dernière étude souligne également une baisse de niveau d'expression musculaire de l'IGF-1, facteur de croissance important dans l'activation de la voie PI3K/Akt/mTOR, dès le 3ème jour de suspension. L'ensemble de ces études permet donc de penser qu'une altération de la balance protéique intervient très tôt lors du déconditionnement musculaire. Cependant d'autres études sont nécessaires afin de caractériser plus précisément les réponses des différents systèmes régissant la balance protéique lors de situations de déconditionnement musculaire, mais également de caractériser la part de chacun d'entre et leur temporalité selon les modèles utilisés.



**En résumé, de nombreuses situations, engendrant principalement une hypoactivité, peuvent provoquer un déconditionnement musculaire caractérisé par une perte de masse et de force musculaire, un shift phénotypique, une augmentation de la fatigabilité, une résistance à l'insuline et un développement d'infiltrations graisseuses. De nombreux modèles expérimentaux, souvent liés aux études des effets néfastes de la microgravité, ont été mis en place afin d'étudier les processus du déconditionnement musculaire. Les mécanismes de perte de masse musculaire sont fortement dépendants d'une altération de la balance protéique, résultant d'une augmentation des processus de dégradation protéique et d'une diminution de ceux régissant la synthèse protéique, et apparaissent de manière très précoce à l'issue d'une situation d'inactivité ou d'hypoactivité.**



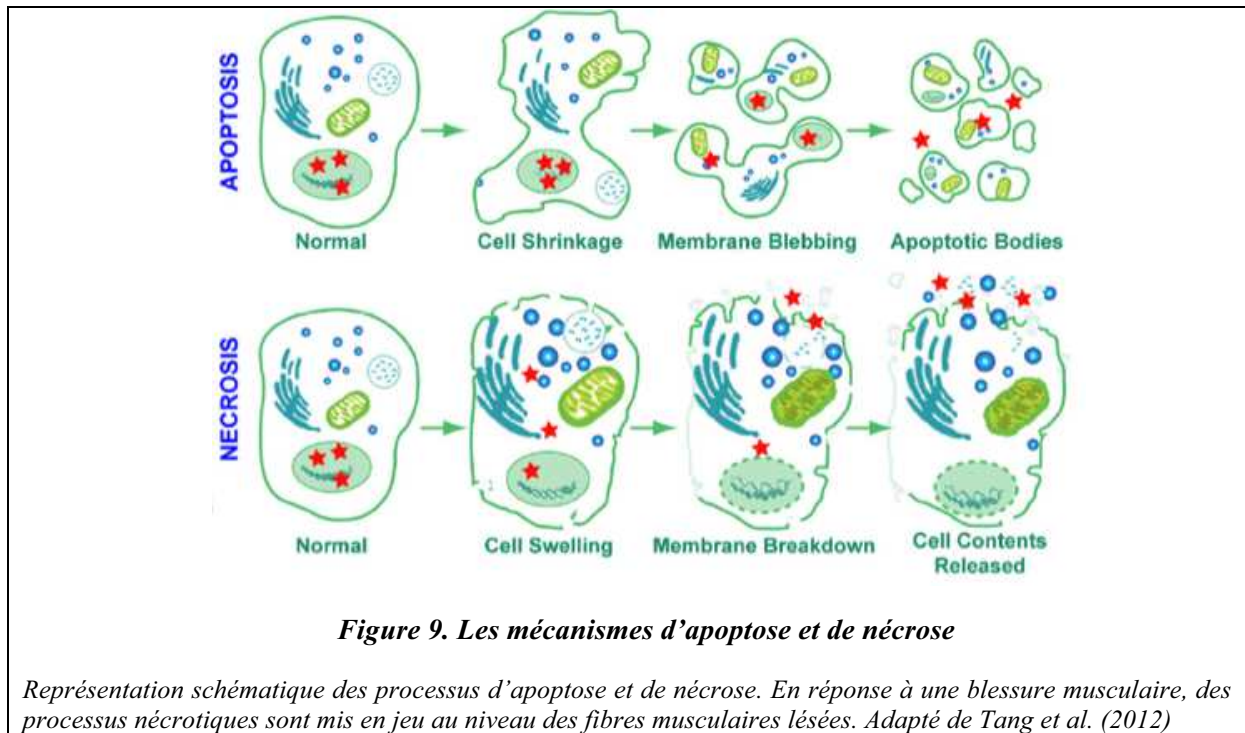
## CHAPITRE 2 : LA REGENERATION MUSCULAIRE

### 2.1. Généralités

Dans des circonstances structurelles et métaboliques normales, les cellules du tissu musculaire (myocytes) sont des cellules stables qui possèdent que très peu de turnover nucléaire. Cependant, après blessure, le muscle strié squelettique possède la capacité d'initier, en quelques heures, une cascade de processus de réparation afin de retrouver l'intégrité originale du muscle lésé. Le rôle fondamental du muscle squelettique est la génération de force, et donc le contrôle des mouvements corporels par contraction, mais les muscles servent aussi de véritables gardes du corps pour les autres organes et sont, le plus souvent, anatomiquement situés immédiatement sous la peau. Ainsi, cette capacité du tissu musculaire squelettique à régénérer est un autre paramètre clé de sa plasticité. Ce phénomène de régénération est indispensable au tissu musculaire dans la mesure où de nombreuses situations, pathologiques ou physiologiques, amènent à des lésions plus ou moins graves des fibres musculaires. Les lésions musculaires sont, le plus fréquemment, la conséquence de blessures sportives et étudier les mécanismes de régénération représente un enjeu majeur dans la médecine sportive. Cependant, la régénération est également indispensable dans la récupération et la guérison de nombreuses situations médicales, et représente donc un élément majeur dans la médecine en général. Les blessures musculaires interviennent dans de nombreuses situations incluant les traumatismes directs (lacérations ou contusions par exemple) ou les causes indirectes (ischémie ou dysfonction neurologique par exemple) (Huard et al., 2002).

Toutes ces situations impliquant une régénération musculaire sont subséquentes à une phase de nécrose d'une ou plusieurs fibres lésées. La nécrose est un processus de mort cellulaire qui intervient suite à des dommages musculaires, elle se caractérise par un gonflement du cytoplasme, une destruction des tous les organites puis une rupture de la membrane plasmique (Kroemer et al., 2009). L'apoptose, ou la mort cellulaire programmée, s'oppose à la nécrose qui constitue un cas de mort « accidentelle ». Contrairement à la nécrose, l'apoptose affecte en général des cellules isolées, aboutissant à un processus de condensation et de fragmentation (Kroemer et al., 2009) (**Fig.9**). Cependant, de nombreuses évidences semblent indiquer que la nécrose pourrait également être promue par des régulations cellulaires programmées, et non plus que par accident : le terme « necroptosis »

est alors employé pour définir ce mécanisme de mort programmée par nécrose, souvent lié à une inhibition de l'activité des caspases (Galluzzi & Kroemer, 2008, Kroemer et al., 2009).



Après blessure, cette phase très rapide et précoce de nécrose des fibres musculaires touchées est suivie par une phase pro-inflammatoire, puis anti-inflammatoire et enfin une phase de réparation du tissu musculaire, incluant l'angiogenèse ou encore la réparation de la matrice extra-cellulaire. L'étude de Sadeh et al. (1985) a particulièrement mis en lumière la remarquable capacité à régénérer du tissu musculaire. En effet, ces auteurs ont montré qu'après plus de 20 lésions, une chaque semaine, le tissu musculaire gardait sa capacité à régénérer. Dans un muscle sain, mais lésé, les processus de régénération permettent donc de réparer les fibres endommagées, ou bien d'en établir de nouvelles, afin de restaurer l'intégrité originale du muscle (Charge & Rudnicki, 2004).

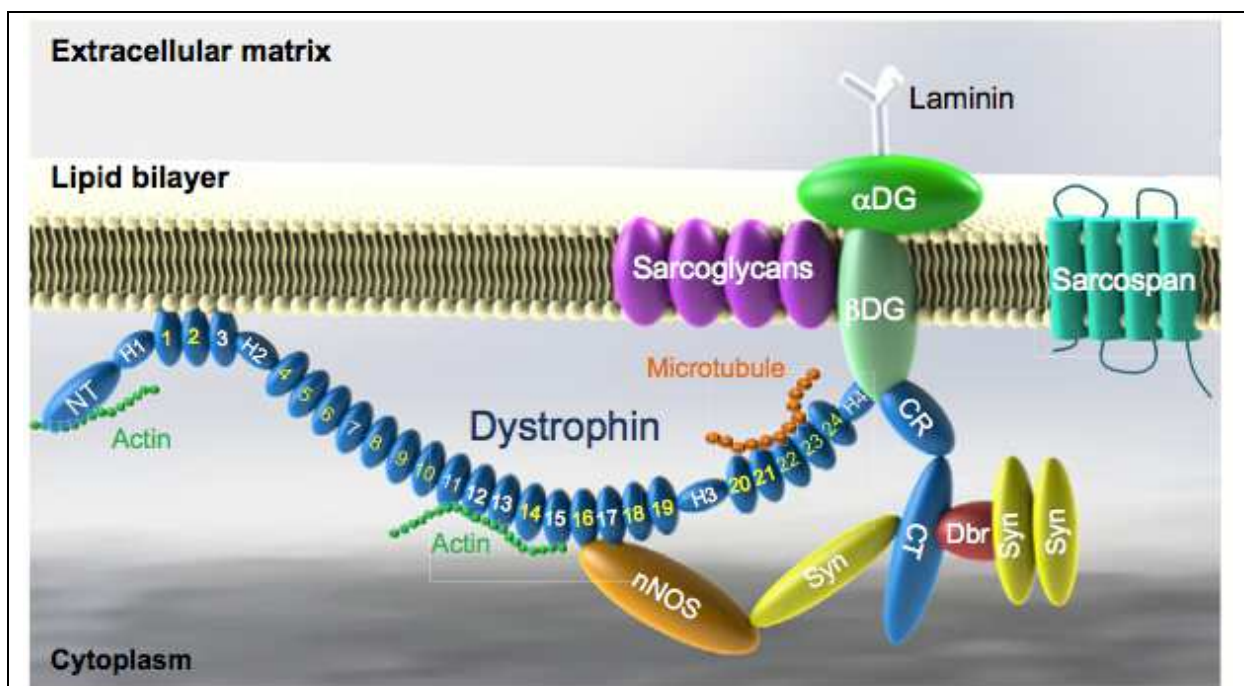
## 2.2. Modèles expérimentaux

### 2.2.1. Modèles humains

Chez l'homme, les modèles expérimentaux concernant la régénération musculaire sont rares et concernent principalement, dans un cas pathologique, les dystrophies musculaires progressives ou alors, dans un cas physiologique, les lésions musculaires induites par l'exercice (notamment en régime excentrique). Dans le cas des dystrophies chez l'homme, ces études sont axées sur les stratégies d'amélioration des symptômes ou de thérapies géniques

mais ne permettent pas réellement d'étudier les mécanismes cellulaires et moléculaires dans un contexte non pathologique. Les études concernant l'exercice en résistance, en particulier avec une dominante excentrique, ont surtout mis en valeur le rôle clé de certains processus de régénération dans les mécanismes d'hypertrophie musculaire.

Les dystrophies musculaires progressives, maladies musculaires primitives, se caractérisent par des cycles continus de dégénérescence/régénération induisant progressivement une faiblesse et une dysfonction musculaire. La Dystrophie de Duchenne (DMD) reste la maladie musculaire dégénérative la plus connue et la plus étudiée mais de nombreuses autres dystrophies, comme par exemple celles de Becker ou facio-scapulo humérale, sont également étudiées. La DMD est caractérisée par une mutation du gène codant pour la dystrophine, une protéine clé liant le cytosquelette et la matrice extracellulaire, induisant un dysfonctionnement et une faiblesse musculaire en cas d'absence ou de mutation (Fig.10).

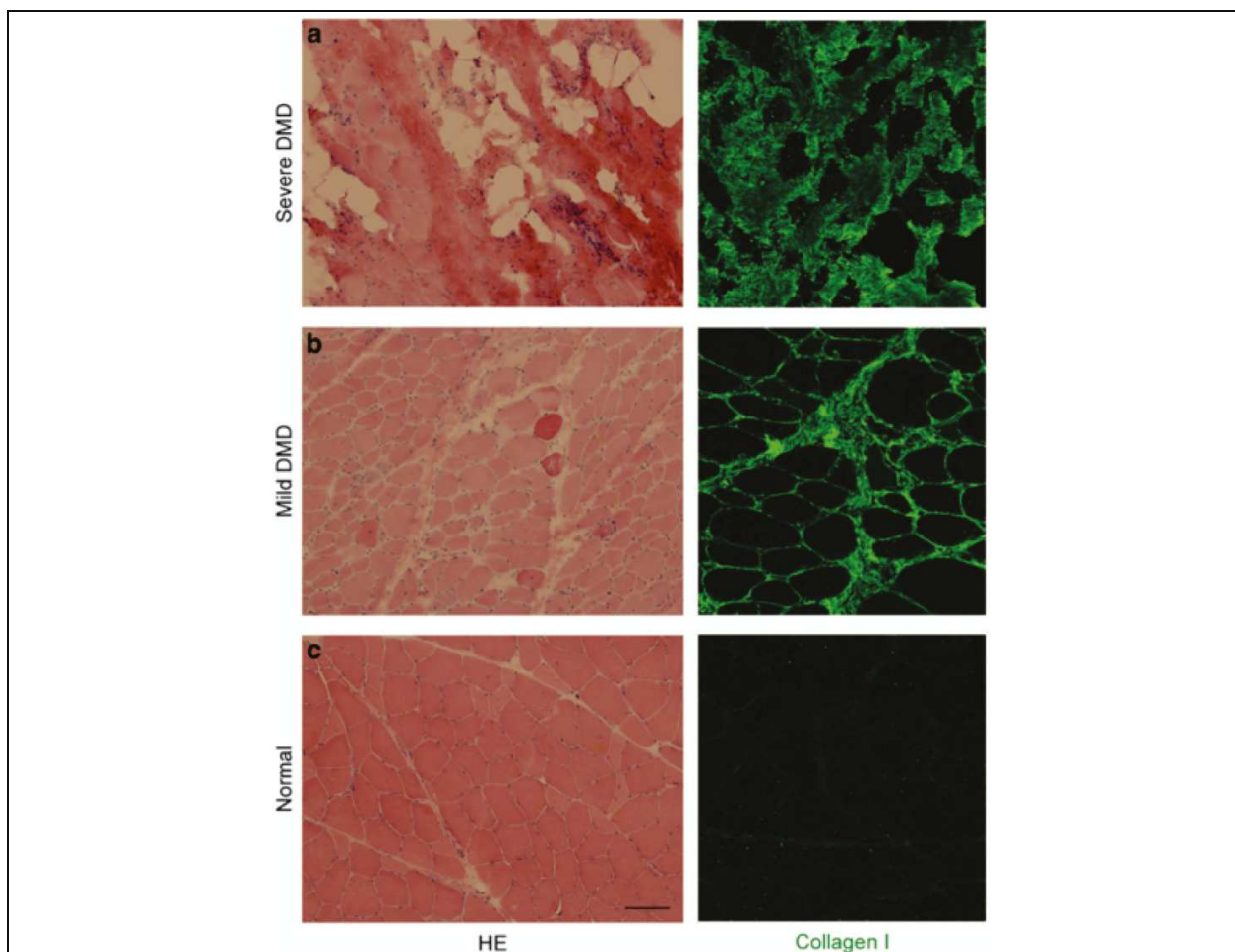


**Figure 10. La dystrophine et les différentes protéines de structure du tissu musculaire**

La dystrophine effectue le lien entre les unités contractiles (actine/myosine) et la matrice extra-cellulaire. L'absence de cette protéine représente induit une faiblesse musculaire majeure, représentée par la DMD chez l'homme. Extrait de McGreevy et al. (2015)

La DMD se caractérise ainsi par des cycles continus de dégénérescence/régénération, un épuisement progressif du potentiel de régénération, une atrophie, et une accumulation de fibrose et d'infiltrations graisseuses (Fig.11). Bien que des biopsies musculaires sur des

patients atteints de dystrophies soient réalisables, ces études sont rares et sont principalement axées sur des analyses histologiques et/ou en culture cellulaire des différentes populations de cellules impliquées dans les mécanismes de régénération (Blau et al., 1983, Yin et al., 2015, Zanotti et al., 2016). Les études réalisées chez des patients atteints de la DMD ne permettent évidemment pas de pouvoir réaliser des cinétiques, afin d’attester de l’évolution des mécanismes de régénération. Bien qu’essentielles afin de trouver des interventions thérapeutiques efficaces, ces études ne représentent pas les meilleurs modèles afin d’étudier précisément les mécanismes de régénération, d’autant plus dans un contexte physiologique et sain.



**Figure 11. Histopathologie musculaire liée à la DMD chez l’homme**

*Les résultats extraits de l’étude de Uezumi et al. (2014a) démontrent les altérations musculaires liées à la DMD chez l’homme. Cette figure souligne particulièrement la formation de fibrose, confirmée avec un immunomarquage du collagène, autour des fibres musculaires qui augmente de manière spectaculaire avec la sévérité de la DMD. Adapté de Uezumi et al. (2014a)*

L’exercice physique, particulièrement lorsqu’il est effectué en mode de contraction excentrique, est également connu pour induire des microlésions responsables de la mise en place de processus similaires à ceux observés lors de la régénération (Grounds, 2014). De très

nombreuses études utilisant ce mode de contraction ont été publiées chez l'homme (mais également chez l'animal) afin de rendre compte du rôle des processus de régénération dans l'hypertrophie induite par l'exercice (Bamman et al., 2004, Begue et al., 2013, Bickel et al., 2005, Costa et al., 2007, Drummond et al., 2010, Fluck, 2006, Kadi et al., 1999, Kadi et al., 2004b, McKay et al., 2009, Philippe et al., 2015, Psilander et al., 2003, Roth et al., 2001). Cependant, il est important de préciser que les lésions qui surviennent de manière physiologique à la suite d'un exercice, quel qu'il soit, n'engendrent pas (ou très peu) de lésions assez importantes pour engendrer une nécrose des fibres musculaires (Grounds, 1998, Jarvinen et al., 2013, Loenneke et al., 2014, Thornell, 2011). En effet, le terme de régénération musculaire est un terme normalement employé lorsqu'un stress engendre la nécrose d'une, ou une partie, des fibres musculaires composant le muscle lésé. Or, l'activité contractile, hormis dans le cas de blessures musculaires et de conditions extrêmes, provoque des lésions structurelles physiologiques liées à l'étirement des sarcomères, qui n'engendrent pas de phénomènes nécrotiques (Grounds, 2014, Jarvinen et al., 2013). Ainsi, il est important de dissocier la régénération musculaire, impliquant donc une nécrose préalable, et les lésions induites par l'exercice.

### 2.2.2. Modèles animaux

Afin d'étudier de manière plus précise les mécanismes cellulaires et moléculaires de la régénération musculaire, de nombreux modèles animaux, notamment chez le rongeur, ont été utilisés. Ces différents modèles sont basés sur des blessures d'ordre physique ou chimique, grâce notamment à l'utilisation de toxines. Enfin de très nombreux modèles murins d'ablation de différentes protéines essentielles à l'intégrité des sarcomères ont été également utilisés.

La littérature récente étudiant la régénération musculaire utilise majoritairement des modèles basés sur des substances chimiques, notamment des toxines. Les plus connues et les plus utilisées étant la cardiotoxine (CTX) et la notexine (NTX), deux composés directement issus du venin du célèbre cobra, pour la CTX, et du serpent-tigre d'Australie pour la NTX ! Ces poisons ont alors la capacité d'induire une nécrose des fibres musculaires de la zone injectée avec par exemple une diminution de 50% de l'expression de la desmine en 1h, de la dystrophine en 5h et de la myosine en 6h seulement (Harris et al., 2003, Ownby et al., 1993). D'autres substances induisant des phénomènes nécrotiques ont été et restent encore utilisés de nos jours, comme la bupivacaine, le chlorure de baryum ou le glycérol (Benoit & Belt, 1970, DalleDonne et al., 1998, Kawai et al., 1990, Pisani et al., 2010a, Zink et al., 2002). Par ailleurs, les principaux modèles physiques utilisés sont l'application de lésions transversales,

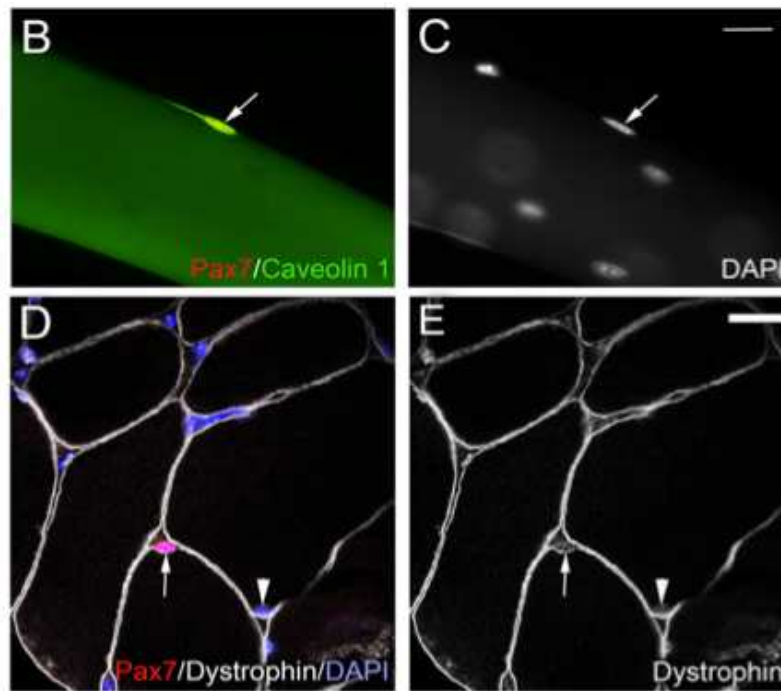
d'écrasements, de lésions par le froid, d'irradiations, d'ischémie par dévascularisation ou encore de la transplantation (Lawson-Smith & McGeachie, 1997). Quelques études ce sont déjà intéressées à comparer différents modèles de régénération et mettent en lumière des différences dans les cinétiques des processus de régénération (Hardy et al., 2016, Lukjanenko et al., 2013, Mahdy et al., 2015, Plant et al., 2006). Malgré ces différences, souvent liées à la sévérité de la blessure ainsi qu'à la dimension de la zone touchée, le tissu musculaire est toujours capable *in fine* de retrouver son intégrité et donc sa fonction originale.

Des nombreux modèles de souris KO (Knock-out ; permettant d'invalider l'expression d'un ou plusieurs gènes), dirigés contre des protéines essentielles à l'intégrité des sarcomères, ont également permis de mettre en lumière d'autres situations faisant intervenir des processus nécrotiques et donc de régénération musculaire. Parmi les protéines invalidées, l'invalidation de la dystrophine est sans aucun doute le modèle le plus connu. En effet, la souris *mdx* (X-linked muscular dystrophy) représente ainsi le modèle permettant d'étudier les effets de l'absence de la dystrophine, représentant la DMD chez l'homme. Ce modèle a été très largement utilisé dans la littérature, bien que le phénotype de la souris *mdx* ne présente que des altérations musculaires mineures comparées à celles observées chez l'homme (Bulfield et al., 1984, Gayraud-Morel et al., 2009). Cette différence peut s'expliquer par l'augmentation de l'expression de l'utrophine A chez la souris (Deconinck et al., 1997, Grady et al., 1997, Tinsley et al., 1996). L'utrophine est une grande protéine du cytosquelette qui affiche un degré élevé de similitude avec la dystrophine. Toutes deux ont une redondance fonctionnelle significative et la surexpression de l'utrophine est essentielle pour atténuer la progression du processus dystrophique (Rafael et al., 1998, Squire et al., 2002). Afin de créer un phénotype musculaire plus sévère chez la souris, donc plus proche de celui de l'homme dans la DMD, la souris double KO dystrophine et utrophine (*mdx*-utrophine dKO) a été développée et étudiée (Al-Rewashdy et al., 2015, Chan et al., 2014, Deconinck et al., 1997, Grady et al., 1997, Li et al., 2012, Lu et al., 2014, Pang et al., 2014, Tinsley et al., 1996). Beaucoup d'autres modèles visant à désorganiser le lien structurel entre les protéines contractiles, le sarcolemme et la matrice extra-cellulaire ont également été développés incluant, de manière non exhaustive, la délétion de la desmine (Li et al., 1997, Smythe et al., 2001), des dystroglycans (Cohn et al., 2002, Williamson et al., 1997), de la syntrophine (Adams et al., 2000, Hosaka et al., 2002, Yokota et al., 2014), des sarcoglycans (Duclos et al., 1998, Kirschner & Lochmuller, 2011), du collagène de type IV (Bonaldo et al., 1998, De Palma et al., 2013, Urciuolo et al., 2013) ou encore de la laminine (Miyagoe et al., 1997, Xu et al., 1994).

## 2.3. Les cellules satellites

### 2.3.1. Un rôle majeur dans la régénération

Les cellules satellites (SCs), initialement décrites par Mauro (1961), sont des progéniteurs myogéniques localisés entre le sarcolemme et la lame basale des fibres musculaires (**Fig.12**). Bien qu'étant maintenues dans un état quiescent à l'âge adulte, ces cellules possèdent une importance primordiale dans la croissance post-natale mais également dans la régénération musculaire (Chang & Rudnicki, 2014, Relaix & Zammit, 2012). En effet, après blessure, les SCs vont sortir de leur état quiescent pour s'engager dans un programme myogénique et ainsi devenir des myoblastes. Ces myoblastes vont proliférer et ensuite pouvoir se différencier pour soit fusionner avec une fibre lésée, et ainsi incorporer de nouveaux noyaux afin de réparer la fibre, ou alors, créer une nouvelle fibre musculaire. Certaines cellules satellites, après avoir proliféré, sont également capables de revenir à l'état quiescent et ainsi de reconstituer le pool de SCs disponible (Collins & Partridge, 2005, Kuang et al., 2008, Motohashi & Asakura, 2014). Cette capacité d'auto-renouvellement est essentielle afin de garder un stock de SCs constant. Ainsi, une SC peut donc engendrer deux SCs filles en se divisant soit de manière symétrique, soit de manière asymétrique. La division asymétrique permettra d'engendrer une cellule fille qui s'engagera dans la lignée myogénique alors que la seconde sera dédiée au retour vers de l'état de quiescence. En ce qui concerne la division symétrique, deux situations sont possibles, une qui permet aux deux cellules filles de retourner à l'état quiescent et l'autre qui au contraire permet l'engagement des deux cellules filles vers la différenciation cellulaire. De nombreux facteurs régulent leur capacité de prolifération, leur type de division ainsi que leur capacité d'auto-renouvellement et seront détaillés dans la partie 2.3.2.



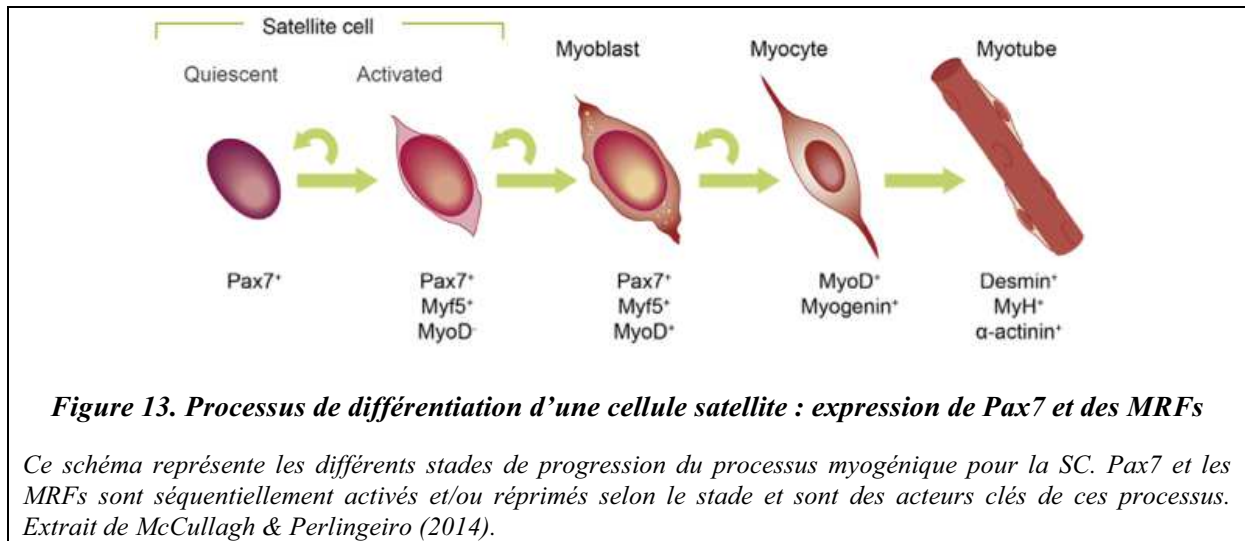
**Figure 12. Localisation des cellules satellites dans le tissu musculaire**

*Localisées entre le sarcolemme et la lame basale des fibres musculaires, les SCs, ici marquées avec l'expression de Pax7, font partie intégrante du tissu musculaire et possèdent une importance majeure dans la croissance post-natale et la régénération musculaire. Extrait de Relaix & Zammit (2012).*

La capacité du tissu musculaire squelettique à régénérer est donc fortement dépendante de du bon déroulement des processus liés aux SCs. Les différentes étapes du cycle cellulaire des SCs sont finement contrôlées par des facteurs de transcription tels que Pax (paired box transcription factor) et les MRFs (myogenic regulatory factors) nommés MyoD (myoblast determination protein 1), Myf5 (myogenic factor 5), myogénine et MRF4 (muscle-specific regulatory factor 4) séquentiellement activés ou réprimés. Les cellules satellites en phase de quiescence ou de prolifération expriment Pax7 et son inactivation induit une sévère déplétion de ces précurseurs myogéniques (Seale et al., 2000). Les processus de prolifération sont déclenchés et maintenus par l'expression de Myf5 puis MyoD (Megency et al., 1996, Ustanina et al., 2007) alors que lorsque le processus de différenciation est engagé, une augmentation de l'expression de la protéine myogénine est observée (Hasty et al., 1993). Durant la phase de maturation, donc dans les processus tardifs de la différenciation, MRF4 est alors activé (Zhang et al., 1995). Les SCs engagées dans le processus de différenciation perdent l'expression de Pax7, or celles continuant à exprimer Pax7 à la fin de la phase de prolifération retournent à l'état de quiescence (Olguin & Olwin, 2004, Olguin et al., 2007) et participent donc à garder le pool de SCs constant. Ainsi la dynamique entière du cycle cellulaire des SCs est modulé au travers des régulations activatrices et/ou inhibitrices de Pax7



et des MRFs (**Fig.13**), y compris des régulations directes entre ces protéines (Olguin et al., 2007, Venuti et al., 1995). En utilisant des souris KO pour les MRFs et Pax7, différentes études ont clairement démontré le rôle primordial des SCs dans la régénération musculaire (Gayraud-Morel et al., 2009). Par exemple, des études ont montré un déficit extrême de régénération avec des souris KO Pax7, donc dépourvues de SCs (Kuang et al., 2006, Lepper et al., 2011, Murphy et al., 2011, Oustanina et al., 2004, Sambasivan et al., 2011, Seale et al., 2000).



### 2.3.2. Les régulateurs de l'activité des cellules satellites

Afin de régénérer du tissu musculaire de manière efficace, les SCs vont donc sortir de leur état de quiescence pour rentrer dans une phase d'activation et de prolifération, suivie par une phase de différenciation. Toutes ces phases sont primordiales pour le bon déroulement du processus de régénération et sont finement régulées par de nombreux facteurs (**Fig.14**).

En ce qui concerne le maintien de l'état de quiescence, la voie Notch semble être une des voies primordiales jouant un rôle de frein sur l'activité des SCs. En effet, différentes études ont montré que la voie Notch était indispensable pour le maintien des SCs dans l'état de quiescence et ont également montré que son inhibition engendrait un engagement direct des SCs dans les processus de différenciation, sans même entrer en phase de prolifération (Bjornson et al., 2012, Mourikis et al., 2012, Pellegrinet et al., 2011). Cette déplétion de la voie Notch altère fortement la régénération musculaire puisque le pool de SCs est diminué. Chez les mammifères, la voie Notch se compose de nombreux acteurs dont 4 récepteurs transmembranaires appelés Notch-1, 2, 3 et 4 ainsi que de 5 ligands transmembranaires

nommés Dll1, Dll3, Dll4, Jag1 et Jag2 (Delta-like1, 3, 4 et Jagged 1, 2 respectivement). Une fois l'interaction ligand-récepteur effectuée, cette interaction conduit à des clivages protéolytiques du récepteur qui libère NICD (Notch intracellular domain). NICD rentre alors dans le noyau, s'associe avec son ADN cible et assemble un complexe de transcription permettant d'activer les gènes cibles de la voie Notch, principalement des gènes appartenant à la famille des *Hes* et *Hey* (Kopan & Ilagan, 2009). Cependant, dans un contexte de blessure musculaire, la voie Notch semble cette fois être nécessaire à la prolifération des SCs (Conboy et al., 2003). En effet, ces auteurs ont démontré qu'à la suite d'une lésion musculaire le récepteur Dll1, faiblement exprimé à l'état normal, voit son expression augmenter et permet de stimuler la prolifération des SCs.

Le facteur de transcription FoxO3, en plus d'être un acteur crucial dans l'activation du SUP et de l'autophagie (voir partie 1.3.4.), est également connu pour promouvoir la quiescence des SCs. En effet, l'étude de Gopinath et al. (2014) a élégamment démontré que l'expression de FoxO3 par les SCs après une blessure musculaire était requise pour leur capacité d'auto-renouvellement. Ces auteurs ont montré, dans des souris KO FoxO3 spécifique aux SCs, une diminution du processus d'auto-renouvellement et une augmentation du potentiel de différenciation. Cette même étude a également démontré que les effets de FoxO3 étaient directement liés à une activation de la voie Notch. Les SCs FoxO<sup>-/-</sup> montrent une diminution d'expression de différents gènes cibles de la voie Notch (*Hes1*, *Hes2*, *Hes6* et *HeyL*), une baisse des niveaux d'expression de Notch-1 et Notch-3 et également une diminution de l'expression de NICD. Ces niveaux d'expression reviennent à des niveaux basaux grâce à une surexpression de FoxO3. Enfin, ils ont également identifié des motifs FREs (FoxO-responsive-elements) dans les gènes promoteurs de *Notch-1* et *Notch-3* et démontré que FoxO3 était bel est bien capable d'interagir avec ces zones cibles, soulignant ainsi le rôle fonctionnel de la régulation de la voie Notch par FoxO3 pendant la régénération musculaire.

La famille des ligands solubles Wnt est également responsable de la quiescence des SCs. En effet, une augmentation de l'expression de la voie Wnt, et donc de la translocation dans le noyau du facteur de transcription  $\beta$ -catenin, augmente l'expression de Pax7 et diminue celle de MyoD (Perez-Ruiz et al., 2008). Le Grand et al. (2009) ont aussi montré que la surexpression de Wnt7a, et de son récepteur Fzd7, permettait la division symétrique des SCs vers un phénotype Pax7<sup>+</sup>/MyoD<sup>-</sup>, aidant ainsi au renouvellement du pool de SCs et au processus d'auto-renouvellement.

Sprouty-1, un inhibiteur de récepteurs à tyrosine kinases, est également un élément essentiel pour la maintenance dans l'état quiescent des SCs (Abou-Khalil & Brack, 2010, Shea et al., 2010). En effet, sprouty-1 est notamment capable d'inhiber FGF2, un facteur de croissance permettant l'entrée dans le cycle cellulaire des SCs, et est exprimé dans un muscle sain et non lésé. L'expression de sprouty-1 décroît fortement lors de la prolifération des SCs induite par une blessure et cette expression est, de manière intéressante, restaurée pour les myoblastes engagés dans le processus d'auto-renouvellement (Shea et al., 2010). Cette même étude a bien montré que sprouty-1 était nécessaire pour le retour à l'état quiescent des SCs et qu'en son absence il n'y avait pas d'altération de la prolifération ou de la différenciation des SCs après blessure. L'étude de Chakkalakal et al. (2012) a montré que l'expression de FGF2 augmente avec l'âge, notamment à cause d'une diminution de l'expression de son inhibiteur sprouty-1, et en résulte une perte de la quiescence des SCs, une déplétion des SCs et *in fine* une altération de la régénération musculaire.

L'interleukine-6 (IL-6) est une cytokine jouant un rôle important dans la prolifération des SCs. Cette protéine a tout d'abord été considérée comme étant une cytokine pro-inflammatoire aux effets néfastes pour la santé humaine car associée à la cachexie notamment (Carson & Baltgalvis, 2010, Haddad et al., 2005). En effet, l'IL-6 est connu pour induire une perte de masse musculaire dans de nombreuses situations de déconditionnement musculaire impliquant une inflammation chronique de bas grade. Cette cytokine est également sécrétée par le tissu musculaire de manière transitoire après un exercice et de ce fait devient par définition une myokine (Keller et al., 2003, Pedersen & Febbraio, 2008). La littérature a également démontré un rôle bien différent pour l'IL-6 sécrétée par le muscle en contraction : cette myokine est dans ce cas capable de stimuler la sensibilité à l'insuline et donc la captation de glucose et, à de faibles doses, l'IL-6 possède même des effets anti-inflammatoires (Pedersen & Febbraio, 2008). Concernant les SCs, l'étude de Serrano et al. (2008) a élégamment mis en évidence le rôle majeur que possède l'IL-6 dans la prolifération des SCs. Ces auteurs ont montré que l'activation de l'axe l'IL-6/STAT3 était nécessaire pour la prolifération des SCs et ainsi pour l'hypertrophie musculaire (dans cette étude une hypertrophie compensatoire). L'étude de Guerci et al. (2012) a également démontré, avec le même modèle d'hypertrophie, le rôle essentiel de cette cytokine dans les processus de prolifération. L'IL-6, en se liant avec son récepteur IL-6R $\alpha$ , engendre une augmentation de la phosphorylation de JAK2 qui, à son tour, phosphoryle STAT3. Grâce à cette phosphorylation, STAT3 est transloqué dans le noyau et peut ainsi induire la transcription des gènes cibles de

la voie de l'IL-6 (Levy & Lee, 2002, Rawlings et al., 2004, Trenerry et al., 2008). Bien que non détectable dans un tissu musculaire au repos et/ou non endommagé, la proportion des SCs exprimant IL-6 est d'environ 70% dès 3h et jusqu'à 24h après un exercice excentrique chez de jeunes hommes en bonne santé (McKay et al., 2009, Toth et al., 2011). De manière intéressante, la prolifération des SCs dans ces études intervient à partir de 24h et jusqu'à trois jours post-exercice. La théorie d'une participation active de l'IL-6 dans les mécanismes de prolifération des SCs est aussi supportée par des données d'études, réalisées chez l'homme, qui montrent une inhibition de la réponse des SCs après un exercice traumatisant par l'administration de drogues anti-inflammatoires qui bloquent notamment la production de cytokines dont l'IL-6 (Mackey et al., 2007, Mikkelsen et al., 2009). Enfin, l'aspect temporel dans la production d'IL-6 semble aussi jouer un rôle important dans son action sur le tissu musculaire. Après un exercice ou une blessure, l'IL-6 est sécrétée de manière rapide et temporaire puisque son expression reste élevée de quelques heures à quelques jours (McKay et al., 2009, Serrano et al., 2008) et cette sécrétion transitoire d'IL-6 apparaît comme une réponse physiologique et bénéfique pour l'organisme (via une augmentation de la captation du glucose et son effet sur les SCs notamment). Au contraire, une élévation chronique mais de bas grade des niveaux circulants d'IL-6 est associée et observée avec de nombreuses situations pathologiques et apparaît donc comme délétère en diminuant la synthèse protéique et en contribuant à la perte de masse musculaire (Carson & Baltgalvis, 2010, Haddad et al., 2005).

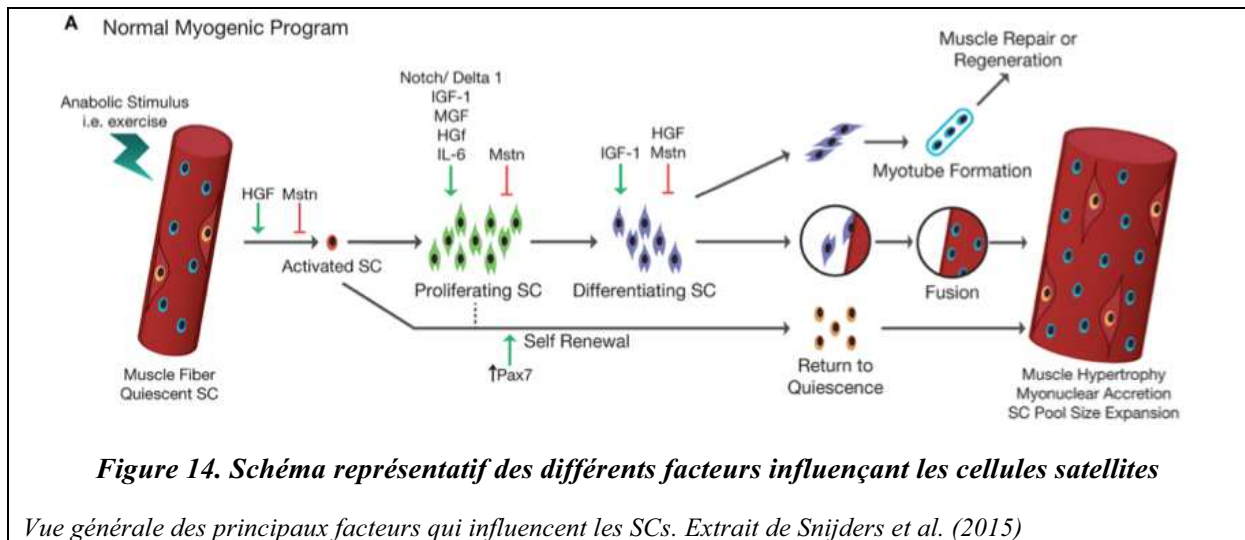
Le facteur de croissance IGF-1, également connu pour promouvoir la synthèse protéique via la voie PI3k/Akt/mTOR (voir partie 1.2.1.), émerge aussi comme étant un modulateur majeur du cycle cellulaire des SCs. Ce facteur de croissance possède la particularité d'être impliqué à la fois dans l'augmentation de l'activation/prolifération des SCs, mais à la fois dans l'augmentation de leur différenciation (Adams & McCue, 1998, Kadi et al., 2005). L'IGF-1 est une hormone qui partage une forte homologie de structure et de cibles avec l'insuline et qui s'associe avec les récepteurs à l'IGF-1 comme à ceux de l'insuline (IGF-1R et IR respectivement). Ce facteur de croissance est produit et sécrété en systémique par le foie et le tissu musculaire principalement. Chez l'homme, l'IGF-1 se compose de trois isoformes différentes (IGF-1Ea, IGF-1Eb et IGF-1Ec) et chaque isoforme est pressentie pour jouer un rôle différent mais complémentaire sur les cellules satellites et donc sur la régénération musculaire (Shefer & Benayahu, 2012, Snijders et al., 2015, Song et al., 2013). L'expression de l'isoforme IGF-1c (également appelé MGF) est fortement augmentée juste après une

blesse musculaire. Son expression est fortement corrélée à l'expression de M-cadherin (un marqueur des SCs), de MyoD et de Myf5. Cette isoforme semble donc être impliquée dans l'activation des SCs ainsi que les premiers processus de prolifération (Hameed et al., 2004, Hameed et al., 2003, Hill & Goldspink, 2003, Hill et al., 2003, McKay et al., 2008, Snijders et al., 2015). L'isoforme IGF-1Ea est elle exprimée plus tardivement lors de la régénération, entre 5 et 10 jours, et pourrait être plus impliquée dans la synthèse protéique des SCs lors des derniers stades de la différenciation puisque son expression protéique est fortement corrélée avec l'expression de MRF4, un marqueur tardif de la différenciation (McKay et al., 2008, Philippou et al., 2009). A ce jour et à ma connaissance, aucune étude ne s'est encore intéressée au rôle que pourrait posséder l'isoforme IGF-1Eb dans la régénération musculaire.

Le couple myostatine/follistatine est aussi connu pour influencer les SCs. La myostatine (Mstn) ou GDF-8 (Growth Differentiation Factor-8), un membre de la famille des TGF- $\beta$  (Transforming Growth Factor- $\beta$ ) est un puissant régulateur négatif de la masse musculaire exprimé de manière prédominante dans le muscle squelettique (Amirouche et al., 2009, Lee, 2004). De nombreuses études ont déjà démontré qu'une inhibition de cette protéine résultait en un phénotype hyper-musclé (McPherron & Lee, 1997, Schuelke et al., 2004, Szabo et al., 1998) et que ce phénotype était lié à une augmentation de la taille de fibres mais aussi de leur nombre (phénomène appelé hyperplasie) (Amthor et al., 2009, Girgenrath et al., 2005, McPherron et al., 2009, McPherron & Lee, 1997, Mendias et al., 2006). Même si la littérature s'est montrée très contradictoire concernant le rôle de la Mstn sur les SCs, son rôle semblerait être situé complètement à l'opposé de l'IGF-1. De nombreuses études ont montré, *in vivo*, que la Mstn inhibait non seulement l'activation et la prolifération des SCs mais également leur différenciation (Jouliia-Ekaza & Cabello, 2006, Lee & McPherron, 2001, McCroskery et al., 2003, McFarlane et al., 2008). En effet, la Mstn est connue pour augmenter l'expression de P21, un inhibiteur du cycle cellulaire qui régule la cyclin-dépendent kinase (Cdk) (McCroskery et al., 2003). Mstn décroît l'expression de Cdk2 et la phosphorylation de la protéine retinoblastoma (Rb) qui sont aussi toutes deux essentielles à la progression du cycle cellulaire (McCroskery et al., 2003). De plus, la Mstn semble pouvoir inhiber directement MyoD, via Smad 3, prévenant ainsi la prolifération des SCs (Langley et al., 2002). Par ailleurs, l'étude de Egerman et al. (2015) a également démontré que GDF11, qui présente 90% d'homologie en acides aminés avec la Mstn (Blau et al., 2015), inhibait la différenciation des myoblastes et ainsi la régénération musculaire. Ces auteurs ont observé que GDF11 et la MSTN possédaient les mêmes cibles cellulaires. La Follistatine (Fstn) est une glycoprotéine

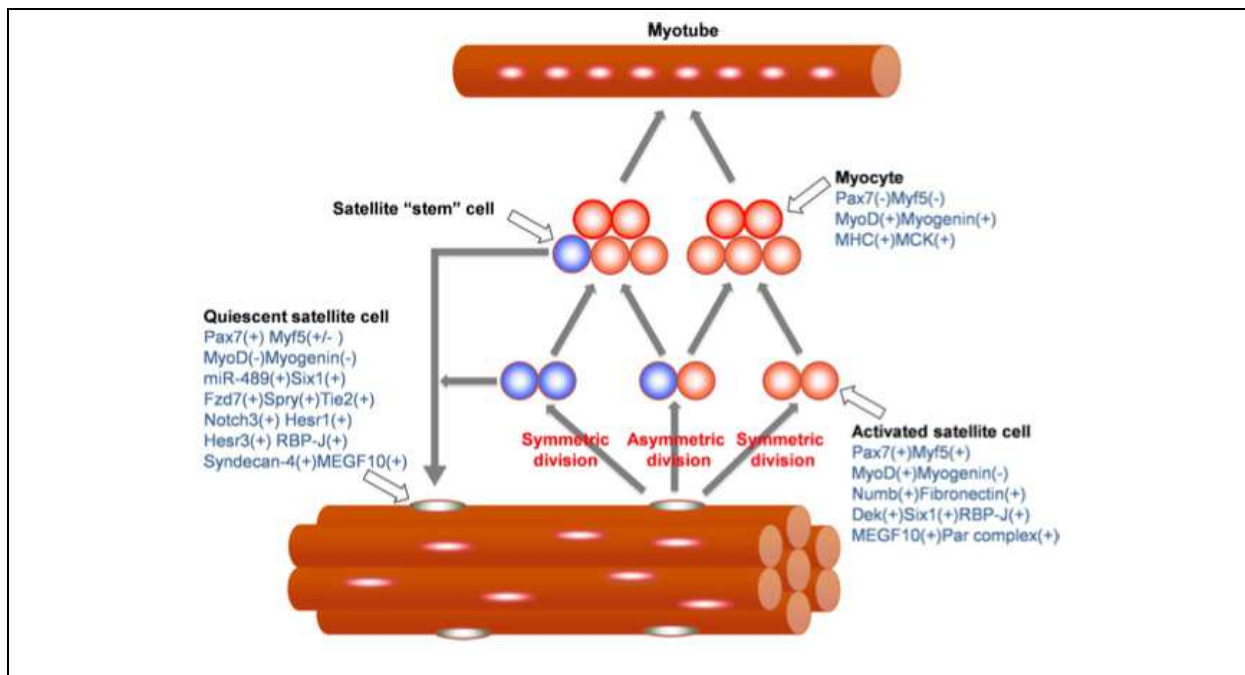
connue pour inhiber plusieurs membres de la famille des TGF- $\beta$  incluant la Mstn et GDF11 (Blau et al., 2015, Hemmati-Brivanlou et al., 1994, Iemura et al., 1998). La surexpression de la Fstn chez la souris résulte en un phénotype hyper-musclé encore plus important que chez la souris KO Mstn (Haidet et al., 2008, Lee & McPherron, 2001). L'étude de Lee (2007) a également couplé le modèle KO pour la Mstn et une surexpression de la Fstn pour le résultat impressionnant d'une masse musculaire quadruplée ! De manière intéressante, cette étude ainsi que la suivante du même groupe (Lee et al., 2010) ont donc montré que la Fstn, malgré l'absence de la Mstn, gardait un impact fonctionnel suggérant ainsi que les mécanismes induisant une prise de masse musculaire par la Fstn ne sont pas totalement Mstn-dépendants. Concernant la régénération musculaire et les SCs, la Fstn joue donc un rôle diamétralement opposé à celui de la Mstn : une augmentation de l'activation/prolifération (Gilson et al., 2009) et de la différenciation (Jones et al., 2015) des SCs et sa surexpression mène donc à une augmentation du potentiel de régénération musculaire (Jeong et al., 2013, Jones et al., 2015, Yaden et al., 2014).

Une autre cytokine faisant partie de la famille des interleukines semble jouer un rôle important dans les processus liés au SCs. En plus d'avoir participé à démontrer le rôle majeur de l'IL-6 dans les processus de prolifération des SCs, l'étude de Guerci et al. (2012) a également montré que l'IL-4 était un élément essentiel pour la fusion des myoblastes. Une étude précédente menée par Horsley et al. (2003) avait aussi mis en lumière le rôle de l'IL-4 dans le recrutement des myoblastes afin de fusionner avec les myotubes. Cette étude a montré qu'une délétion chez la souris pour les gènes codants pour l'IL-4 et son récepteur (IL-4R $\alpha$ ) entraînait une diminution de la croissance des cellules musculaires après leur formation lors de la régénération. Cependant, l'étude récente de Heredia et al. (2013) a démontré que l'ablation spécifique de l'IL-4R $\alpha$  sur les SCs n'engendrait aucune modification sur la régénération musculaire liée à une injection de CTX. D'autres études sont donc clairement nécessaires pour définir le lien entre l'IL-4 et la fusion des cellules satellites.



### 2.3.3. Une population hétérogène

Les SCs étant toutes des progéniteurs exprimant Pax7, ces cellules ont tout d'abord été considérées comme étant une population homogène. Pourtant, une accumulation d'évidences démontre leur hétérogénéité qui se traduit par des SCs se comportant différemment après une blessure musculaire (Almeida et al., 2016, Motohashi & Asakura, 2014, Tierney & Sacco, 2016). En effet, le simple fait qu'après avoir proliféré, certaines SCs soient capables de revenir à l'état quiescent alors que d'autres continuent dans le cycle cellulaire, est une preuve de leur hétérogénéité (**Fig.15**).



*Ce schéma représente les 3 divisions possibles d'une SC avec les marqueurs exprimés. Extrait de Motohashi & Asakura (2014)*

Dès 2007, l'étude de Kuang et al. (2007) a mis en évidence que l'expression du marqueur Myf5 était absente de près de 10% de la population totale en SCs. Ainsi, la population Pax7<sup>+</sup>/Myf5<sup>-</sup> est une population engagée dans le processus d'auto-renouvellement et permet donc de garder le pool de SCs constant. Ces SCs expriment également de forts niveaux d'expression de Wnt7a et Fzd7 (Le Grand et al., 2009) et cette voie est directement liée à une division symétrique des SCs permettant l'auto-renouvellement. Par contre, les SCs exprimant Myf5 s'engageront plutôt dans le processus de différenciation cellulaire. L'étude de Rocheteau et al. (2012) a par ailleurs démontré que les SCs exprimaient également des niveaux d'expression protéique différents pour Pax7. En effet, cette étude montre que les SCs exprimant fortement Pax7 (Pax7<sup>Hi</sup>) possèdent un métabolisme de base de faible intensité et s'orientent également vers une division permettant le retour à l'état quiescent, l'inverse est évidemment retrouvé pour les SCs Pax7<sup>Lo</sup>.

Des différences en terme de vitesse de prolifération ont également été reportées pour les SCs puisque deux populations coexistent : certaines avec un potentiel rapide de prolifération, et d'autres avec un potentiel lent (Schultz, 1996). De manière logique avec les résultats des études exposées précédemment, les SCs à faible taux de prolifération sont des cellules liées au processus d'auto-renouvellement (cependant ces cellules gardent la capacité de s'engager dans la lignée myogénique via une division asymétrique) alors que les cellules à fort taux de prolifération s'engagent rapidement dans le cycle cellulaire et vers une différenciation rapide (Ono et al., 2012). Ainsi ces cellules à fort potentiel de prolifération et différenciation sont les premières SCs permettant une mise en place précoce des processus de régénération. L'étude de Rantanen et al. (1995) a parfaitement mis en lumière une augmentation des mRNA codant pour la protéine myogénine dès 4h et 8h après blessure ! Ainsi, dès les premières heures après une lésion, des SCs sont déjà capables de se différencier alors que dans cette même étude les premiers signes de prolifération de SCs n'apparaissent qu'à partir de 24h post-blessure. Drummond et al. (2010) ont également montré une augmentation de l'expression protéique de la myogénine dès 6h après un exercice en résistance (engendrant des microlésions), alors que l'expression de la protéine MyoD reste inchangée jusqu'à au moins 48h après l'exercice. Ainsi, ces études mettent en avant le fait qu'il puisse y avoir une sous-population de SCs directement prête à se différencier, sans même avoir proliféré, et diffère donc des populations capables de proliférer rapidement ou lentement.

Les SCs présentent également une hétérogénéité évidente en fonction du type de muscle étudié (Biressi & Rando, 2010, Harel et al., 2009, Randolph & Pavlath, 2015). Deux études

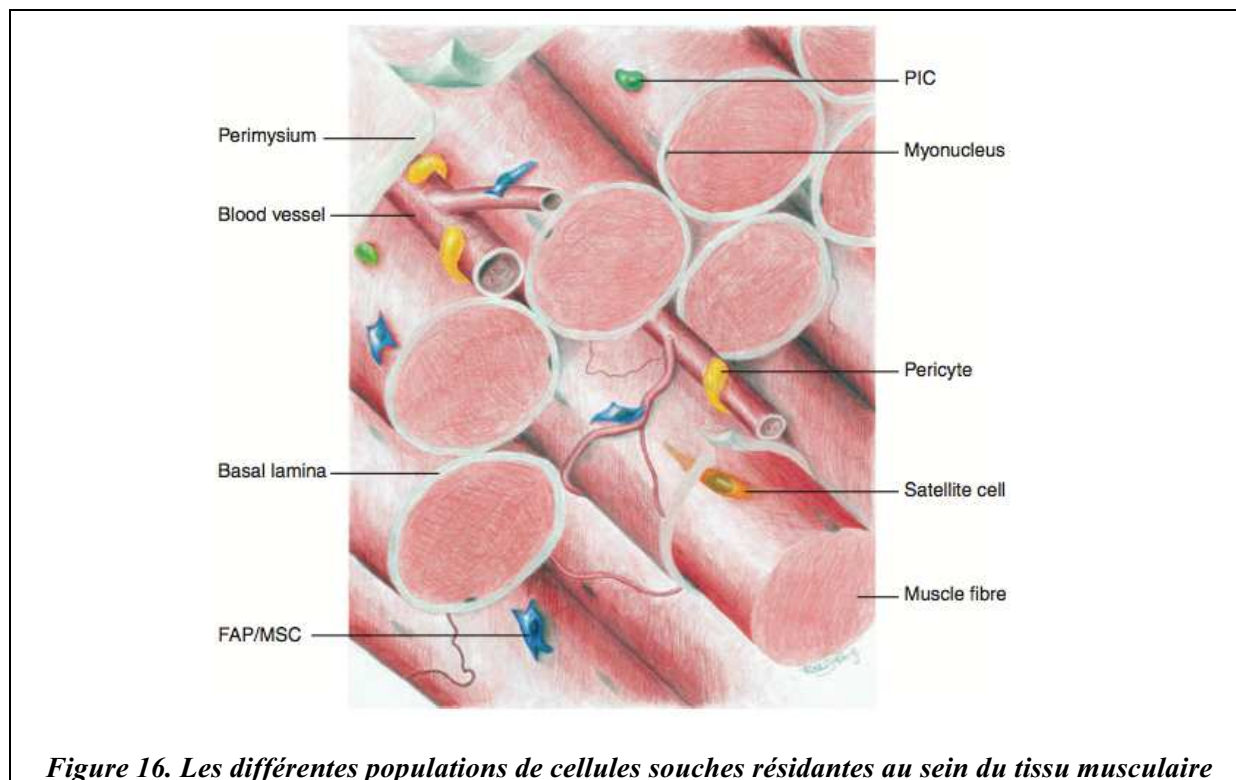


ont par exemple montré des niveaux d'expression de Pax3 différents selon le muscle étudié (Buckingham et al., 2003, Relaix et al., 2006). Collins et al. (2005) ont aussi observé un comportement différent de SCs issues de différents muscles pourtant transplantées dans le même environnement hôte. Deux travaux de recherche différents ont été également menés afin de déterminer si cette hétérogénéité en fonction du type de muscle était liée à l'environnement musculaire ou alors aux propriétés intrinsèques des SCs (Porter et al., 2006, Sambasivan et al., 2009). Ces deux études apportent des évidences pour les deux hypothèses et d'autres études sont donc nécessaires.

Enfin, des différences morphologiques ont également été observées au sein des SCs et ont été associées à différents potentiels myogéniques (Hashimoto et al., 2004). Certaines observations suggèrent que deux sous-populations ont des rôles différents en fonction de leur morphologie : une population dédiée à la croissance musculaire dont le nombre décroît avec l'avancée en âge, et une dédiée à la régénération musculaire après blessure qui est globalement maintenue pendant notre vie (Neal et al., 2012).

#### **2.4. Autres cellules souches dans l'environnement musculaire**

Si les SCs s'imposent comme les cellules souches ayant le rôle le plus important dans la régénération musculaire, beaucoup d'autres cellules souches présentes dans l'environnement musculaire possèdent également un rôle désormais bien établi (**Fig.16**). En effet, de nombreuses populations de cellules souches d'origine mésenchymateuse (MSCs) ou endothéliale ont été étudiées et possèdent un rôle non négligeable dans les processus de régénération. Si, dans un contexte sain, elles agissent de concert avec les SCs et la réponse immunitaire pour promouvoir une régénération efficace, dans un contexte pathologique, elles sont également responsables du développement d'infiltrations graisseuses et de fibrose qui altèrent la fonction musculaire (Boppart et al., 2013, Judson et al., 2013, Uezumi et al., 2014b).

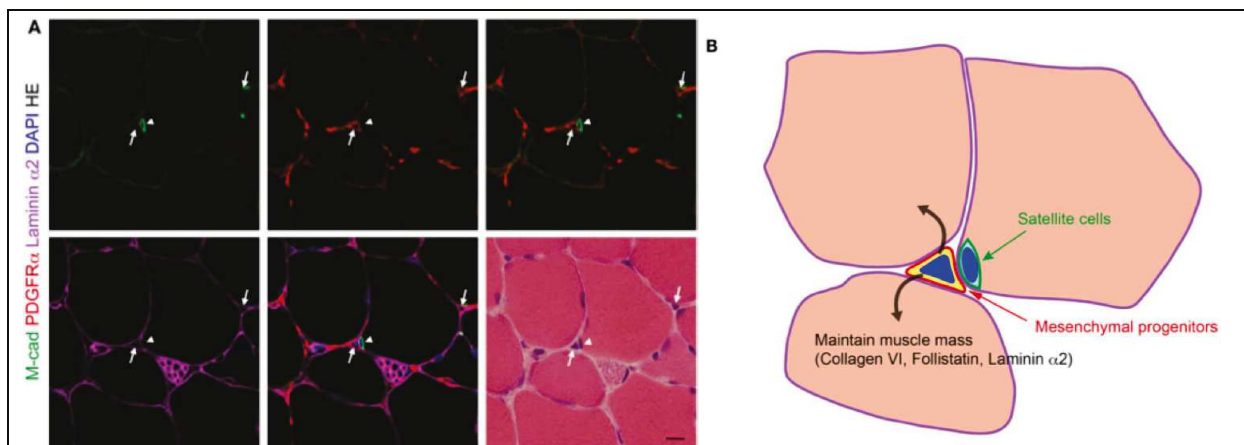


**Figure 16.** Les différentes populations de cellules souches résidentes au sein du tissu musculaire

#### 2.4.1. Les fibro-adipogenic progenitors : FAPs

Les FAPs (pour fibro/adipogenic progenitors) sont des cellules souches multipotentes d'origine mésenchymateuse et représentent la population de cellules souches la plus étudiée à ce jour, au sein du tissu musculaire, après les SCs. Les FAPs ont été identifiés en 2010 par deux études quasi-simultanées (Joe et al., 2010, Uezumi et al., 2010). Celle réalisée par Joe et al. (2010) a montré que les FAPs, isolés chez la souris et identifiés comme  $\text{lin}^{-}\alpha\text{integrin}^{-}\text{Sca1}^{+}\text{CD34}^{+}\text{PDGFR}\alpha^{+}$ , sont capables de donner, *in vitro*, des fibroblastes ou des adipocytes mais ne présentent aucune capacité myogénique. Cette même étude a également mis en lumière la prolifération rapide des FAPs après blessure musculaire (avec un pic entre 2 et 3 jours) et l'augmentation de la myogénicité des SCs observée lors de co-cultures myoblastes/FAPs. Les FAPs semblent donc avoir un effet bénéfique sur l'aspect myogénique des SCs et ces auteurs ont montré qu'ils étaient capables de sécréter différents facteurs améliorant la prolifération et différenciation des SCs comme IL-6 ou IGF-1. De manière intéressante, l'étude de Uezumi et al. (2010) a par ailleurs montré en culture que les fibres musculaires possédaient un rôle majeur dans l'inhibition de la différenciation des FAPs en adipocytes. Cette étude a aussi été la première à localiser clairement les FAPs dans l'environnement musculaire et a montré que ces cellules souches étaient localisées dans l'espace interstitiel du tissu musculaire (**Fig.17**). Cette étude précise aussi que les FAPs sont

plus fréquemment observés dans l'espace interstitiel du pérимыsium que dans celui de l'endomysium et que les FAPs tendent à se localiser préférentiellement autour des vaisseaux sanguins. Ces deux études princeps ont donc montré que les FAPs, résidants dans le tissu musculaire, influençaient positivement et indirectement la régénération musculaire et que, à l'inverse, l'environnement musculaire était également capable d'inhiber leur différenciation en tissu fibreux ou adipeux. Un autre article du même groupe a ensuite caractérisé, cette fois chez l'homme, les mêmes cellules souches d'origine mésenchymateuse (MSCs) exprimant le récepteur membranaire PDGFR $\alpha$  (Uezumi et al., 2014a). Cette étude a montré que ces cellules souches possédaient la même localisation et les mêmes propriétés que celles isolées chez la souris et ont donc bien logiquement été appelées FAPs également.



**Figure 17. Localisation des FAPs au sein du tissu musculaire**

*Les travaux de l'équipe de Uezumi et al., ont permis d'identifier la localisation des FAPs grâce à l'expression spécifique du marqueur de surface PDGFR $\alpha$ . Contrairement aux SCs, les FAPs sont localisés à l'extérieur de la lame basale, dans l'espace interstitiel. Adapté de Uezumi et al. (2014b)*

De manière cohérente avec l'étude de Joe et al. (2010), les travaux récents réalisés par Lemos et al. (2015) ont aussi montré, après injection de CTX, une prolifération rapide des FAPs atteignant un pic à 3 jours post-blessure. Cette étude démontre aussi le retour, entre 8 et 9 jours, à des valeurs de base par apoptose, concernant le nombre de FAPs. Enfin, l'étude de Heredia et al. (2013) a élégamment démontré que les FAPs possèdent une importante activité de phagocytose des débris nécrotiques dans les premiers temps de la régénération musculaire. En effet, cette étude a même conclu que les FAPs possédaient une activité de phagocytose 4 fois plus importante que les macrophages pourtant étiquetés maîtres dans l'art de la phagocytose ! Ainsi, en plus de leurs effets sur les SCs en facilitant les processus de myogenèse, les FAPs sont aussi essentiels dans le nettoyage clairement nécessaire pour rebâtir l'intégrité du tissu musculaire lésé.

#### 2.4.2. PW1<sup>+</sup>/Pax7<sup>-</sup> interstitial cells : PICs

L'étude de Mitchell et al. (2010) a été la première à identifier des cellules souches localisées dans l'espace interstitiel du tissu musculaire exprimant PW1, comme les SCs, mais qui n'expriment ni Pax7, ni Pax3. Ces MSCs (car exprimant Sca1 notamment) appelées PICs (pour PW1<sup>+</sup>/Pax7<sup>-</sup> Interstitial Cells) ont un potentiel myogénique et adipogénique *in vitro* et *in vivo* (Mitchell et al., 2010, Pannerec et al., 2013). Les travaux menés par Pannerec et al. (2013) ont également montré que cette population de cellules souches était une population hétérogène. En effet, il existe deux populations majeures de PICs : une exprimant PDGFR $\alpha$  et l'autre non. De ce fait, les auteurs ont donc conclu que la population de PICs exprimant PDGFR $\alpha$  représente certainement les FAPs décrits plus haut. De plus, cette population ne possède aucun potentiel myogénique, confirmant ainsi le très possible croisement avec une sous-population des FAPs. La deuxième sous-population des PICs n'exprimant pas PDGFR $\alpha$  possède un potentiel myogénique clairement établi.

#### 2.4.3. Les péricytes

Caractérisés par une morphologie très distincte, les péricytes sont des cellules souches localisées à l'intérieur de la membrane basale des vaisseaux sanguins et expriment un unique panel de marqueurs de surface : NG2<sup>+</sup>/CD146<sup>+</sup>/PDGFR $\beta$ <sup>+</sup>/ALP<sup>+</sup>. Cette population de cellules souches est également une population hétérogène et comporte deux grandes sous-populations : les péricytes de type 1 caractérisés par l'expression de PDGFR $\alpha$  et les péricytes de type 2 qui n'expriment pas PDGFR $\alpha$ . Alors que les deux types vont proliférer à la suite d'une blessure, les péricytes de type 1 se différencient en tissu adipeux et fibreux alors que ceux du type 2 peuvent s'engager dans la lignée myogénique de façon Pax7-dépendante (Birbrair et al., 2013, Birbrair et al., 2014, Birbrair et al., 2015). Malgré le fait que certaines revues récentes émettent l'idée que les péricytes de type 1 soient une sous-population des FAPs (Boppart et al., 2013, Sciorati et al., 2015), l'étude de Uezumi et al. (2010) a pourtant clairement défini que les FAPs étaient localisés, contrairement aux péricytes, à l'extérieur de la membrane basale des capillaires et de plus n'exprimaient pas NG2, un marqueur des péricytes. Ainsi, le rôle des péricytes, et de ses différentes sous-populations, dans la régénération musculaire reste encore de nos jours à définir clairement.

#### 2.4.4. Les myo-endothelial progenitors: MECs

Les Myo-endothelial progenitors (MECs) sont des cellules souches résidant également dans l'espace interstitiel du tissu musculaire. Ces cellules souches sont principalement isolées comme étant Sca1<sup>+</sup>/CD31<sup>+</sup>/CD45<sup>-</sup>/PDGFR $\alpha$ <sup>-</sup>. L'étude de Huang et al. (2014) a brillamment mis en évidence le rôle important des MECs lors de la régénération musculaire. En effet, ces auteurs ont tout d'abord montré que les MECs se différenciaient en myotubes (de manière Pax7 et MRFs dépendante), *in vitro*, aussi bien dans un milieu de culture favorisant la myogenèse que dans celui favorisant l'adipogenèse. De manière intéressante, ces auteurs ont également démontré que les MECs pouvaient se différencier en myotubes *in vivo* un mois après une transplantation dans le tibialis antérieur chez la souris. Enfin, cette même étude a également montré le rôle primordial du récepteur Bmpr1a (Bmp-receptor 1a) et de ses effecteurs (Smad1/5 et 8) dans le maintien de la capacité myogénique des MECs mais également dans leur capacité de prolifération. En effet, grâce à une suppression de ce récepteur, ces travaux ont démontré le potentiel adipogénique des MECs *in vitro*, mais également *in vivo* et montré de larges infiltrations graisseuses au sein des muscles des souris mutantes pour ce récepteur. Enfin, ces travaux ont également conclu que les MECs ne participaient pas, malgré leur capacité myogénique, à la formation de nouvelles fibres lors de la régénération musculaire mais par contre que ces cellules souches possédaient un important rôle d'inhibition des processus d'adipogenèse. En effet, les MECs possèdent un rôle majeur dans l'inhibition de la différenciation en adipocytes des FAPs et des expérimentations de co-culture cellulaire ont montré un effet dose réponse : plus le nombre de MECs en co-culture avec des FAPs est important, plus l'adipogenèse est inhibée et ce lien n'est retrouvé que lors d'un contact physique entre ces deux populations (donc non retrouvé en utilisant les milieux de culture). Cette étude est donc la première à mettre en évidence un rôle clé pour ces progéniteurs dans la régénération musculaire et d'autres études sont nécessaires pour confirmer et continuer à explorer ces différents mécanismes (Huang et al., 2014).

#### 2.5. Rôle clé du système immunitaire

Si les cellules souches de l'environnement musculaire, et notamment les cellules satellites et les FAPs, possèdent un rôle majeur pour régénérer un muscle lésé, le système immunitaire joue également un rôle clé pour une régénération efficace. De nombreuses revues de littérature sur le sujet ont été publiées très récemment et démontrent clairement l'impact du système immunitaire dans la régénération (Aurora & Olson, 2014, Chazaud, 2016, Farup et al., 2015b, Madaro & Bouche, 2014, Maffioletti et al., 2014, Schiaffino et al., 2016).

### 2.5.1. Les éosinophiles et neutrophiles

Les éosinophiles et neutrophiles représentent les deux premières populations du système immunitaire à investir le tissu musculaire dans la zone lésée.

Les éosinophiles, à l'origine associés à la réponse immunitaire liée à une infection par un parasite ou aux réactions allergiques (Silberstein & David, 1986), possèdent un rôle important dans la régénération musculaire. L'ablation génétique de la protéine cytosolique MBP-1 (major binding protein-1), exprimée par les éosinophiles, cause par exemple une aggravation de l'état des souris *mdx* liée à une apoptose précoce des fibres musculaires (Wehling-Henricks et al., 2008). Cette population du système immunitaire secrète également une substance clé pour la régénération musculaire : l'IL-4 (Guerci et al., 2012, Heredia et al., 2013, Horsley et al., 2003). Alors que les deux études les plus anciennes ont montré un rôle de l'IL-4 dans la fusion des cellules satellites, l'étude de Heredia et al. (2013) n'en a trouvé aucun et au contraire, cette dernière étude montre un rôle, dans les premiers temps de la régénération, de l'IL-4 sécrétée par les éosinophiles sur les FAPs. En effet, cette étude démontre clairement que l'IL-4 augmente d'une part la prolifération des FAPs durant les premiers jours de la régénération musculaire (via sa fixation sur son récepteur IL-4R $\alpha$  et la subséquente phosphorylation de STAT6) et d'autre part inhibe la différenciation de ces FAPs en adipocytes. Cette étude est donc la première à mettre clairement en évidence un rôle clé concernant les éosinophiles sur la régénération musculaire et plus spécifiquement sur le contrôle de la prolifération et de la différenciation des FAPs.

Les neutrophiles, identifiés comme étant Ly6C<sup>+</sup>/F4/80<sup>-</sup>, envahissent la zone lésée dès les premières heures après une blessure musculaire et leur concentration atteint son pic aux alentours de 24h après la blessure puis décline rapidement (Orimo et al., 1991, Papadimitriou et al., 1990, Tidball, 1995). Les neutrophiles possèdent une fonction de lyse des membranes cellulaires, via la libération de TNF- $\alpha$  et de myeloperoxydase (MPO), amplifient donc la réponse inflammatoire et, en apparence, l'ampleur des dégâts (Hodgetts et al., 2006, Nguyen & Tidball, 2003b). Cependant, cette activité de lyse des neutrophiles, couplée avec celle de phagocytose des débris nécrotiques, semble primordiale pour une bonne régénération puisque, lorsque l'activité des neutrophiles est perturbée, la régénération musculaire est beaucoup plus lente (Hodgetts et al., 2006, Teixeira et al., 2003).

### 2.5.2. Les macrophages

Si l'infiltration de neutrophiles et éosinophiles est rapide et transitoire, celle des macrophages intervenant peu de temps après, persiste et est nécessaire pour une régénération efficace. De nombreuses études ont déjà démontré que la perturbation du recrutement des macrophages, provenant majoritairement de la circulation sanguine, engendrait une forte diminution du potentiel de régénération musculaire et une augmentation drastique des signes de régénération perturbée comme les infiltrations graisseuses et la fibrose (Arnold et al., 2007, Contreras-Shannon et al., 2007, Segawa et al., 2008, Shireman et al., 2007, Summan et al., 2006). Tout d'abord, la littérature scientifique a identifié de nombreuses sous-populations de macrophages allant du type M1 (exprimant fortement Ly-6C ou exprimé Ly-6C<sup>high</sup>), pro-inflammatoire, au type M2 anti-inflammatoire Ly-6C<sup>low</sup> (incluant des types de macrophages intermédiaires tels que M2a, M2b, M2c) (Madaró & Bouche, 2014). Ces différentes polarisations des macrophages restent complexes et peu étudiées, et la littérature récente se contente, pour l'instant, d'utiliser la nomenclature M1 et M2 pour identifier les macrophages.

La première fonction identifiée pour les macrophages dans les premières études était leur rôle de phagocytose des débris nécrotiques. Cependant, les macrophages sont maintenant connus pour avoir de nombreux autres rôles et notamment sur les cellules souches de l'environnement musculaire. Des expérimentations réalisées *in vitro* ont tout d'abord démontré que la prolifération et la différenciation des myoblastes était augmentée en condition de co-culture avec des macrophages. Ce lien est également retrouvé avec des expérimentations utilisant le milieu de culture suggérant donc un effet de différentes substances sécrétées par les macrophages (Cantini & Carraro, 1995, Cantini et al., 1994, Saclier et al., 2013).

Après une blessure musculaire, les macrophages recrutés dès le premier jour sont de type pro-inflammatoire, donc des M1 macrophages (M1), et vont connaître leur pic d'expression environ 3 jours après blessure (Lemos et al., 2015, Perdiguero et al., 2011). Les M1 sont caractérisés par une expression de iNOS et sécrètent de nombreuses cytokines pro-inflammatoires telles que TNF- $\alpha$ , IL-1 $\beta$ , INF $\gamma$ , IL-6 ou encore du NO. Toutes ces cytokines vont aggraver l'inflammation, promouvoir la lyse des membranes, aider au processus de phagocytose des débris générés mais également vont promouvoir la prolifération des SCs (Arnold et al., 2007, Kharraz et al., 2013, Lemos et al., 2015, Perdiguero et al., 2011, Saclier et al., 2013, Villalta et al., 2009). Aux alentours du 3<sup>ème</sup>/5<sup>ème</sup> jour après blessure, un shift des M1 vers du M2 a lieu progressivement (Lemos et al., 2015, Perdiguero et al., 2011). Les

M2 possèdent un rôle diamétralement opposé à celui des M1 : ils ont en effet un rôle anti-inflammatoire et pro-régénératif. Les M2 vont sécréter à leur tour différentes cytokines dont l'IGF-1, TGF- $\beta$ , IL-10 ou encore de l'IL-4 (bien que controversé) qui vont toutes promouvoir la différenciation/fusion des SCs ainsi que la croissance musculaire nécessaire pour la « re-croissance » des nouvelles fibres formées ou celles régénérées (Arnold et al., 2007, Gordon, 2003, Lu et al., 2011, Perdiguero et al., 2011).

Si la présence des macrophages, des deux types, est primordiale pour les processus de régénération, le timing du shift phénotype du M1 vers du M2 semble essentiel. Si la première phase d'inflammation est nécessaire, elle ne doit pas perdurer et le shift vers une phase anti-inflammatoire doit s'opérer. Au contraire, si le shift phénotypique vers des M2 s'opère trop vite, la régénération musculaire sera également perturbée (Aurora & Olson, 2014). L'AMPK apparaît comme un régulateur puissant de la balance entre M1/M2 et semble, elle aussi, être une protéine primordiale dans le processus de régénération (Mounier et al., 2013). En effet, ces auteurs ont montré qu'une déficience en AMPK $\alpha$ 1 résultait en une diminution du shift vers du M2, une déficience de l'activité de phagocytose et donc d'une régénération largement perturbée. L'IL-10 possède également un rôle dans le shift phénotypique ou plutôt dans son déroulement. Une fois celui-ci engagé, les macrophages M2b sécrètent de hauts niveaux d'IL-10, induisant un shift vers des macrophages de type M2c qui possèdent un effet anti-inflammatoire majeur comparé aux autres types de macrophages (Gordon, 2003, Madaro & Bouche, 2014).

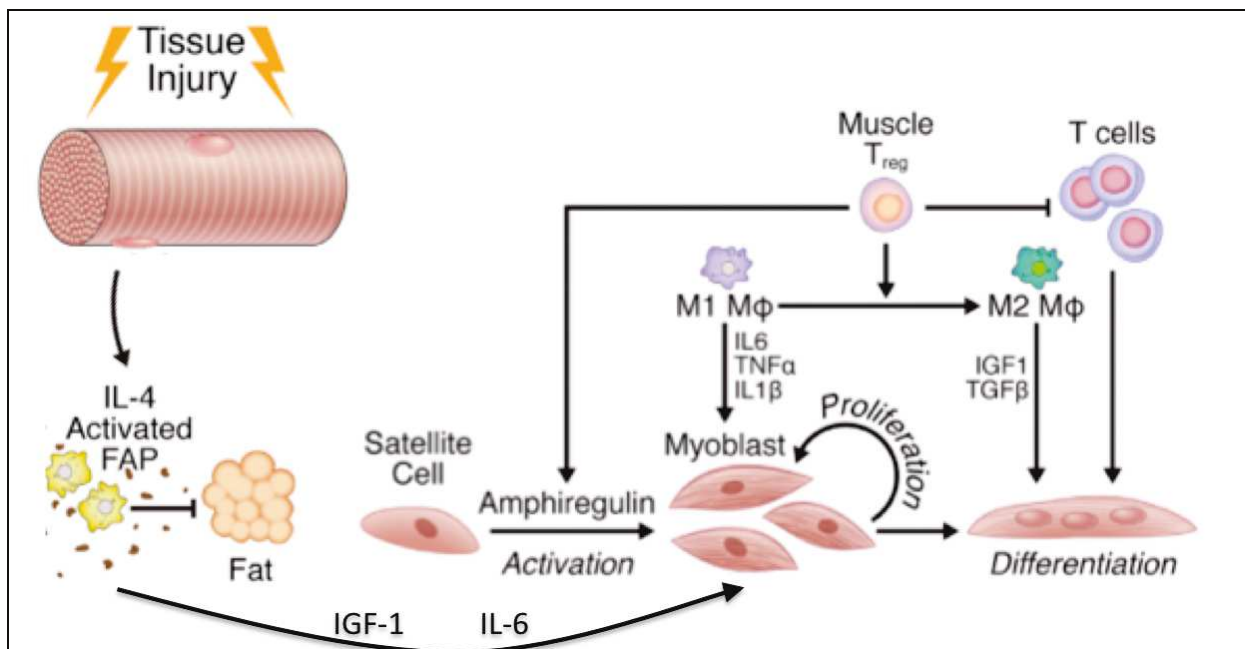
### 2.5.3. Les regulatory T cells : Treg

Les Treg font partie de la famille des lymphocytes T et sont définis par l'expression de marqueurs de surface tels que CD4 et CD25 ainsi que par l'expression du facteur de transcription forkhead box P3 (Foxp3) (Fontenot et al., 2003, Hori et al., 2003). Ces cellules exercent des actions immunosuppressives, anti-inflammatoires et maintiennent la tolérance immunitaire (Josefowicz et al., 2012). Une déficience congénitale en Treg, causée par des mutations de Foxp3, cause des phénotypes auto-immuns graves chez la souris et chez l'homme (Bacchetta et al., 2016, Ramsdell & Ziegler, 2014).

L'étude de Burzyn et al. (2013) a souligné le rôle des Treg dans la régulation des mécanismes de régénération musculaire. Ces travaux ont tout d'abord démontré que les Treg musculaires représentaient une sous-population de Treg totalement unique (analyse transcriptomique entre les différents Treg situés dans d'autres organes) et s'accumulaient



après blessure musculaire exactement au même moment qu'avait lieu le shift phénotypique des macrophages. Après avoir ensuite montré qu'une absence de Treg compromettrait clairement la régénération musculaire (avec la présence d'une accumulation anormale de fibrose), les auteurs ont ensuite démontré que les Treg régulaient le shift phénotypique des macrophages M1 vers du M2 mais aussi que les Tregs sécrétaient un facteur important pour la régénération : l'amphiréguline. Outre le fait que les Treg peuvent augmenter directement la prolifération des SCs *in vitro* (Castiglioni et al., 2015), un traitement avec de l'amphiréguline sur les souris dont les Treg ont été invalidés permet de globalement restaurer l'analyse transcriptomique retrouvée chez les souris WT. Enfin, cette étude a également démontré que l'amphiréguline permettait d'augmenter le potentiel de différenciation des SCs *in vitro*. Une étude sortie peu de temps après confirmera le rôle important de l'amphiréguline (dans les poumons) grâce à une ablation spécifique de cet facteur de croissance dans les Treg (Arpaia et al., 2015).



**Figure 18. L'implication majeure du système immunitaire dans la régénération musculaire**

Ce schéma représente le rôle des différentes cellules du système immunitaire, accompagné des cytokines impliquées, dans les processus de myogenèse des SC et donc dans la régénération musculaire. Il est à noter l'absence des éosinophiles et des neutrophiles sur ce schéma, qui participent également à la régénération musculaire comme précédemment décrit dans la partie 2.5.1. Adapté de Aurora & Olson (2014)

## **2.6. Suppléments alimentaires et optimisation du processus de régénération**

Si la régénération musculaire fait intervenir de nombreux acteurs cellulaires et moléculaires, l'alimentation permet également d'influencer ces acteurs et ainsi la régénération. Les études scientifiques sont encore peu nombreuses, mais la revue récente de Domingues-Faria et al. (2016) a parfaitement résumé l'apport de nombreuses suppléments alimentaires sur la régénération musculaire. Tout d'abord, une supplémentation en acides aminés branchés (issus de la prise de WHEY protéines), riche en leucine, permet d'améliorer les processus de régénération post-exercice excentrique ou à la suite d'une cryolésion (Farup et al., 2014, Pereira et al., 2014, Pereira et al., 2015). Ces études ont montré une augmentation de la prolifération des SCs, de la surface de section des fibres mais également une amélioration de la fonction musculaire associée à diminution de l'apparition de fibrose. La consommation de nutriments riches en polyphénols, comme des extraits de thé vert ou une supplémentation en « proanthocyanidolic oligomer », est également connue pour améliorer les processus de régénération musculaire. Les polyphénols, qui font partie d'une classe de nutriments ayant des capacités anti-inflammatoires, permettent notamment une activation plus précoce des SCs dans un contexte de blessure par contusion chez le rat (Myburgh et al., 2012) et améliorent le phénotype de la souris *mdx* (Evans et al., 2010). L'intérêt d'une supplémentation en vitamine D pour améliorer les processus de régénération est aussi soulevé dans la littérature scientifique. Une supplémentation en vitamine D chez l'animal permet d'augmenter la prolifération et de diminuer l'apoptose (Stratos et al., 2013). Chez l'homme, lors d'une carence en vitamine D, une supplémentation de cette vitamine améliore la capacité migratoire, la fusion en myotubes, la différenciation et l'hypertrophie des myoblastes isolés après biopsie musculaire (Owens et al., 2015).

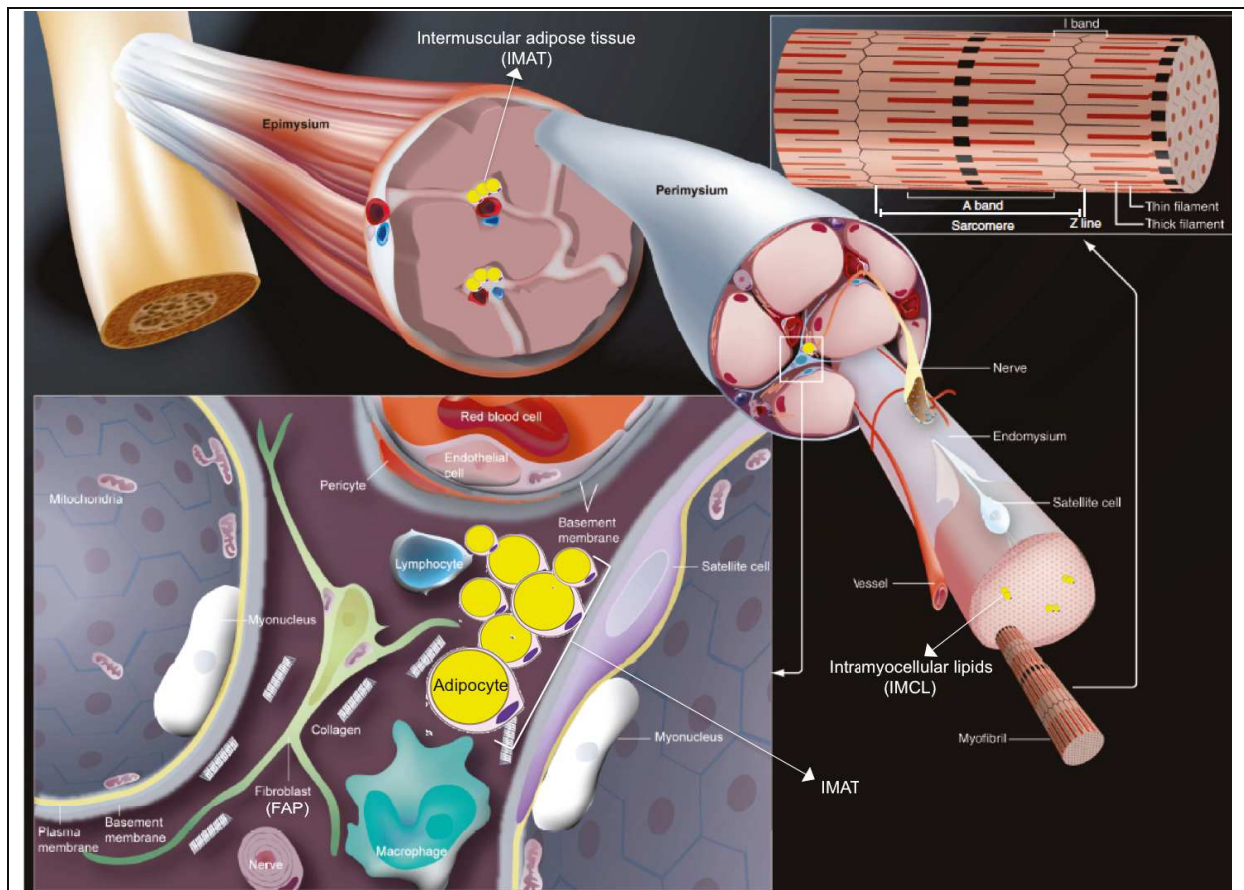
L'ensemble des stratégies nutritionnelles décrites précédemment possède également un rôle dans le dialogue entre les cellules du système immunitaire et la régénération musculaire. En effet, les polyphénols semblent jouer un rôle sur la modulation de la réponse inflammatoire en régulant négativement la voie NF- $\kappa$ B alors qu'une déficience en vitamine D diminue les sécrétions d'IL-10 et IL-13 (Domingues-Faria et al., 2016). Dans leur ensemble, ces différentes études mettent en évidence un champ disciplinaire encore trop peu étudié dans la régénération musculaire. Clairement, d'autres études sont nécessaires afin de caractériser les mécanismes cellulaires et moléculaires des différentes stratégies nutritionnelles afin d'améliorer la régénération musculaire.

**En résumé, la capacité du tissu musculaire squelettique à régénérer est un autre paramètre clé de sa plasticité. Ce phénomène de régénération est indispensable au tissu musculaire dans la mesure où de nombreuses situations, pathologiques ou physiologiques, amènent à des lésions plus ou moins graves des fibres musculaires. Les processus de régénération permettent donc de réparer les fibres endommagées, ou bien d'en établir de nouvelles, afin de restaurer l'intégrité originelle du muscle. Afin d'étudier de manière plus précise les mécanismes cellulaires et moléculaires de la régénération musculaire, de nombreux modèles animaux, notamment chez le rongeur, ont été utilisés. Ces différents modèles ont été principalement basés sur des blessures d'ordre physique ou chimique, grâce notamment à l'utilisation de toxines. Si les SCs s'imposent comme les cellules souches ayant le rôle le plus important dans la régénération musculaire, beaucoup d'autres cellules souches résidentes dans l'environnement musculaire possèdent également un rôle désormais bien établi et notamment les FAPs qui représentent la population la plus étudiée. Enfin cette partie a également souligné le rôle majeur joué par le système immunitaire dans les processus d'une régénération efficiente.**

## CHAPITRE 3 : LES INFILTRATIONS GRAISSEUSES

### **3.1. Définition et localisation**

Comme nous l'avons déjà précisé précédemment, la littérature soulève que la perte de force excède la perte de masse observée dans différentes situations de déconditionnement musculaire (Berg et al., 1997, Delmonico et al., 2009, Di Prampero & Narici, 2003, Jubrias et al., 1997). Ce paramètre du déconditionnement met ainsi en lumière le rôle de différents facteurs permettant d'expliquer la perte de force accrue. Par exemple, une réorganisation de l'appareil neuromusculaire explique en partie une diminution de la force observée dans des situations d'hypoactivité et/ou d'avancée en âge (Arentson-Lantz et al., 2016, Clark et al., 2008, McNeil et al., 2005, Ohira et al., 2015). D'une part, une diminution, de l'activation du système nerveux central et, d'autre part, l'apoptose de motoneurones, impactent négativement la force développée. Par ailleurs, la littérature soulève également que les infiltrations graisseuses du tissu musculaire semblent jouer un rôle non négligeable dans le processus de déconditionnement musculaire. Plus communément appelées IMAT pour « Inter-Muscular Adipose Tissue », ces adipocytes sont localisés sous l'épimysium du muscle, entre les fibres et/ou les faisceaux de fibres musculaires, et représentent une véritable accumulation ectopique de lipides située à l'extérieur de la cellule musculaire (**Fig.19**). Il faut donc clairement différencier les IMAT des gouttelettes lipidiques, qui elles, représentent de réels réservoirs d'énergie, présentes au sein même de la cellule musculaire (Addison et al., 2014, Karampinos et al., 2012, Vettor et al., 2009). L'accumulation de ces IMAT est intimement liée aux situations d'hypokinésie et/ou hypodynamie, ainsi qu'à certaines pathologies, et, dans ce contexte, un nombre croissant d'études soulignent le rôle prépondérant de l'accumulation d'IMAT sur l'altération de la fonction musculaire.



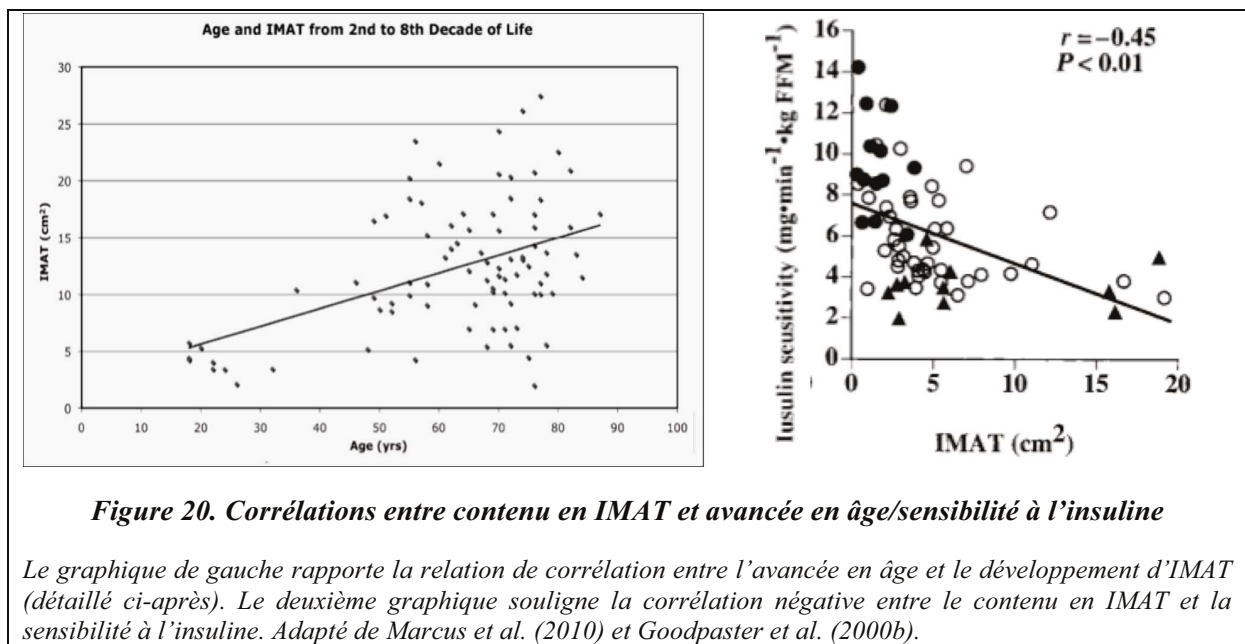
**Figure 19. Illustration de la localisation des IMAT au sein de l'environnement musculaire**

Ce schéma représente la structure du tissu musculaire avec ces principaux constituants ainsi que la localisation des IMAT. Les IMAT sont constitués d'adipocytes localisés sous l'épimysium du muscle, entre les faisceaux de fibres, et sous le périmysium entre les fibres. La localisation des gouttelettes lipidiques intramyocellulaires (IMCL) est également représentée. De nombreux acteurs impliqués dans l'homéostasie musculaire sont aussi présentés de manière non exhaustive : les FAPs, les macrophages, les SCs ou encore les péricytes. Adapté de Gayraud-Morel et al. (2009).

### 3.2. Altération de la fonction musculaire

Pourtant naturellement présents mais de manière variable chez l'homme, la littérature scientifique souligne que le développement et l'accumulation d'IMAT apparaît dans de nombreuses situations, pathologiques ou non, toutes intimement liées au phénomène de déconditionnement musculaire : sarcopénie (Brioché et al., 2016, Marcus et al., 2010), inactivité (Manini et al., 2007), obésité et diabète de type II (Gallagher et al., 2014, Goodpaster et al., 1999, Karampinos et al., 2012), dénervation (Ceylan et al., 2014, Dulor et al., 1998), rupture tendineuse (Farshad et al., 2011, Kuzel et al., 2013), régénération perturbée (Contreras-Shannon et al., 2007, Shireman et al., 2007) ou encore DMD (Uezumi et al., 2014a, Wren et al., 2008). Les connaissances relatives aux infiltrations graisseuses ne cessent

d'augmenter et soulignent leur rôle prépondérant sur la fonction musculaire. En effet, de nombreuses études ont déjà démontré de fortes corrélations entre l'accumulation d'IMAT et la perte de masse musculaire, de force et d'insulino-sensibilité (Delmonico et al., 2009, Goodpaster et al., 2000a, Miljkovic-Gacic et al., 2008, Visser et al., 2005) (**Fig.20**). A ma connaissance, Aherne (1965) a conduit la première étude décrivant des infiltrations graisseuses dans de nombreux tissus, incluant le muscle strié squelettique, chez des nouveau-nés et cette étude fut suivie par de nombreuses autres (Birkbeck, 1970, Kannan et al., 1976, Kelley et al., 1991, Nordal et al., 1988).



La majorité des études portant sur les IMAT chez l'homme ont été réalisées chez la personne âgée ou chez la personne obèse et/ou insulino-résistante. Il est actuellement clairement établi que les IMAT augmentent avec l'avancée en âge (Addison et al., 2014, Brioché et al., 2016, Kirkland et al., 2002). Les premières études ayant démontré une augmentation du contenu en IMAT ont été réalisées chez des hommes (Borkan et al., 1983, Rice et al., 1989) et femmes (Ryan & Nicklas, 1999, Song et al., 2004) d'âge avancé, en utilisant de la tomographie par densité osseuse ou, plus récemment, de l'imagerie par résonance magnétique (IRM). Deux études, réalisées sur la même cohorte de personnes âgées, ont ensuite éclairci le rôle des IMAT dans la fonction musculaire (Goodpaster et al., 2001, Visser et al., 2002). En effet, ces deux études ont été les premières à mettre en évidence une corrélation entre l'accumulation d'IMAT et la perte de masse mais aussi la perte de force musculaire. L'étude de Visser et al. (2002) a également démontré une diminution de la performance aux tests de marche de 6 mètres et levers de chaise enchaînés, avec une

corrélation entre les niveaux d'accumulation d'IMAT et les performances aux deux tests. Quelques années après, une étude longitudinale de 5 ans réalisée par Delmonico et al. (2009) confirmera une nouvelle fois la corrélation entre le contenu en IMAT et la perte de force liée à l'avancée en âge. Dans le contexte spécifique de la sarcopénie, d'autres études ont également montré que l'accumulation d'IMAT représentait un risque indépendant de la réduction importante de la mobilité (Beavers et al., 2013, Marcus et al., 2012, Murphy et al., 2014, Tuttle et al., 2012, Visser et al., 2005) mais également un bon prédicteur du risque de fracture (Schafer et al., 2010). Une augmentation du contenu en IMAT est également associée avec une altération de l'angle de pennation. Cette altération pouvant alors résulter en un angle mécanique défavorable et une diminution des niveaux de forces produits (Meyer et al., 2004). Les premières observations concernant le lien entre infiltrations graisseuses et obésité/insulino-résistance sont également apparues très tôt dans la communauté scientifique. En effet, dès les années 1991, l'étude de Kelley et al. (1991) a été la première à mettre en évidence un lien entre obésité et composition du tissu musculaire. D'autres études ont ensuite vérifié et démontré le lien entre obésité, insulino-résistance et infiltrations graisseuses (Goodpaster et al., 1999, Goodpaster et al., 2000a, Goodpaster et al., 1997, Simoneau et al., 1995).

### **3.3. Les modèles expérimentaux**

Différents modèles expérimentaux permettent d'étudier la problématique des IMAT. Chez l'homme, les infiltrations graisseuses sont naturellement présentes au sein du tissu musculaire et ainsi chaque étude réalisée chez l'homme peut potentiellement évaluer la quantité d'IMAT par des techniques de tomodensitométrie ou d'IRM. Chez l'animal, et notamment chez le rongeur, que très peu d'IMAT sont naturellement présents dans le tissu musculaire. Ainsi, de façon à pouvoir étudier les mécanismes du développement des IMAT, de nombreux modèles faisant intervenir les mécanismes de régénération se sont développés. Si les modèles de régénération permettent d'étudier le développement des IMAT, il est important de noter que les résultats de ces études restent dans le contexte d'une régénération musculaire, ce qui ne permet pas d'extrapoler les résultats obtenus dans des contextes ne présentant pas de régénération musculaire, à l'augmentation du contenu en IMAT avec l'avancée en âge par exemple.

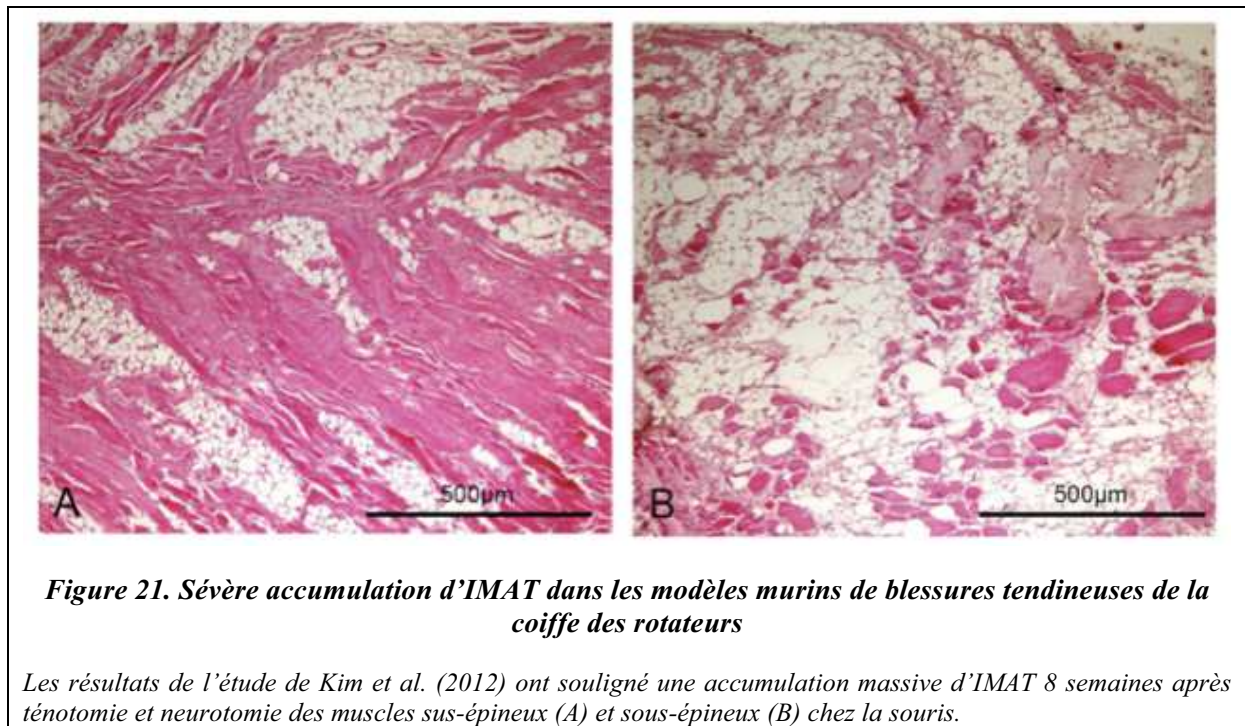
### 3.3.1. Modèles sans régénération musculaire

Comme évoqué dans le paragraphe 3.2., les infiltrations graisseuses ont été étudiées de manière intensive chez la personne âgées ainsi que chez la population obèse.

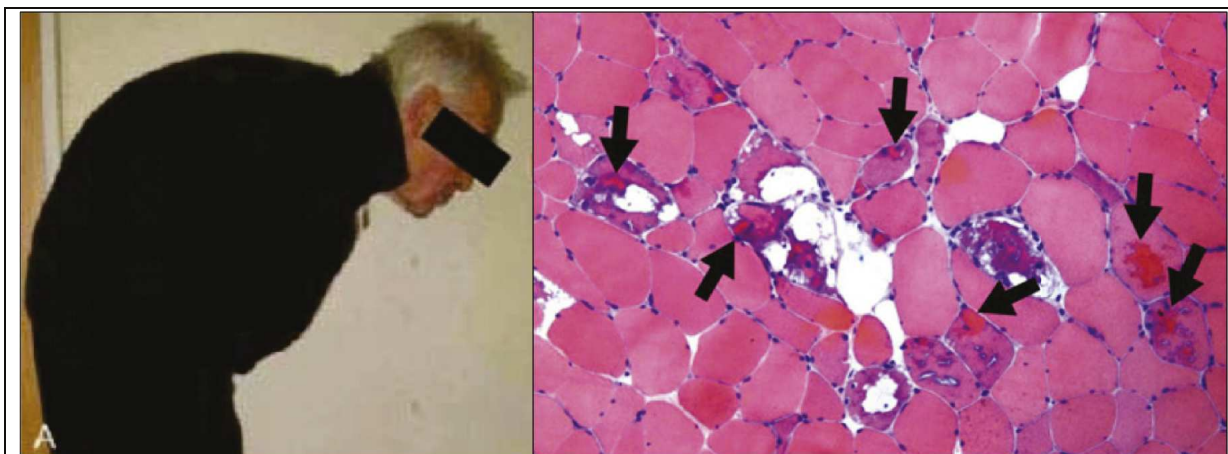
La littérature scientifique a aussi mis en lumière depuis de nombreuses années un développement d'IMAT massif lors des déchirures des tendons de la coiffe des rotateurs (au niveau de l'épaule) (Kuzel et al., 2013, Laron et al., 2012). Les dysfonctions et douleurs liées à l'articulation de l'épaule, et majoritairement concernant la coiffe des rotateurs, représentent un trouble musculo-squelettique majeur chez l'Homme. Par exemple, des évidences obtenues grâce aux IRM ont révélé que 4% des individus âgés de moins de 40 ans et 50% de ceux âgés de plus de 60 ans présentent certains degrés pathologiques de la coiffe des rotateurs (Shin, 2011). De très nombreux articles, présentés de manière complète dans les revues de Laron et al. (2012) et Kuzel et al. (2013), ont montré qu'une déchirure des tendons de la coiffe des rotateurs amènent au développement massif d'infiltrations graisseuses dans les tendons lésés mais également des muscles associés. Ces infiltrations graisseuses sont accompagnées d'une atrophie musculaire marquée. L'étude de Goutallier et al. (1994) a été la première à proposer une classification de ces infiltrations graisseuses basées sur le ratio IMAT/volume musculaire. Cette classification se révèle alors importante puisque corrélée avec la diminution de la fonction musculaire et la sévérité de la déchirure mais montrant également que ces infiltrations graisseuses semblent irréversibles même après chirurgie. Des modèles animaux ont ensuite été développés afin de pouvoir étudier de manière plus complète et plus fondamentale l'apparition d'IMAT liée aux déchirures de la coiffe des rotateurs. De nombreux animaux tels que les chèvres, les chiens ou les lapins ont été utilisés et peuvent recréer avec succès le développement pathologique d'IMAT et l'atrophie musculaire observés chez l'homme. Ces études ont permis d'engager un débat concernant la cause de l'apparition de ces IMAT : certains pensent que les infiltrations graisseuses se développent à cause à l'absence de contraintes mécaniques alors que d'autres groupes postulent que la blessure tendineuse induit également une blessure neurologique entraînant par la suite le développement d'infiltrations graisseuses (Gerber et al., 2004, Grumet et al., 2009, Gupta & Lee, 2007, Rowshan et al., 2010, Rubino et al., 2007, Safran et al., 2005). Les études réalisées chez la chèvre et le lapin ont aussi permis de confirmer que la chirurgie de la coiffe des rotateurs ne permettait pas de diminuer la quantité d'IMAT déjà présents. Des modèles ont été également développés chez le rat (Kim et al., 2012, Liu et al., 2011) et chez la souris (Kim et



al., 2012) permettant par la suite l'utilisation de souris transgéniques dans l'optique de déchiffrer les mécanismes moléculaires impliqués (**Fig.21**).



Une autre manifestation impressionnante de la dysfonction causée par les IMAT est représentée par la camptocormie. Ce syndrome de la colonne vertébrale courbée, appelé également « syndrome de la tour de Pise », représente l'acquisition d'une position anormale du buste en flexion vers l'avant ou latéralement. Ce syndrome est intimement lié à une dysfonction des muscles paravertébraux (spinaux), situés de part et d'autre de la colonne vertébrale. Souvent associé à la maladie de Parkinson (Findlay et al., 2013, Finsterer & Strobl, 2011, Margraf et al., 2010, Spuler et al., 2010), les biopsies réalisées, ainsi que les images IRM acquises sur les muscles paravertébraux des personnes atteintes de camptocormie montrent le développement d'IMAT et des signes liés à différentes myopathies (Findlay et al., 2013, Gomez-Puerta et al., 2007, Kuo et al., 2009, Renard et al., 2012) (**Fig.22**). Même si la littérature suspecte des troubles neurologiques comme étant à l'origine de la camptocormie, les causes restent encore floues et des études sont donc nécessaires pour comprendre l'apparition de ce syndrome. Enfin l'impact des infiltrations graisseuses dans ce syndrome reste également à définir.



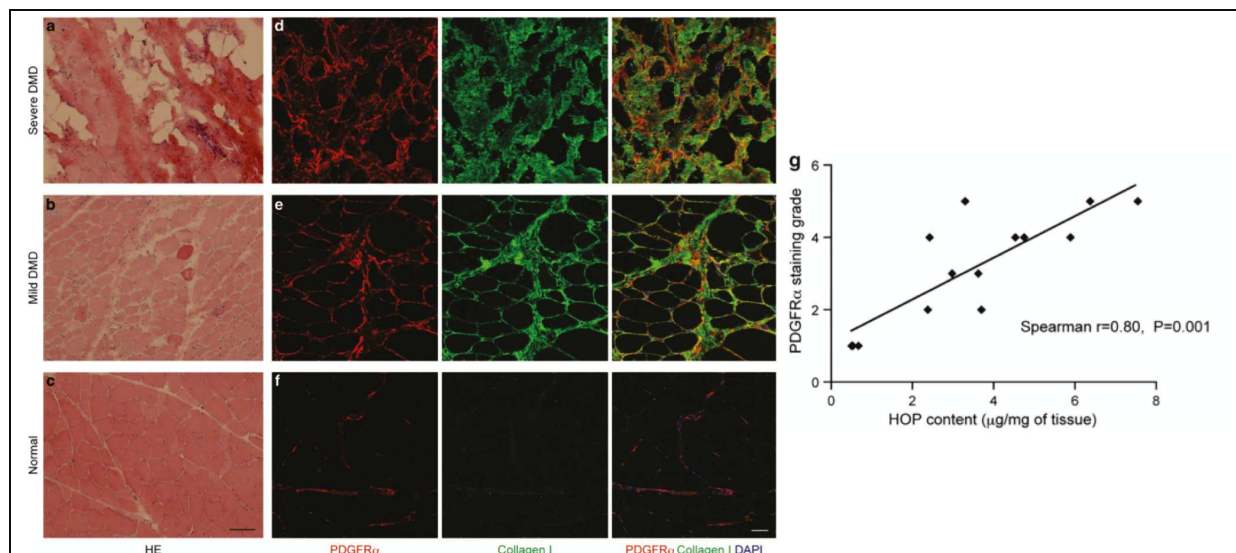
**Figure 22. Posture d'un patient atteint de camptocormie et histopathologie du muscle strié squelettique**

*Biopsie issue du quadriceps. Adapté de Renard et al. (2012) et Spuler et al. (2010)*

### 3.3.2. Modèles avec régénération musculaire

Contrairement à l'homme, les rongeurs ont très peu, voire aucun IMAT naturellement présents au sein du tissu musculaire. Ainsi, de façon à pouvoir étudier les mécanismes de développement d'IMAT, de nombreux modèles faisant intervenir les mécanismes de régénération se sont développés. Plus précisément, la plupart de ces modèles consistent en une perturbation des mécanismes de régénération induisant le plus souvent un développement d'IMAT et de fibrose dans le muscle lésé.

Comme développé dans la partie 2.2.1., les dystrophies musculaires progressives se caractérisent par des cycles continus de dégénérescence/régénération induisant progressivement une perte du potentiel de régénération et un développement massif de fibrose et d'IMAT (Blau et al., 1983, Ieronimakis et al., 2016, Wren et al., 2008). Ainsi le tissu musculaire d'un patient atteint de la DMD (ou dans une moindre mesure d'une souris *mdx*) est caractérisé par de larges infiltrations graisseuses et fait l'objet d'un modèle expérimental pour les étudier. L'étude de Uezumi et al. (2014a) a par exemple observé que les FAPs étaient majoritairement exprimés autour des zones de fibrose et d'infiltrations graisseuses mais aussi que l'expression de PDGFR $\alpha$  était fortement corrélée à la sévérité de la dystrophie (**Fig.23**). Des résultats similaires avaient été déjà retrouvés par le même groupe dans le diaphragme de souris *mdx* (Uezumi et al., 2011). Par ailleurs, l'étude de Wren et al. (2008) avait démontré une forte corrélation entre le pourcentage d'IMAT et la sévérité de la DMD chez l'homme.



**Figure 23. Corrélation positive entre l'expression de PDGFR $\alpha$  et le degré de sévérité de la DMD**

Grâce à différentes expérimentations histologiques et d'immunofluorescence, l'étude de Uezumi et al. (2014a) a démontré une importante corrélation positive entre l'expression de PDGFR $\alpha$ , le contenu en collagène et donc la sévérité des symptômes liés à la DMD. « HOP content » correspond au degré de sévérité de la DMD.

La plupart des modèles de régénération musculaire utilisés afin d'étudier les mécanismes de régénération (chez les rongeurs notamment) permettent une régénération efficace, accompagnée d'une restauration de l'intégrité d'origine sans apparition anormale d'IMAT et de fibrose. Le modèle « glycérol », qui consiste en une injection de glycérol intra-musculaire, est un modèle de régénération qui, au contraire, possède la particularité de permettre le développement et l'accumulation d'IMAT dans le muscle lésé (**Fig.19**). La première étude utilisant ce modèle de régénération, menée chez le lapin par Kawai et al. (1990), a permis d'identifier un modèle de régénération musculaire que les auteurs ont appelé « experimental glycerol myopathy » en raison de la ressemblance des mécanismes de dégénérescence avec ceux de la DMD y compris en ce qui concerne l'apparition d'IMAT. Ce modèle glycérol a ensuite été utilisé dans de nombreuses études afin d'étudier les mécanismes cellulaires et moléculaires de l'apparition d'IMAT (Arsic et al., 2004, Joe et al., 2010, Lukjanenko et al., 2013, Pisani et al., 2010a, Uezumi et al., 2010). L'étude de Pisani et al. (2010a) a cependant été la première à caractériser et à quantifier de manière précise, chez la souris, les effets du modèle glycérol en tant que modèle de régénération musculaire permettant l'apparition d'IMAT. Ces travaux ont en effet caractérisé de manière précise le développement d'IMAT en fonction de la concentration du glycérol injecté, l'âge, le genre et la souche des souris utilisés.

### 3.4. L'origine des IMAT

#### 3.4.1. L'implication majeure des FAPs

Si de nombreuses cellules souches de l'environnement musculaire peuvent potentiellement s'engager dans un lignage adipogénique, les FAPs représentent pour l'instant la population qui semble jouer un rôle majeur dans le développement des IMAT. L'étude de Uezumi et al. (2010) a d'ailleurs clairement mis en évidence que seuls des progéniteurs exprimant le récepteur de surface PDGFR $\alpha$ , représentant les FAPs dans cette étude, étaient capables de se différencier en adipocytes à la suite d'une blessure musculaire induite par le modèle glycérol. De manière importante, l'étude de Heredia et al. (2013) a par la suite confirmé, qu'après blessure musculaire, PDGFR $\alpha$  était exclusivement exprimé par les FAPs. L'étude de Uezumi et al. (2011) a également confirmé le potentiel adipogénique, *in vivo*, des FAPs. Cependant, si l'implication des FAPs dans l'apparition des IMAT chez la souris est admis et prouvé dans la littérature, aucune étude n'a pour l'instant clairement démontré le rôle des FAPs dans le développement d'IMAT chez l'homme. Même si l'étude de Uezumi et al. (2014a) a déjà démontré une accumulation anormale de FAPs autour des zones de fibrose et d'IMAT chez des patients atteints de la DMD, cette étude ne donne aucune information concernant la provenance de ces IMAT. Ainsi des études sont encore nécessaires afin de démontrer clairement, chez l'homme, que les FAPs sont les cellules souches à l'origine des IMAT. Par ailleurs, si la trans-différenciation des SCs (Vettor et al., 2009) reste encore de nos jours débattue dans la littérature scientifique, les études récentes utilisant des outils d'isolation plus spécifiques et plus performants semblent démontrer que les SCs ne peuvent s'orienter que vers des processus myogéniques (Sciorati et al., 2015).

#### 3.4.2. PDGFR $\alpha$ : un récepteur clé

Si l'on admet que les FAPs semblent être les cellules souches à l'origine des IMAT, il est donc important de connaître quels sont les mécanismes cellulaires et moléculaires modulant leur activité. Tout d'abord, avant même que les FAPs ne soient caractérisés dans le tissu musculaire, l'étude de Olson & Soriano (2009) avait montré qu'une augmentation de l'activité de la voie liée à l'interaction de PDGFR $\alpha$  avec son ligand PDGF-AA engendrait une augmentation du développement de tissu conjonctif et donc de fibrose dans de nombreux organes, dont le muscle squelettique. Ainsi, l'activation du récepteur PDGFR $\alpha$  spécifique aux FAPs dans le muscle squelettique en régénération semblerait donc être une piste sérieuse dans l'évolution des processus de fibrose observés lors d'une régénération perturbée.

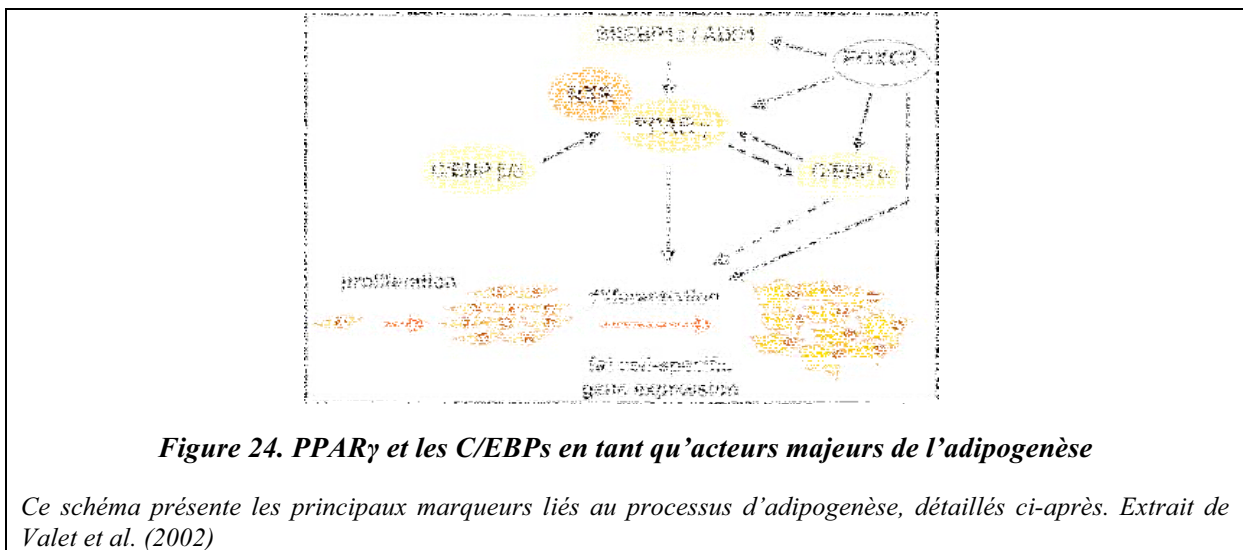
Le facteur de croissance PDGF a été identifié il y a plus de 40 ans et défini comme un facteur permettant la croissance des fibroblastes (Kohler & Lipton, 1974). Chez l'homme, la famille des facteurs de croissance PDGF comporte 5 isoformes de protéines (PDGF-A, -B, -C et -D) qui peuvent former grâce à des ponts disulfures des homodimères (PDGF-AA, -BB, -CC et -DD) et un hétérodimère (PDGF-AB). Ces nombreuses isoformes peuvent ainsi interagir avec leurs récepteurs (également sous forme de dimères) PDGFR $\alpha$  et PDGFR $\beta$  qui, eux aussi, peuvent former un récepteur hétérodimère PDGFR $\alpha\beta$ . Cependant, si les expérimentations réalisées *in vitro* démontrent une complexité majeure concernant les différentes interactions ligands/récepteurs, les expérimentations *in vivo* ont pour l'instant démontré que PDGFR $\alpha$  interagissait avec PDGF-AA et PDGF-CC, alors que le récepteur PDGFR $\beta$  interagissait avec PDGF-BB (Andrae et al., 2008, Heldin & Westermark, 1999). Une fois l'interaction ligand/récepteur effectuée, le récepteur s'auto-phosphoryle et active différentes voies de signalisation : la voie des Ras-MAPK, de PI3K, de la phospholipase C $\gamma$  ou encore des intégrines (Andrae et al., 2008, Heldin & Westermark, 1999).

La première étude concernant les FAPs et le rôle de son récepteur PDGFR $\alpha$  a été effectuée par Uezumi et al. (2011) et a, dans un premier temps, montré le rôle majeur de l'interaction PDGF-AA et PDGFR $\alpha$  dans la prolifération des FAPs *in vitro*. Cette même étude a dans un second temps mis en lumière qu'une activation de ce récepteur augmentait aussi l'expression de nombreux gènes impliqués dans le développement de la fibrose comme TIMP1,  $\alpha$ -SMA et Col3a1. Ce même groupe a ensuite voulu explorer ces mécanismes chez l'homme et ont également démontré qu'une stimulation de PDGFR $\alpha$  augmentait la prolifération des FAPs et que cet effet était complètement annihilé avec des inhibiteurs spécifiques de l'activation de PDGFR $\alpha$  (Uezumi et al., 2014a). Ensuite, en utilisant un jeu d'inhibiteurs des différents acteurs des voies de signalisation induites par l'activation de PDGFR $\alpha$ , cette étude a par la suite souligné que PDGFR $\alpha$  induisait la prolifération des FAPs de manière PI3K-Akt et MEK2-MAPK dépendante. Enfin ces travaux ont aussi corrélé les niveaux d'expression de PDGFR $\alpha$  à la sévérité de la DMD chez l'homme et pointent donc du doigt le rôle négatif des FAPs dans un environnement musculaire pathologique. Enfin, une étude très récente a de nouveau mis en évidence le rôle majeur joué par PDGFR $\alpha$  (Ieronimakakis et al., 2016). Cette étude a en effet souligné que la surexpression de ce récepteur engendrait une accumulation anormale de fibrose après blessure musculaire et au contraire que son inhibition, *via* un inhibiteur spécifique de la liaison PDGF-AA/PDGFR $\alpha$ , permettait une amélioration du phénotype de la souris *mdx*.

### 3.4.3. Du progéniteur à l'adipocyte mature

De nombreuses études ont déjà montré que les FAPs, qui sont donc des progéniteurs au potentiel adipogénique, allaient successivement exprimer plusieurs marqueurs clés impliqués dans les processus d'adipogenèse pour *in fine* former des adipocytes matures exprimant également des marqueurs clés les définissant.

Les mécanismes inhérents à la différenciation des préadipocytes en adipocytes matures ont été étudiés de manière intensive et sont relativement bien connus. La littérature scientifique a notamment prouvé le rôle majeur que possédait deux familles de facteurs de transcriptions essentiels aux processus d'adipogenèse : la famille des CCAAT-enhancer binding proteins (C/EBPs) et celle des peroxisome-proliférateur-activés récepteurs (PPARs). Même si d'autres études ont également démontré le rôle de beaucoup d'autres voies impliquées dans l'adipogenèse (SREBP, STAT, KLFs, FOXC2, HMG1c ou encore les microRNAs), ces deux familles restent les plus sondées dans les études concernant les infiltrations graisseuses (Tang & Lane, 2012, Valet et al., 2002, White & Stephens, 2010) (**Fig.24**).



La famille des C/EBPs contient 4 isoformes nommés C/EBP $\alpha$ , C/EBP $\beta$ , C/EBP $\delta$  et CHOP10 (oui CHOP10), qui jouent un rôle majeur dans le processus de différenciation adipogénique. Premièrement, C/EBP $\beta$  et C/EBP $\delta$  sont très rapidement exprimés (dans les 4 premières heures) dans les processus précoces de la différenciation (Tang & Lane, 1999). C/EBP $\beta$  reste le plus étudié de ces deux facteurs précoces et malgré l'augmentation de son expression dès les premières heures, son activité transcriptionnelle n'augmente qu'à partir de 16h après l'induction de la différenciation (Tang & Lane, 1999). Si l'expression de C/EBP $\beta$  est nécessaire *in vitro* pour la différenciation cellulaire des adipocytes (Tanaka et al., 1997,

Tang et al., 2005, Tang & Lane, 1999, Tang et al., 2003), les résultats *in vivo* obtenus dans l'étude de Tanaka et al. (1997) démontrent que la synergie C/EBP $\beta$  et C/EBP $\delta$  est nécessaire pour le développement normal du tissu adipeux. C/EBP $\beta$  est également régulé de manière post-translacionnelle afin de réguler son activité. Dans les premières heures de la différenciation, C/EBP $\beta$  est dans un premier temps (environ 4h) phosphorylé par les MAP kinases sur la Thr188. Cette phosphorylation, qui n'est pas suffisante pour augmenter son activité transcriptionnelle, est suivie par une phosphorylation de GSK-3 $\beta$  sur la Ser184 et/ou sur la Thr179 aux alentours de 12 à 16h après induction de la différenciation (Kim et al., 2007, Tang et al., 2005). Ces phosphorylations successives permettent de changer la conformation spatiale de C/EBP $\beta$ , augmentant ainsi son activité transcriptionnelle (Kim et al., 2007, Vinson et al., 1989). En ce qui concerne C/EBP $\alpha$ , le phénotype de la souris KO C/EBP $\alpha$  est bien plus sévère que celle de la souris C/EBP $\beta$ . Ces souris ne sont pas capables d'accumuler des lipides dans le tissu adipeux et meurent prématurément. Différentes études ont donc démontré, grâce à ces souris, que C/EBP $\alpha$  était nécessaire et essentiel pour la différenciation des préadipocytes en tissu adipeux blanc (Linhart et al., 2001, Wu et al., 1999). Si C/EBP $\beta$  et C/EBP $\delta$  sont activés rapidement, C/EBP $\alpha$  augmente son activité plus tardivement après l'initiation de la différenciation (Cao et al., 1991). L'étude de Yeh et al. (1995) a également mis en lumière que C/EBP $\beta$  et C/EBP $\delta$  étaient responsables de l'augmentation de l'expression de C/EBP $\alpha$  et qu'une fois activé, C/EBP $\alpha$  pouvait maintenir lui-même son activité transcriptionnelle (Christy et al., 1991). CHOP10 possède le rôle inverse de ses homologues C/EBPs. En effet, CHOP10 joue le rôle d'un dominant négatif pour C/EBP $\beta$  et inhibe donc l'adipogenèse (Batchvarova et al., 1995, Ron & Habener, 1992). CHOP10 est donc fortement exprimé dans des préadipocytes dont le cycle cellulaire est en arrêt en phase G1 (Tang & Lane, 2000).

Malgré les nombreux facteurs de transcription régulant l'adipogenèse, incluant ceux cités auparavant, la littérature scientifique a également démontré qu'un seul facteur de transcription était absolument nécessaire pour l'adipogenèse : PPAR $\gamma$ . PPAR $\gamma$  est un membre de la superfamille des récepteurs nucléaires hormonaux requis pour le développement des adipocytes. Une délétion de PPAR $\gamma$  chez la souris résulte en une dysfonction placentaire et une létalité observée chez l'embryon (Barak et al., 1999, Rosen et al., 1999). Il existe 3 isoformes de PPAR $\gamma$  (1, 2 et 3) qui sont transcrits par le même gène (Fajas et al., 1998, Zhu et al., 1995) et l'isoforme PPAR $\gamma$ 2 représente celle spécifique à l'adipocyte (Tontonoz & Spiegelman, 2008). PPAR $\gamma$ 2 représente donc le « master regulator » du programme

adipogénique et l'augmentation de son expression dans des préadipocytes ou fibroblastes *in vitro* promeut l'expression des gènes adipogéniques ainsi que la différenciation en adipocytes (Egan et al., 1992, Tontonoz et al., 1994, Tontonoz & Spiegelman, 2008). L'étude de He et al. (2003) a supprimé spécifiquement l'expression de PPAR $\gamma$  dans le tissu adipeux et démontré une forte diminution de la taille du tissu adipeux alors qu'une délétion globale engendre une létalité de l'embryon. Comme indiqué ci-dessus, C/EBP $\beta$  et C/EBP $\delta$  sont rapidement exprimés après l'induction de la différenciation et, dans un délai d'environ 16h, C/EBP $\beta$  active de manière coordonnée l'expression de PPAR $\gamma$ 2 et C/EBP $\alpha$  qui à leur tour vont activer un large groupe de gènes impliqués dans la maturation et le phénotype de l'adipocyte (Davis & Zur Nieden, 2008, Morrison & Farmer, 1999, Tang & Lane, 1999).

Une fois les adipocytes matures formés, de nombreux marqueurs sont utilisés dans la littérature afin de quantifier leur présence, notamment au sein du tissu musculaire dans l'optique de quantifier les IMAT. Le transporteur d'acides gras FABP4, le régulateur de la lipolyse périlipine, ou encore de nombreuses adipokines comme la leptine, adiponectine ou adiposine sont ainsi couramment utilisés (Heredia et al., 2013, Lukjanenko et al., 2013, Pisani et al., 2010a, Uezumi et al., 2014a, Uezumi et al., 2010).

### **3.5. IMAT et fibrose : signe d'une régénération musculaire perturbée**

#### **3.5.2. Perturbation de la régénération musculaire avec l'avancée en âge**

Le déconditionnement musculaire apparaissant avec l'âge est caractérisé par une diminution de la masse musculaire, de la force musculaire, de l'endurance, du potentiel de régénération et est accompagné d'une augmentation de la quantité d'IMAT, le tout étant considéré comme un syndrome gériatrique appelé sarcopénie (Cruz-Jentoft et al., 2010, Fielding et al., 2011, Morley et al., 2011, Muscaritoli et al., 2010). Le terme dynapénie est également employé afin de décrire la diminution de force et de fonction musculaire avec l'avancée en âge, tout en prenant en compte les altérations de l'ensemble du système neuromusculaire (Clark & Manini, 2008, Clark & Manini, 2012, Manini & Clark, 2012).

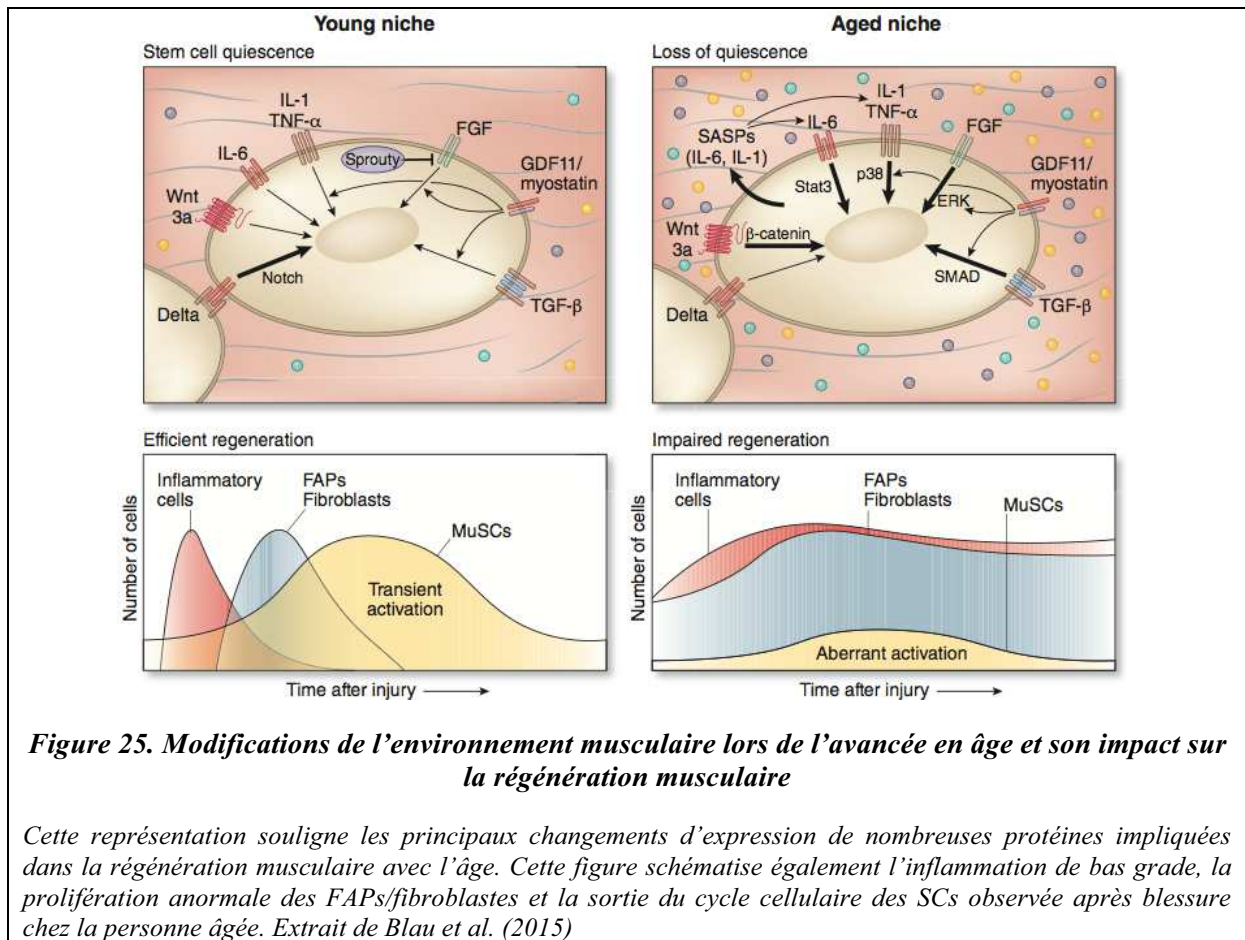
La perte du potentiel de régénération musculaire, qui nous intéresse particulièrement dans cette partie, peut être attribué à de nombreux facteurs incluant les SCs et le système immunitaire. En effet, concernant les SCs, il apparaît que le pool de SCs est maintenu jusqu'à 70 ans (Dreyer et al., 2006, Roth et al., 2000) puis décline considérablement (Kadi et al., 2004a, Renault et al., 2002, Sajko et al., 2004, Verdijk et al., 2014). Ce déclin étant plus



marqué pour les SCs étant situées sous la lame basale des fibres de type II (Verdijk et al., 2012, Verdijk et al., 2007, Verdijk et al., 2014), ce qui coïncide parfaitement avec le développement préférentiel de la sarcopénie pour les fibres de type II. Il est également important de noter que l'avancée en âge est associée avec une capacité altérée de prolifération des SCs (Barani et al., 2003, Conboy et al., 2003, Schultz & Lipton, 1982, Suetta et al., 2013) logiquement concomitante avec la perturbation retrouvée de la capacité de régénération (Collins-Hooper et al., 2012, Gallegly et al., 2004, Marsh et al., 1997, Renault et al., 2002). De nombreuses études basées sur des expérimentations de parabiose et de greffes musculaires ont montré que ce déclin de la fonction des SCs observé avec l'avancée en âge était largement attribuable à des facteurs extrinsèques, donc à des facteurs liés à l'environnement musculaire (Brack et al., 2007, Carlson & Faulkner, 1989, Conboy et al., 2005, Harrison, 1983, Villeda et al., 2011). Cependant, des études plus récentes, utilisant des techniques plus puissantes et plus spécifiques d'isolation des SCs ont aussi confirmé des altérations intrinsèques (Bernet et al., 2014, Chakkalakal et al., 2012, Cosgrove et al., 2014, Price et al., 2014, Sousa-Victor et al., 2014). La récente revue de littérature publiée par Brack & Munoz-Canoves (2015) souligne l'importance de l'hétérogénéité des SCs (détaillée dans la partie 2.3.3.) comme la principale cause des divergences retrouvées dans la littérature concernant l'altération de la fonction des SCs avec la sarcopénie. Ainsi, les différentes sous-populations de SCs sont différentiellement sensibles aux facteurs intrinsèques et extrinsèques sondés dans ces études. Enfin, malgré ce déclin de la fonction des SCs avec l'âge, la littérature récente semble prouver que les SCs ne possèdent qu'un lignage myogénique, ne permettant donc pas de contribuer au développement d'IMAT observé avec l'avancée en âge (Blau et al., 2015, Sciorati et al., 2015). Par contre, cet environnement défavorable pour une régénération musculaire pourrait potentiellement impacter d'autres cellules souches, comme les FAPs par exemple, qui sont reconnues pour s'engager vers l'adipogenèse dans des conditions défavorables.

Malgré le potentiel adipogénique reconnu des FAPs dans la littérature, aucune étude n'a pour l'instant caractérisé un rôle, pourtant fortement probable, des FAPs dans le phénomène d'apparition d'infiltrations graisseuses avec l'avancée en âge (Blau et al., 2015, Farup et al., 2015b) (**Fig.25**). De plus, deux études réalisées *in vitro* ont démontré que les adipocytes dérivés de FAPs isolés du tissu musculaire chez l'homme sont insulino-résistants et induisent également, par des expérimentations de co-culture, une insulino-résistance des fibres musculaires (Arrighi et al., 2015, Laurens et al., 2015). Ces études supportent donc, d'une part, les corrélations observées entre l'accumulation d'IMAT et diminution de la sensibilité à

l'insuline chez l'homme (Goodpaster et al., 1999, Goodpaster et al., 2000a, Goodpaster et al., 1997, Ryan & Nicklas, 1999, Simoneau et al., 1995), et d'autre part, le fait que les FAPs puissent être la population responsable du développement d'IMAT dans la sarcopénie. Même si aucune étude n'est disponible concernant les mécanismes cellulaires et moléculaires menant à l'apparition d'infiltrations graisseuses avec l'avancée en âge, de nombreuses pistes peuvent être soulevées.



De nombreuses études du même groupe ont évalué l'effet d'inhibiteurs d'histone déacetylases (HDACi) sur des souris *mdx*. Ces chercheurs ont montré qu'un traitement avec des HDACi sur des souris *mdx* permettait un recouvrement morphologique et fonctionnel du muscle strié squelettique, certainement médié par une augmentation de l'expression de la FSTN (Minetti et al., 2006). Ce groupe a ensuite montré que, chez des jeunes souris *mdx*, les FAPs étaient sensibles à une inhibition de l'adipogenèse provoquée par ce traitement aux HDACi et que ces FAPs supportaient donc la myogenèse (Mozzetta et al., 2013). Cependant, ces auteurs ont également mis en lumière que le traitement avec les HDACi n'était pas efficace chez des souris *mdx* plus âgées. De plus, une autre étude a aussi démontré qu'une intervention épigénétique, encore avec des HDACi, en plus d'inhiber le potentiel

adipogénique des FAPs, pouvait de manière très surprenante promouvoir un lignage myogénique (Faralli & Dilworth, 2014, Saccone et al., 2014). Un résultat de nouveau non retrouvé chez des souris *mdx* plus âgées mettant en avant le fait que les FAPs sont fortement influencés par leur environnement et notamment dans le cas du vieillissement. Il est quand même très important de noter que la population de FAPs isolée dans ces études n'était pas pure et pourrait également contenir des PICs ou des péricytes de type 1. Ce point méthodologique explique donc le développement des FAPs vers un lignage myogénique dans cette étude qui n'a été retrouvé dans aucune étude auparavant.

La voie Notch semble aussi être impliquée dans le possible rôle des FAPs dans le développement des IMAT avec l'avancée en âge. Cette voie, détaillée dans la partie 2.3.2., est majoritairement impliquée dans la maintenance dans un état quiescent des SCs et dans l'augmentation des processus de prolifération après blessure (Bjornson et al., 2012, Conboy et al., 2003, Mourikis et al., 2012, Pellegrinet et al., 2011, Wen et al., 2012). Cependant, avec l'âge, une atténuation de la réponse de la voie Nocth résulte en une inhibition de la prolifération des SCs et ainsi une altération de la régénération musculaire. De manière intéressante, l'expression de TGF- $\beta$  qui est un inhibiteur de la voie Notch est augmentée avec l'avancée en âge (Buas & Kadesch, 2010, Carlson et al., 2008, Carlson et al., 2009) et peut expliquer, dans un contexte dénué de blessure musculaire, l'entrée spontanée dans le cycle cellulaire des SCs et ainsi une diminution du pool de SCs (Conboy et al., 2003, Conboy et al., 2005). De plus, deux études ont également démontré qu'une altération de la voie Notch dans le muscle cardiaque entraînait un développement anormal de fibrose (Croquelois et al., 2008, Nemir et al., 2014). Ces études ont donc démontré et conclu que la voie Notch régulait des mécanismes clés concernant les progéniteurs cardiaques d'origine mésenchymateuse et contrôlait donc la balance entre le développement de fibrose et la régénération cardiaque. Cependant, aucune étude n'est disponible concernant la voie Notch, les FAPs et la sarcopénie afin d'identifier un rôle de cette voie dans la régénération perturbée par l'avancée en âge.

De très nombreuses études ont également déjà prouvé le rôle majeur de la voie des Wnt dans l'inhibition de la différenciation des MSCs en adipocytes en plus de la promotion de leur potentiel myogénique (Bennett et al., 2002, Brunt et al., 2012, Chung et al., 2012, Moldes et al., 2003, Ross et al., 2000, Shang et al., 2007, Zaragosi et al., 2008). L'étude de Vertino et al. (2005) a particulièrement souligné le rôle de Wnt10b et a conclu que, lors de l'avancée en âge, une altération de l'expression de Wnt10b augmentait l'expression de gènes clés

impliqués dans l'adipogenèse. D'autres études sont nécessaires pour élucider le rôle des Wnt, une voie très complexe, sur le développement des IMAT durant la sarcopénie.

La sarcopénie est également associée à une altération de la synthèse de NO (nitric oxide) dans le muscle squelettique (Nyberg et al., 2012, Samengo et al., 2012). Une étude menée par Cordani et al. (2014) a démontré, *in vitro*, que le NO inhibait la différenciation en adipocytes des FAPs et soulève ainsi un nouveau mécanisme potentiel par lequel les FAPs pourraient mener au développement d'IMAT chez la personne âgée.

Enfin, le facteur de croissance FGF2 (fibroblast growth factor 2) semble jouer un rôle dans l'altération de la régénération musculaire liée à l'âge. L'expression de FGF2 augmente avec l'avancée en âge, notamment à cause d'une diminution de l'expression de son inhibiteur sprouty-1, et ce mécanisme induit une sortie des SCs de leur état de quiescence, menant donc à leur entrée dans le cycle cellulaire et engendrant une baisse du nombre de SCs (Chakkalakal et al., 2012). Certaines études ont montré que FGF2 possédait également une fonction dans la différenciation des MSCs (Bae et al., 2015, Cai et al., 2013, Lee et al., 2013). D'autres études sont donc nécessaires pour déterminer quel pourrait être le rôle de FGF2 sur les FAPs.

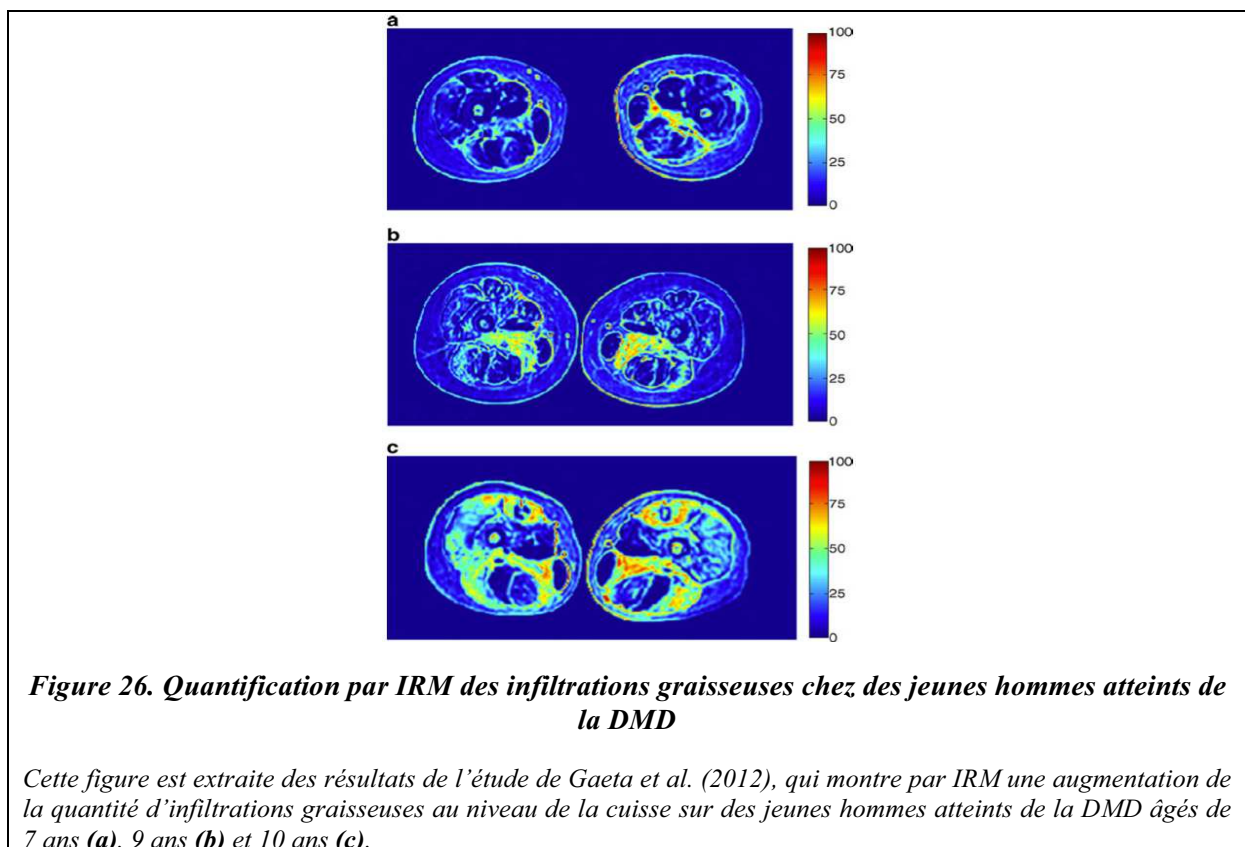
### 3.5.3. Altération du recrutement et de la fonction des macrophages

Comme développé dans la partie 2.5., le système immunitaire joue un grand rôle dans les processus de régénération musculaire. Les macrophages sont particulièrement impliqués et leur recrutement est essentiel dans le bon déroulement de la régénération (Chazaud, 2016). En effet, à la suite d'une blessure impliquant une nécrose des fibres musculaires lésées, des chimiokines de la famille des CC chimiokines (CCL2/3/4 et 5) sont principalement impliquées dans le recrutement des macrophages du système vasculaire au niveau du site de la lésion. Le couple CCL2 et son récepteur CCR2 représente le système de recrutement des macrophages le plus étudié. Malgré le fait que la source de CCL2 après une blessure musculaire reste à élucider, de nombreuses études ont déjà démontré qu'une perturbation de cet axe engendrait une altération majeure de la régénération musculaire (Contreras-Shannon et al., 2007, Ochoa et al., 2007, Shireman et al., 2007, Sun et al., 2009). D'autres études ont directement montré qu'une déplétion spécifique des macrophages (Arnold et al., 2007, Summan et al., 2006), ou une perturbation de leur infiltration via le récepteur M-CSFR (Segawa et al., 2008), perturbait également fortement la régénération musculaire. Parmi l'ensemble de ces études altérant la fonction et le recrutement des macrophages, de nombreuses ont retrouvé un développement majeur d'IMAT, accompagnant la perturbation de

la régénération musculaire, à la suite de l'utilisation de différents modèles de régénération : blessure par le froid (Summan et al., 2006), ischémie (Contreras-Shannon et al., 2007, Shireman et al., 2007), injection de NTX (Arnold et al., 2007) ou encore de CTX (Segawa et al., 2008). Prises dans leur ensemble, ces études semblent démontrer que, malgré les différents modèles expérimentaux utilisés, une altération de la cinétique d'apparition sur le site lésé et/ou de la fonction des macrophages est concomitante avec l'apparition des infiltrations graisseuses lors de la régénération.

#### 3.5.4. Exemple de la dystrophie de Duchenne

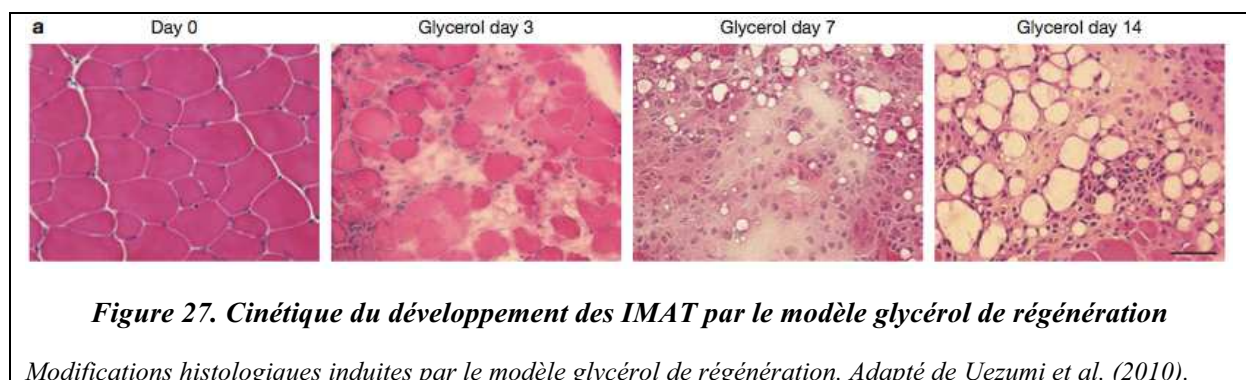
Les patients et/ou souris présentant une déficience en dystrophine, représentant la DMD, sont le parfait exemple d'un tissu musculaire pour lequel les mécanismes de régénération sont complètement perturbés. Cette maladie se caractérise par des cycles continus de dégénérescence/régénération et un épuisement progressif du potentiel de régénération. La principale caractéristique d'une régénération perturbée est représentée par le développement anormal de fibrose et d'infiltrations graisseuses et l'étude de Gaeta et al. (2012) a même montré que les IMAT pouvaient occuper jusqu'à 50% du volume musculaire chez des jeunes hommes atteints de la DMD (**Fig.26**). Les IMAT ont même été décrits comme permettant une mesure fiable de la sévérité de la DMD (Wren et al., 2008).



Une autre caractéristique d'un muscle déficient en dystrophine est la perturbation des mécanismes inflammatoires avec une invasion massive du tissu musculaire par les macrophages (Spencer et al., 2001, Spencer & Tidball, 2001, Wehling et al., 2001). La récurrence des lésions induites par l'absence de dystrophine perturbe grandement la réponse des macrophages et notamment le temporalité du recrutement et de leur shift phénotypique. Ainsi, les mécanismes cellulaires et moléculaires intervenant en réponse à une blessure musculaire se répètent indéfiniment et se confondent, donnant une réponse temporelle du système immunitaire complètement désordonnée. Les macrophages, des deux phénotypes, sont présents en grand nombre et contribuent ainsi à la physiopathologie de la souris *mdx* (Lemos et al., 2015, Mojumdar et al., 2014, Villalta et al., 2014). Les infiltrations graisseuses, omniprésentes dans le tissu musculaire déficient en dystrophine, peuvent donc être, au regard des nombreuses études attestant du développement d'IMAT lors d'une perturbation du bon fonctionnement du système immunitaire, le résultat d'une dérégulation de la fonction des macrophages. De plus, une étude récente a démontré que des souris *mdx* déficientes en CCR2 présentaient une diminution du nombre de macrophages associé à une forte amélioration du phénotype musculaire.

### 3.5.5. Le modèle glycérol : pourquoi davantage d'IMAT ?

Si la plupart des modèles de régénération utilisés chez le petit rongeur ne permettent pas (dans un état normal) le développement et l'accumulation d'infiltrations graisseuses, le modèle glycérol est bien connu pour permettre leur apparition (Pisani et al., 2010a) (**Fig.27**). Si le résidu glycérol constitue l'articulation centrale de tous les lipides de la classe des triglycérides et des phosphoglycérides, aucune étude n'est cependant disponible dans la littérature pour expliquer ce phénomène.



L'étude de Uezumi et al. (2010) a parfaitement mis en exergue le fait que les FAPs sont les cellules souches à l'origine des IMAT dans le modèle glycérol, et cette étude a également

montré que cette différenciation des FAPs en adipocyte était bien dépendante de l'environnement musculaire et donc du type de blessure. En effet, les auteurs ont isolé des FAPs (notamment caractérisés par l'expression de PDGFR $\alpha$ ) à la suite d'une blessure induite par de la CTX et du glycérol et ont injecté ces derniers dans l'autre système de blessure. Les FAPs isolés d'un muscle lésé par le modèle glycérol ne se différencient pas en adipocytes une fois injectés dans un muscle précédemment injecté avec de la CTX, et à l'inverse les FAPs isolés après injection de CTX se différencient en adipocytes une fois injectés dans un muscle lésé par le modèle glycérol. Ces résultats confirment donc que le modèle glycérol induit un environnement musculaire défavorable et que les FAPs qui se différencient donc en adipocytes contribuent au développement d'IMAT. Au regard des nombreuses études ayant montré l'apparition d'infiltrations graisseuses avec une altération du recrutement et de la fonction des macrophages (voir le 3.3.3.), les auteurs de l'étude de Pisani et al. (2010a) discutent de la potentielle dérégulation de la fonction des macrophages dans ce modèle spécifique de régénération menant à l'apparition d'IMAT.

De manière intéressante, l'étude de Lukjanenko et al. (2013) a confirmé cette hypothèse en comparant, grâce à des analyses transcriptomiques et histologiques, le modèle de régénération utilisant le glycérol à celui de la CTX. Cette étude démontre dans un premier temps que les processus de dégénérescence et de régénération semblaient similaires dans les deux modèles mais que les IMAT étaient beaucoup plus présents et persistants dans le modèle glycérol. Ces travaux démontrent également que la réponse anti-inflammatoire, médiée principalement par les macrophages de type M2, est exprimée beaucoup plus tôt dans le modèle glycérol. Ce résultat permet d'émettre l'hypothèse d'une altération du bon fonctionnement des macrophages et notamment du timing du shift phénotypique des macrophages, ici trop précoce, perturbant les processus de régénération dans le modèle glycérol et favorisant ainsi le développement d'IMAT.

### **3.6. L'activité physique comme principale mesure pour lutter contre l'apparition d'IMAT**

Malgré le fait que les IMAT soient fortement liés à la sarcopénie et à l'obésité, les études suivantes ont également suggéré que l'accumulation d'IMAT pouvait, dans un contexte dénué de mécanismes de régénération, être le résultat d'une inactivité musculaire plus que de l'avancée en âge. En effet, l'étude de Manini et al. (2007) a démontré que seulement 4 semaines d'inactivité induisaient une accumulation d'IMAT, de l'ordre de 15% pour la cuisse

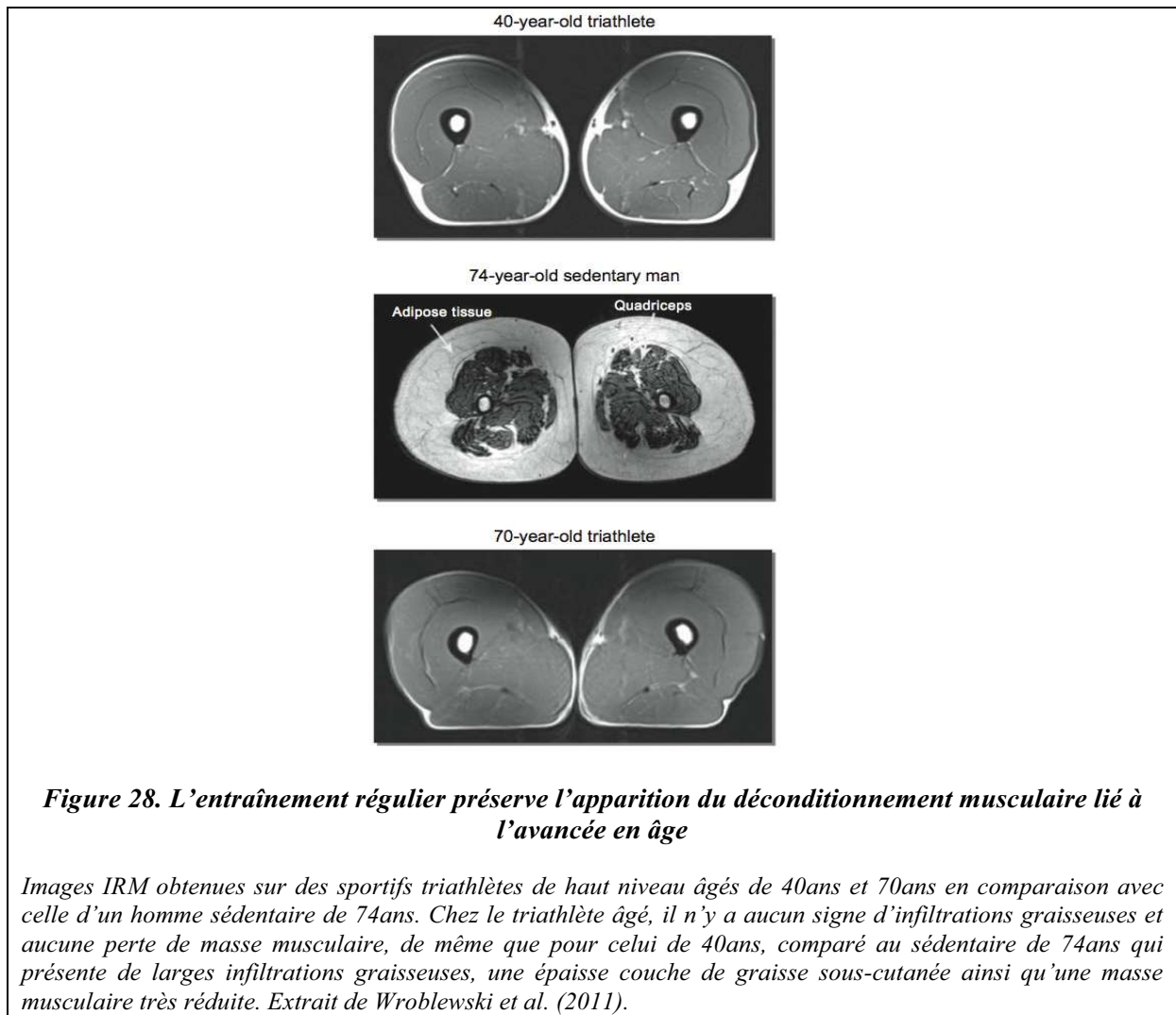
et 20% pour le mollet, chez des sujets jeunes en bonne santé. De plus, une étude longitudinale étalée sur 32 ans impliquant des jumeaux, a également démontré que le jumeau inactif avait une augmentation de 54% du volume occupé par les IMAT en comparaison avec son jumeau physiquement actif (Leskinen et al., 2009). Enfin, l'étude de Tuttle et al. (2011) a également observé une corrélation entre le nombre de pas moyen par jour et l'accumulation d'IMAT. Toutes ces études soulignent donc le rôle primordial que semble avoir l'inactivité sur le développement des infiltrations graisseuses liée à l'avancée en âge.

A ma connaissance, l'étude réalisée par Goodpaster et al. (2008) a été la première à détailler de manière précise l'effet d'une augmentation de l'activité physique pendant 1 an chez des personnes âgées. Cette étude a démontré un effet protecteur de cette activité physique contre le développement d'IMAT mais aussi face à une diminution de la force produite par les muscles extenseurs du genou. D'autres travaux, publiés pratiquement en même temps par Marcus et al. (2008), ont révélé des données similaires avec soit un entraînement combiné en aérobie et en résistance, soit un entraînement uniquement en aérobie, sur des personnes atteintes de diabète de type 2. Deux ans plus tard, ce même groupe a ensuite investigué les effets de 12 semaines d'entraînement en résistance chez la personne âgée et a reporté une diminution de 11% des IMAT observés dans la cuisse (Marcus et al., 2010). Dans des conditions d'augmentation d'activité physique quasi-similaires, Ryan et al. (2011) ont aussi observé une diminution de la présence d'IMAT chez des personnes âgées ayant survécu à un AVC (accident vasculaire cérébral). Une autre étude a également examiné les effets de 8 mois d'exercice en endurance sur des sujets sédentaires et montré une diminution de l'accumulation d'IMAT (Durheim et al., 2008). Deux études différentes ont aussi prévenu le développement d'IMAT avec l'âge soit avec un programme de diète et d'exercice modéré étalé sur 1 an chez des personnes en surpoids ou obèses atteintes de diabète de type 2 (Gallagher et al., 2014), soit par un programme plus intensif d'exercice physique mais plus court chez des personnes âgées sujettes aux chutes (Jacobs et al., 2014). Une étude plus récente a également retrouvé une diminution de l'aire occupée par les IMAT après 5 mois d'entraînement en résistance modéré chez des sujets âgés en surpoids (Nicklas et al., 2015). Les travaux de Taaffe et al. (2009) ont observé une augmentation du contenu en IMAT chez des personnes âgées à la suite d'une session de « désentraînement » suivi d'une diminution de ces niveaux d'IMAT après un réentraînement en résistance. Enfin, l'étude très intéressante menée par Wroblewski et al. (2011) sur des athlètes âgés de haut niveau, entraînés pour différentes compétitions au moins 4 ou 5 fois par semaine, a montré que ces



athlètes ne présentaient aucune diminution de masse musculaire ni aucune augmentation du contenu en IMAT avec l'âge (**Fig.28**). Ces deux dernières études, en plus de montrer, comme toutes les autres, que l'augmentation de l'activité physique peut diminuer l'accumulation d'IMAT, soulignent que les altérations musculaires observées durant la sarcopénie peuvent être en grande partie une conséquence directe d'une diminution progressive de l'activité physique chez les personnes âgées. Prises dans leur ensemble, toutes ces études démontrent et révèlent l'impact positif d'une augmentation de l'activité physique, et au contraire de l'effet négatif de l'inactivité, sur le développement des IMAT avec l'avancée en âge. Si toutes ces études possèdent un impact non négligeable sur le rôle de l'activité physique sur l'apparition des IMAT, aucune ne soulève de mécanismes cellulaires ou moléculaires impliqués. Clairement, des études sont nécessaires afin de comprendre par quels mécanismes l'absence ou au contraire l'application de contraintes mécaniques sur le tissu musculaire module le développement des IMAT.

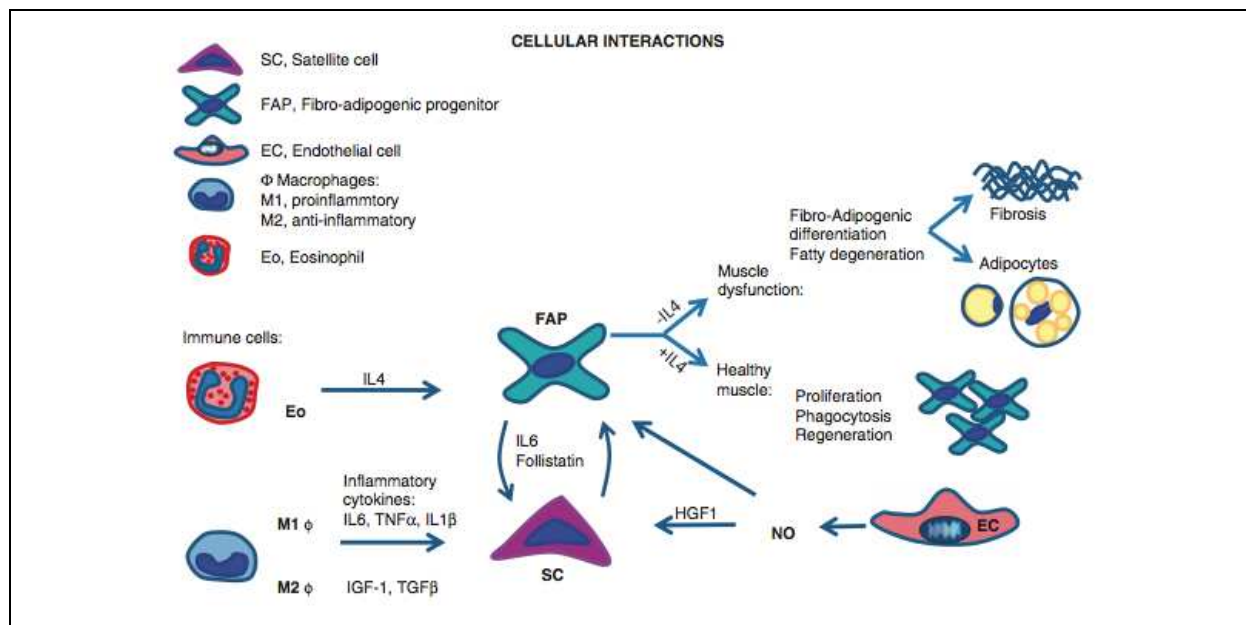
Souvent associé à une augmentation de l'activité physique, l'impact des régimes restrictifs sur le contenu en IMAT chez des personnes obèses a également été étudié (Avila et al., 2010, Christiansen et al., 2009, Gallagher et al., 2014, Gower & Goss, 2015, Mojtahedi et al., 2011, Ryan & Harduarsingh-Permaul, 2014, Ryan & Nicklas, 1999). L'ensemble de ces études indique clairement i) qu'un régime hypo-calorique permet de diminuer le contenu en IMAT et que ii) un régime hypo-calorique riche en protéines ou pauvre en carbohydrates permet également de diminuer le contenu en infiltrations graisseuses (Gower & Goss, 2015, Mojtahedi et al., 2011). D'autres études sont nécessaires afin de déterminer la meilleure stratégie nutritionnelle (couplée, de manière optimale, avec une augmentation de l'activité physique) afin d'optimiser la réduction du contenu en IMAT chez les personnes obèses mais également afin de limiter le développement des IMAT dans de nombreuses situations de déconditionnement musculaire, y compris dans le contexte de la sarcopénie.



### 3.7. Interactions FAPs et cellules satellites/immunitaires

#### 3.7.1. Un système complexe pour une régénération efficace

Si dans un contexte dénué de régénération musculaire les mécanismes cellulaires et moléculaires modulant le développement d'IMAT restent complètement à élucider, ceux impliqués lors de la régénération ont été plus largement étudiés. L'interaction entre FAPs et cellules satellites/immunitaires semble être un trio indispensable pour une régénération efficace (**Fig.29**). En effet, si les FAPs sont une population de cellules souches capables d'aider à la régénération musculaire, c'est avant tout grâce aux différentes interactions, souvent par l'intermédiaire de cytokines, qu'elles entretiennent tout au long du processus de régénération avec les différents acteurs présents dans l'environnement musculaire. Ces différentes interactions permettent dans un premier temps aux FAPs de proliférer et d'aider à la régénération musculaire et, dans un second temps, permettent aux FAPs de rentrer dans une phase apoptotique inhibant leur potentiel adipogénique.



**Figure 29. Illustration des différentes interactions entre cellules immunitaires, endothéliales, satellites et FAPs lors de la régénération musculaire**

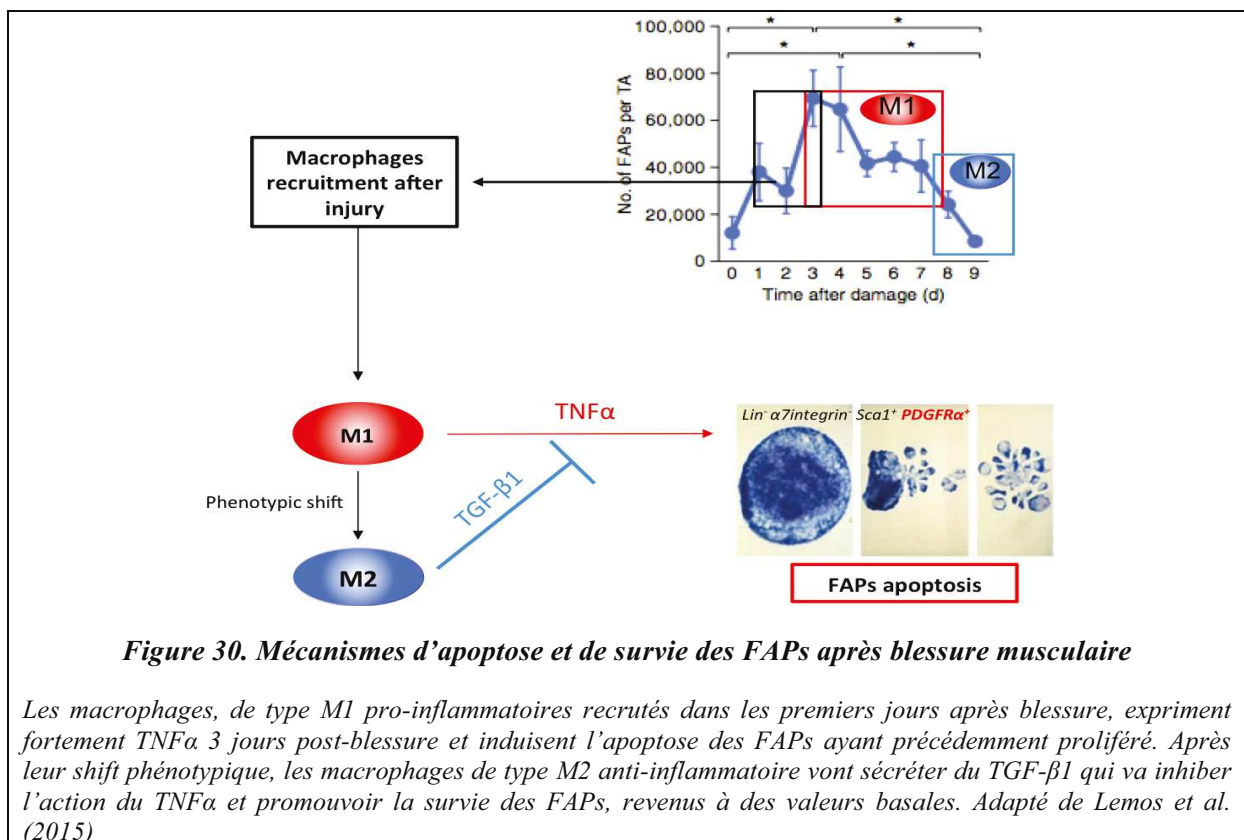
Ce schéma représente les différents acteurs de la régénération musculaire et les différentes cytokines impliquées. Il est à noter que l'action des macrophages sur les FAPs, via le TNF $\alpha$  et le TGF- $\beta$ 1 n'est pas illustrée ici (mécanismes connus à posteriori et présentés ci-après). Extrait de Farup et al. (2015b).

Tout d'abord, des évidences ont été démontrées *in vitro* concernant les interactions entre les FAPs et les différents acteurs cellulaires de la régénération. L'étude de Uezumi et al. (2010) a montré que les SCs étaient capables d'inhiber, par interaction directe, l'adipogenèse des FAPs. Alors que de son côté, l'étude de Joe et al. (2010) a mis en lumière la capacité des FAPs à augmenter la différenciation des SCs de manière dose dépendante. Ces travaux ont également souligné que les FAPs, après avoir proliféré, étaient capables de sécréter des facteurs clés concernant la myogenèse tel que IL-6 et IGF-1. Les travaux réalisés par Mozzetta et al. (2013) ont aussi confirmé le rôle important des FAPs dans la myogenèse en situation de co-culture. Cette étude a par ailleurs démontré que la Fstn, exprimée par les FAPs, était essentielle pour médier l'effet de FAPs sur les SCs. Concernant les macrophages, les premières expérimentations réalisées *in vitro* ont tout d'abord démontré que la prolifération et la différenciation des myoblastes était augmentée en condition de co-culture. Ce lien est également retrouvé avec des expérimentations utilisant le milieu de culture suggérant donc un effet de différentes substances sécrétées par les macrophages (Cantini & Carraro, 1995, Cantini et al., 1994, Saclier et al., 2013). Deux études majeures ont par la suite établi le rôle primordial de certaines cytokines sur les FAPs lors de la régénération musculaire *in vivo*.

L'étude de Heredia et al. (2013) a démontré que l'IL-4 (et l'IL-13) sécrétée par les éosinophiles dans les premières heures/jours de la régénération musculaire était essentielle. En effet, l'IL-4 stimule la prolifération des FAPs *in vivo* et les auteurs ont montré, grâce à différents inhibiteurs et des souris KO IL-4R $\alpha$  et STAT6, que cet effet passait via STAT6. Enfin ces travaux ont également démontré que l'IL-4 inhibait la différenciation des FAPs en adipocytes. Après avoir montré que cette inhibition nécessitait le récepteur à l'IL-4 et STAT6 *in vitro*, les auteurs ont mis en lumière le rôle primordial de l'IL-4 *in vivo* pour prévenir l'apparition d'IMAT. Dans un modèle de régénération avec de la CTX, pour lequel il n'y a pas ou très peu d'IMAT, les souris KO pour l'IL-4R $\alpha$  et STAT6 présentaient de larges infiltrations graisseuses. Au contraire, avec l'utilisation du modèle glycérol permettant le développement d'IMAT, deux injections intra-péritonéales d'IL-4 à 1 et 4 jours après lésion ont suffi pour diminuer le développement des IMAT ainsi que l'expression de différents marqueurs clés d'adipocytes matures. Cette étude clé a donc démontré de manière rigoureuse que l'IL-4, sécrétée par les éosinophiles lors de la régénération, avait un rôle primordial dans le devenir des FAPs lors de la régénération musculaire.

Si l'étude de Heredia et al. (2013) a souligné les acteurs permettant la prolifération des FAPs et l'inhibition de leur potentiel adipogénique, celle de Lemos et al. (2015) a par contre mis en lumière les acteurs régulant leur apoptose. Ces auteurs ont d'abord quantifié la présence des FAPs durant les neuf premiers jours après une injection de CTX. Ces expérimentations ont révélé, de manière cohérente avec les travaux de Joe et al. (2010) que les FAPs proliféraient et atteignaient un pic entre le 3<sup>ème</sup> et le 4<sup>ème</sup> jour avant de rapidement rentrer dans une phase de mort programmée (d'apoptose) pour revenir à des valeurs basales entre 7 et 9 jours. Cette étude a ensuite montré que le TNF $\alpha$ , sécrété par les macrophages de type M1, était essentiel pour induire l'apoptose des FAPs. Après avoir proliféré et aidé à la prolifération des SCs, les FAPs rentrent donc, à partir du 3<sup>ème</sup> jour de la régénération, dans une phase d'apoptose qui permet alors de prévenir l'excès de fibrose et hypothétiquement d'IMAT. Cette étude a également montré que TGF- $\beta$ 1, sécrété par les macrophages de type M2, permettait au contraire de promouvoir la survie cellulaire des FAPs et inhibait donc l'action du TNF $\alpha$ . Un traitement avec du TGF- $\beta$ 1 pendant le pic d'expression du TNF $\alpha$  réduit l'apoptose des FAPs alors que l'inhibition de TGF- $\beta$ 1 permet l'effet inverse. Cette étude permet donc d'affirmer que le recrutement et la temporalité du shift phénotypique des macrophages durant la régénération musculaire permet de passer d'un environnement riche en TNF $\alpha$  à un environnement riche en TGF- $\beta$ 1, permettant ainsi de moduler la réponse des

FAPs. Enfin, après avoir révélé le potentiel pro-apoptotique du TNF $\alpha$  sécrété par les M1 macrophages sur les FAPs, les travaux réalisés dans cette étude ont également vérifié le rôle du lien entre macrophages et FAPs chez la souris *mdx*. De manière très intéressante, les macrophages de type M1 et M2 coexistent (35% de type M1) dans le tissu musculaire de la souris *mdx* et ainsi, dans ce modèle de blessure chronique, les macrophages seraient présents dans un état non fonctionnel. Cette étude a ainsi vérifié cette hypothèse en observant, en co-culture, que les macrophages purifiés chez la souris *mdx* échouaient à provoquer l'apoptose des FAPs. Les auteurs ont donc conclu que la transition d'un environnement riche en TNF $\alpha$  puis en TGF- $\beta$  observée dans un modèle de régénération classique n'était pas respectée avec des dommages chroniques, et que la présence simultanée des deux types de macrophages, et des deux types de cytokines, permettait la survie des FAPs et l'apparition de fibrose/IMAT. Cette étude a, pour finir, traité des souris *mdx* (qui subissaient en plus des micro-lésions de manière journalière) avec un inhibiteur de TGF- $\beta$  (nilotinib) et observé une augmentation de l'apoptose des FAPs et une diminution de l'expression du collagène de type I. Pour conclure, cette étude a donc mis en lumière le rôle majeur du TNF $\alpha$ , sécrété par les M1 macrophages, et du TGF- $\beta$ 1, sécrété par les M2 macrophages, dans le processus d'apoptose des FAPs nécessaire pour une régénération sans développement de fibrose et probablement d'IMAT (Fig.30).



### 3.7.2. Effets d'une modulation de l'activité contractile ?

Les effets de l'activité physique sur l'organisme entier ou directement sur le tissu musculaire, que ce soit un exercice aigu ou un entraînement régulier, sont déjà très bien caractérisés dans la littérature scientifique. D'excellentes revues de littérature très récentes résument de manière complète ces effets (Allen et al., 2015, Atherton et al., 2015, Atherton & Smith, 2012, Booth et al., 2015, Egan & Zierath, 2013, Ferraro et al., 2014, Gorgens et al., 2015, Hargreaves, 2015, Hawley et al., 2014, Hood et al., 2015, Kjaer et al., 2015, McGlory & Phillips, 2015, Mul et al., 2015, Russell & Lamon, 2015, Tsiloulis & Watt, 2015).

Par contre, en ce qui concerne les effets de l'exercice sur les cellules souches d'origine mésenchymateuse (incluant les FAPs), les études sont encore peu nombreuses (Boppert et al., 2015). L'étude de Valero et al. (2012) a mis en évidence que la quantité de cellules souches localisées dans l'espace interstitiel, près des vaisseaux sanguins et des terminaisons nerveuses identifiées comme étant Sca1<sup>+</sup>CD45<sup>-</sup> (incluant les FAPs et les péricytes par exemple), était doublée après un exercice aigu excentrique chez la souris. De manière intéressante, après transplantation, ces cellules souches isolées 1h après l'exercice possèdent la capacité de promouvoir la prolifération des SCs ainsi que la formation de nouvelles fibres musculaires à la suite d'un exercice également chez la souris (Valero et al., 2012). De plus, l'étude de Zou et al. (2014), issue du même groupe de recherche, a ensuite démontré que la transplantation de ces MSCs avant un entraînement excentrique chez la souris augmentait la quantité de SCs, le domaine myonucléaire, la CSA moyenne et la force musculaire. Ces études ont donc démontré que les MSCs contribuent aux adaptations bénéfiques de l'entraînement excentrique et d'autres études sont nécessaires afin d'isoler de manière plus spécifique les différentes populations de MSCs. Une étude récente, réalisée par Farup et al. (2015a) chez l'homme, a démontré une augmentation de la quantité de FAPs en état de prolifération à la suite d'un entraînement concentrique ou excentrique de 12 semaines. La quantité de péricytes a par ailleurs globalement diminué avec les deux modes d'entraînement. Les FAPs semblent donc être sensibles aux contraintes mécaniques liées à une augmentation de l'activité contractile. Aucune étude n'est pour l'instant disponible afin d'expliquer les mécanismes menant à la prolifération des MSCs avec l'exercice. Cependant, le tissu musculaire à l'exercice devenant un véritable organe endocrine, il est possible d'entrevoir certaines pistes afin d'expliquer cette prolifération *via* les nombreuses cytokines ayant un rôle sur les FAPs notamment.

En ce qui concerne les différentes cytokines ayant un rôle majeur sur la régénération musculaire et/ou sur les FAPs, la revue très complète de Gorgens et al. (2015) permet

d'identifier les nombreuses adipokines, myokines et adipomyokines régulées par l'exercice aigu ou l'entraînement. Parmi elles, on retrouve par exemple une diminution de l'expression de la Mstn ainsi qu'une augmentation de l'expression de PDGF-AA, IL-6, TNF $\alpha$ , IL-4, IL-13, IL-15, Fstn ou encore CCR2 (Czarkowska-Paczek et al., 2010, Gorgens et al., 2015, Prokopchuk et al., 2007). De plus toutes les études présentées dans la partie 3.4., soulignant le rôle majeur de l'exercice physique dans la diminution du développement des IMAT avec l'âge, ajoutées à celles montrant une régulation par l'exercice de nombreuses cytokines impliquées dans la modulation de l'activité des FAPs, semblent démontrer un rôle important de l'activité contractile dans les processus menant au développement et à l'accumulation d'IMAT. Cette modulation de l'apparition d'IMAT par l'activité contractile étant potentiellement possible dans un contexte de déconditionnement musculaire mais également dans un contexte de régénération musculaire.

**En résumé, la littérature a soulevé que la perte de force excède la perte de masse observée dans différentes situations de déconditionnement musculaire, mettant ainsi en lumière le rôle des infiltrations graisseuses (IMAT) dans cette perte de force accrue. Les IMAT sont intimement liés au déconditionnement musculaire retrouvé dans de nombreuses situations pathologiques ou physiologiques et la littérature souligne leur rôle prépondérant dans l'altération de la fonction musculaire. De nombreuses études ont démontré l'impact positif d'une augmentation de l'activité physique, et au contraire l'effet négatif de l'inactivité ou l'hypoactivité, sur le développement des IMAT avec l'avancée en âge. Si de nombreuses cellules souches de l'environnement musculaire peuvent potentiellement s'engager dans un lignage adipogénique, les FAPs représentent pour l'instant la population qui semble jouer un rôle majeur dans le développement d'IMAT. L'interaction entre FAPs et cellules satellites/immunitaires semble être un trio indispensable pour une régénération efficace. En effet, si les FAPs sont une population de cellules souches capables d'aider la régénération musculaire, c'est avant tout grâce aux différentes interactions, souvent par l'intermédiaire de cytokines, qu'elles entretiennent tout au long du processus de régénération avec les différents acteurs présents dans l'environnement musculaire. Ces différentes interactions permettent dans un premier temps aux FAPs de proliférer et d'aider à la régénération musculaire et, dans un second temps, de rentrer dans une phase apoptotique inhibant leur potentiel adipogénique. L'ensemble de ces interactions semble également pouvoir être modulé par une variation du niveau d'activité contractile.**

# OBJECTIFS DE LA THESE

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Au regard de la littérature scientifique, une modulation du niveau d'activité contractile permet de faire varier le niveau d'expression de nombreuses cytokines clés impliquées dans le devenir des FAPs et donc dans l'apparition d'IMAT. Cependant, aucune étude n'est disponible concernant les effets d'une modulation de l'activité contractile lors de la régénération musculaire et sur le développement d'IMAT. Nous avons émis l'hypothèse qu'une modulation de cette activité contractile permettrait de modifier l'apparition d'IMAT lors de la régénération musculaire induite par le modèle glycérol.

Dans un autre contexte, celui du déconditionnement musculaire dénué de processus de régénération, seule l'étude de Manini et al. (2007) a observé l'apparition d'infiltrations graisseuses chez l'homme jeune et sain après seulement 4 semaines d'hypoactivité. Cependant, aucune étude n'est disponible sur des temps plus courts de déconditionnement ainsi que sur les mécanismes impliqués dans le processus d'accumulation d'IMAT. Concernant cet axe de recherche, nous avons émis l'hypothèse que les processus d'adipogenèse et d'accumulation d'IMAT pouvaient être augmentés dès les premiers jours de microgravité simulée.

Ainsi, mon travail de thèse a été centré sur deux grands axes afin d'étudier le développement d'infiltrations graisseuses :

- Le premier objectif de ce travail de thèse a été d'étudier le rôle du niveau d'activité contractile sur l'apparition d'IMAT dans le contexte spécifique de la régénération musculaire induite par le modèle glycérol chez la souris et également de mettre en lumière les mécanismes impliqués. Dans un premier temps, nous avons étudié la régénération musculaire sous l'effet d'une situation de microgravité simulée, donc de diminution de contraintes mécaniques via le modèle HU (étude 1). Dans cette situation, proche de celle d'une diminution des contraintes mécaniques appliquées au muscle lésé chez le sportif blessé par exemple, l'hypoactivité induite par le modèle HU juste après lésion musculaire permettrait une régénération plus efficiente, sans apparition d'IMAT. Dans un second temps, nous avons caractérisé les mécanismes impliqués dans l'inhibition de l'apparition des IMAT avec l'hypoactivité (étude 2). Au regard de l'importance des mécanismes pro-inflammatoires dans la régulation de la différenciation des FAPs en adipocytes, nous avons donc estimé que ces processus seraient potentiellement impliqués dans l'inhibition du développement des IMAT.



- Le deuxième objectif de ce travail de thèse a été d'étudier l'effet de 3 jours de microgravité simulée, par le modèle novateur de dry immersion, sur le développement des infiltrations graisseuses (étude 3). Notre étude, impliquant des jeunes hommes sains non-sédentaires, devrait permettre de mettre en évidence si les mécanismes cellulaires et moléculaires menant à l'apparition d'IMAT, liée à une situation de déconditionnement musculaire, se mettent en place de manière précoce.

## RESULTATS ET DISCUSSION

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**ETUDE 1 : LA REGENERATION MUSCULAIRE ET  
L'ACCUMULATION D'IMAT EST MODULEE PAR LES CONTRAINTES  
MECANIQUES**

Article 1

**Muscle Regeneration with Intermuscular Adipose Tissue (IMAT)  
Accumulation Is Modulated by Mechanical Constraints**

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## **Contexte de l'étude 1**

Après blessure, le muscle strié squelettique possède la capacité d'initier, en quelques heures, une cascade de processus de réparation afin de retrouver l'intégrité originale du muscle lésé. Cette capacité du tissu musculaire squelettique à régénérer est un autre paramètre clé de sa plasticité. Ce phénomène de régénération est indispensable au tissu musculaire dans la mesure où de nombreuses situations, pathologiques ou physiologiques, amènent à des lésions plus ou moins graves des fibres musculaires. Les lésions musculaires sont, le plus fréquemment, la conséquence de blessures sportives et étudier les mécanismes de régénération représente un enjeu majeur dans la médecine sportive. La littérature scientifique a clairement indiqué qu'une perturbation de la régénération musculaire engendrait un développement anormal de fibrose et d'IMAT. Le modèle glycérol est de ce fait utilisé dans notre étude car il permet l'apparition d'IMAT dans le muscle lésé, contrairement aux autres modèles de régénération qui n'en démontrent que très peu.

Par ailleurs, après blessure, le sportif est normalement amené, de manière logique, à diminuer les contraintes mécaniques et donc l'activité musculaire du muscle lésé (à l'aide de béquilles, par alitement prolongé, ou encore par immobilisation d'une articulation). Les mécanismes et les effets de cette diminution d'activité contractile sur le muscle lésé ne sont à ce jour pas totalement étudiés et élucidés. De plus, aucune donnée n'est disponible concernant la relation entre la diminution des contraintes mécaniques lors des processus de régénération et le développement et l'accumulation des IMAT. Ainsi, nous avons tout d'abord utilisé le modèle glycérol, chez la souris, afin d'induire une régénération musculaire accompagnée d'un développement d'IMAT et immédiatement suspendu les souris par le train arrière (modèle hindlimb unloading) afin d'induire une réduction du niveau d'activité contractile sur les membres postérieurs. Cette combinaison de modèles expérimentaux nous a donc permis d'étudier la régénération sous l'effet d'une situation de microgravité simulée, donc de diminution de contraintes mécaniques.

RESEARCH ARTICLE

# Muscle Regeneration with Intermuscular Adipose Tissue (IMAT) Accumulation Is Modulated by Mechanical Constraints

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## Abstract

Sports trauma are able to induce muscle injury with fibrosis and accumulation of intermuscular adipose tissue (IMAT), which affect muscle function. This study was designed to investigate whether hypoactivity would influence IMAT accumulation in regenerating mouse skeletal muscle using the glycerol model of muscle regeneration. The animals were immediately hindlimb unloaded for 21 days after glycerol injection into the tibialis anterior (TA) muscle. Muscle fiber and adipocyte cross-sectional area (CSA) and IMAT accumulation were determined by histomorphometric analysis. Adipogenesis during regenerative processes was examined using RT-qPCR and Western blot quantification. Twenty-one days of hindlimb unloading resulted in decreases of 38% and 50.6% in the muscle weight/body weight ratio and CSA, respectively, in soleus muscle. Glycerol injection into TA induced IMAT accumulation, reaching 3% of control normal-loading muscle area. This IMAT accumulation was largely inhibited in unloading conditions (0.09%) and concomitant with a marked reduction in perilipin and FABP4 protein content, two key markers of mature adipocytes. Induction of PPAR $\gamma$  and C/EBP $\alpha$  mRNA, two markers of adipogenesis, was also decreased. Furthermore, the protein expression of PDGFR $\alpha$ , a cell surface marker of fibro/adipogenic progenitors, was much lower in regenerating TA from the unloaded group. Exposure of regenerating muscle to hypoactivity severely reduces IMAT development and accumulation. These results provide new insight into the mechanisms regulating IMAT development in skeletal muscle and highlight the importance of taking into account the level of mechanical constraint imposed on skeletal muscle during the regeneration processes.

## OPEN ACCESS

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## Introduction

The capacity of skeletal muscle to regenerate is a key parameter of its plasticity. A wide variety of stress can induce muscle injuries, including sport traumas, prolonged blood flow disruption or even muscle diseases. After injury, skeletal muscle is able to regenerate through various and high coordinated stages including degeneration, inflammation, and regeneration process [1]. These steps include recruitment of satellite cells (SCs), which are localized between the sarcolemma and the basal lamina of myofibers [2]. Indeed, it is now well known that quiescent satellite cells proliferate, migrate and differentiate into mature myofibers to regenerate injured muscle tissue [3–5]. Numerous studies have already shown that hindlimb unloading (HU), commonly used to mimic hypoactivity and also microgravity [6], induces a loss in SC content and mitotic activity, which disturbs muscle regeneration by reducing growth of the newly formed myofibers [7–9].

The research literature also indicates that abnormal fibrosis and intermuscular adipose tissue (IMAT) accumulation occur, particularly when early regeneration processes are altered, and that this in turn alters muscle function. IMAT is defined as adipocyte accumulation between muscle cells and beneath the muscle fascia, and it should not be confused with intramyocellular triglyceride accumulation [10]. Studies have shown that impaired macrophage function is linked to poor muscle regeneration and IMAT accumulation after freeze-induced [11], ischemic [12, 13], notexin-induced [14] and cardiotoxin-induced [15] injury.

In these regeneration models, little or no IMAT accumulation is naturally observed. Although IMAT does not occur naturally in rodent skeletal muscles, a skeletal muscle regeneration model with IMAT accumulation was developed in rabbit by Kawai et al. [16] and was later used in mice in several studies [17–20]. This regeneration model consists of injecting glycerol into skeletal muscle, and Dani's group was the first to present a detailed characterization of the "glycerol approach" [21]. The model has been used in several studies to investigate IMAT development and its related adipogenic processes and, more recently, to better characterize muscle-resident adipocyte precursors [19, 20, 22]. To our knowledge, the study of Lukjanenko et al. [22] has been the only one to provide a detailed characterization of some of the cellular responses related to this regeneration model in comparison with the more classic cardiotoxin model. Their study clearly showed that the two models induced similar kinetics of skeletal muscle degeneration and regeneration, but they differed with regard to the adipogenic response amplitude. The glycerol model was therefore associated with more mature adipocytes accumulation.

Recently, studies have highlighted the growing importance of muscle-resident mesenchymal stem cells in the regeneration process of skeletal muscle [23, 24]. In particular, fibro/adipogenic progenitors (FAPs), which are mainly positive for the cell surface marker platelet-derived growth factor receptor alpha (PDGFR $\alpha$  or CD140a), play an important role in efficient regeneration. In a healthy but damaged muscle, FAPs proliferate, phagocytize necrotic debris, and increase the proliferation of SCs without differentiating into adipocytes [19, 25]. In muscle disuse or pathological conditions, such as Duchenne muscular dystrophy, FAPs proliferate and differentiate into adipose and/or fibrous tissue [26, 27]. Parallel to the decrease in SC content [28, 29], FAPs in this case lead to an accumulation of IMAT [10, 20].

Although it is clear that disturbed regeneration promotes IMAT accumulation and impaired skeletal muscle function [30], there is a lack of data evaluating the effects of prolonged hypoactivity on IMAT accumulation during regeneration processes. In a closed context, the study of Jarvinen and Lehto [31] conducted in rats showed that immobilization following contusion injury limited the size of the connective tissue area formed within the injury site. One might therefore ask whether a period of hypoactivity would modulate IMAT development in a

regeneration model characterized by IMAT development. Our study was designed to investigate and characterize the effects of prolonged hypoactivity induced by hindlimb unloading on IMAT development and accumulation in the glycerol model of muscle regeneration. Rodent HU was set up immediately after glycerol injection to ensure hypoactivity during the entire period of the regeneration process. Our results clearly showed that IMAT accumulation in regenerating muscle was substantially lowered with unloading conditions, with a subsequent decrease in FAP recruitment.

## Materials and Methods

### Ethics statement

This study was approved by the Committee on the Ethics of Animal Experiments of Languedoc Roussillon in accordance with the guidelines from the French National Research Council for the Care and Use of Laboratory Animals (CEEA-LR-14002). All efforts were made to minimize animal suffering.

### Animals

Experiments were carried out on 6-month-old C57BL6J/CBA female mice ( $n = 36$ ; mean body mass =  $25.4\text{g} \pm 0.44$ ) from our own stock. Animals were maintained on a 12h/12h light–dark cycle and provided with food and water ad libitum. Experiments were performed at  $22^\circ\text{C}$ .

### Experimental procedures and muscle sampling

Experimental procedures were performed under anesthesia using isoflurane inhalation. In accordance with the study of Pisani et al. [21], the mice were injected with  $25\mu\text{l}$  of 50% v/v glycerol in the right tibialis anterior (TAg) and with saline solution in the contralateral tibialis anterior (TA). Two experimental groups were formed: hindlimb-unloaded (HU,  $n = 6$ ) and control (CTL,  $n = 6$ ), for 21 days. Tail-suspended experiments were conducted with suspension cages and the protocol used in other studies [32, 33]. At the end of the 21 days of unloading or control conditions, *soleus* (SOL) and *extensor digitorum longus* (EDL) hindlimb muscles were rapidly dissected out and immediately frozen in isopentane cooled with liquid nitrogen and then stored at  $-80^\circ\text{C}$ . For the first set of experiments, TAg and TA muscles were also rapidly dissected out and immediately fixed overnight in 4% paraformaldehyde solution at room temperature and then paraffin-embedded. For the second and third set of experiments, TAg and TA from CTL and HU animals were dissected out and rapidly frozen in liquid nitrogen for quantification of mRNA and protein content, as described elsewhere.

### Histology

Transverse serial sections of SOL and EDL muscles ( $9\mu\text{m}$  thick) were obtained at  $-20^\circ\text{C}$  using a cryostat and sections were subjected to hematoxylin-eosin staining for subsequent cross-sectional area (CSA) measurements.

TA and TAg muscles were fixed in 4% neutral-buffered formalin (24h) and paraffin-embedded. The paraffin-embedded tissues were sectioned ( $3\mu\text{m}$  thick) every  $50\mu\text{m}$  over the entire muscle depth, and sections were processed by hematoxylin/eosin/saffron (H/E/S) staining. Stained slides were digitalized with the NanoZoomer slide scanner with a x40 objective (Hamamatsu) for subsequent muscle fibers and adipocytes CSA evaluation. Images were analyzed with ImageJ<sup>®</sup> (1.46r version) software to measure the CSA.

## Immunohistochemistry

The immunohistochemistry protocol was globally performed as previously described [34]. Briefly, mouse colon (positive control) and TAg muscle sections were deparaffinized, rehydrated, and incubated for antigen retrieval in EDTA buffer at 100°C for 10 min. Sections were incubated in 0.3% H<sub>2</sub>O<sub>2</sub> for 20 min and endogenous biotins were blocked using the Avidin-Biotin Blocking kit (Vector Laboratories, CliniSciences). Nonspecific antibody binding was blocked by incubation with TBS containing 20% normal goat serum for 30 min at RT. Sections were then incubated ON at 4°C with rabbit anti-PDGFR $\alpha$  diluted at 1:250 or non-specific rabbit IgG (Vector Laboratories, CliniSciences) at the same concentration. Antibody binding was revealed by the streptavidin/biotin-peroxydase complex method using ABC Vectastain kit and the peroxidase substrate DAB (Vector Laboratories, Clinisciences).

## RNA extraction and real-time polymerase chain reaction (RT-qPCR)

Total RNAs were isolated from homogenate muscle samples using the RNeasy Fibrous Tissue Mini Kit following the manufacturer's instructions (Qiagen). RNA concentration was determined by spectrophotometric analysis (Eppendorf AG, Hamburg, Germany), and integrity was checked by the OD<sub>260nm</sub>/OD<sub>280nm</sub> absorption ratio (>1.7). Reverse transcription reaction was performed with 2 $\mu$ g of total RNA using the RevertAid First Strand cDNA Synthesis kit (Thermo Scientific) according to the manufacturer's instructions. qPCR analysis was performed in a MiniOpticon detection system (Bio-Rad, Hercules, CA) with 10 $\mu$ L of KAPA SYBR Fast Universal Readymix (CliniSciences), 300nM of both forward and reverse primers, 2 $\mu$ L of diluted cDNA template and water to a final volume of 20 $\mu$ L. The forward and reverse primers used to amplify genes are listed in Table 1. All PCRs were performed in duplicate using the following cycle parameters: 30s at 98°C, 40 cycles of 1s at 95°C and 15s at 60°C. Relative mRNA levels were normalized to ribosomal protein S9 (rpS9) and cyclophilin A housekeeping gene levels, which were unaffected by treatments. Results are expressed using the comparative cycle threshold (C<sub>T</sub>). The relative changes in the level of a specific gene were calculated with the  $\Delta\Delta C_T$  formula.

## Protein isolation and Western blotting

The Western blot protocol was performed as previously described [35]. Briefly, muscle samples were homogenized in 10 volumes of lysis buffer (50mM Tris-HCl pH7.5, 150mM NaCl, 1mM EGTA, 100mM NaF, 5mM Na<sub>3</sub>VO<sub>4</sub>, 1% Triton X-100, 40mM  $\beta$ -glycerophosphate and protease inhibitor mixture (P8340; Sigma-Aldrich)) and centrifuged at 10.000g for 10 min (4°C). 60 $\mu$ g of protein extracts were loaded on SDS-polyacrylamide gels before electrophoretic transfer onto a nitrocellulose membrane (Bio-Rad). After transfer, membranes were blocked with 50mM Tris-HCl pH7.5, 150mM NaCl, and 0.1% Tween 20 (TBS-T) containing 5% skimmed milk or BSA and incubated overnight at 4°C with primary antibodies. Membranes were incubated for 1 h with a peroxidase-conjugated secondary antibody. Immunoblots were revealed using a Pierce ECL kit (32106; Thermo Scientific) and proteins were visualized by enhanced chemiluminescence and quantified with ImageJ<sup>®</sup> (1.46r version) software.  $\beta$ -actin was used as a loading control.

## Antibodies

Anti-PDGFR $\alpha$  (#3174), anti-perilipin (#9349), anti-FABP4 (#3544) and anti-adiponectin (#2789) primary antibodies were purchased from Cell Signaling and used at 1:500. Anti- $\beta$ -actin (sc-81178) primary antibody was purchased from Santa Cruz and used at 1:2000. Anti-



**Table 1. Real-time PCR primers.**

Gene	Forward	Reverse	Amplicon size
C/EBP $\alpha$	GACCAGAAAGCTGAGTTGTGAG	CCACAAAGCCCAGAAACCTA	69 bp
C/EBP $\beta$	CTCCAGGTAGGGGTGAAGT	TTTAGACCCATGGAAGTGCC	150 bp
Cyclophilin A	TTCTCCTTTCACAGAATTATTCCA	CCGCCAGTGCCATTATGG	75 bp
MyoD	AGCACTACAGTGGCGACTCA	GGCCGCTGTAATCCATCAT	75 bp
myogenin	ACAGGCCTTGCTCAGCTC	CGCTGTGGGAGTTGCATT	102 bp
Pax7	GCTACCAGTACAGCCAGTATG	GTCACTAAGCATGGGTAGATG	328 bp
PDGFR $\alpha$	AAGACCTGGGCAAGAGGAAC	GAACCTGTCTCGATGGCACT	67 bp
PPAR $\gamma$	GTGCCAGTTTCGATCCGTAGA	GGCCAGCATCGTGTAGATGA	142 bp
rpS9	CGGCCCGGGAGCTGTTGACG	CTGCTTGC GGACCCTAATGT	247 bp

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mouse (NA931V) and anti-rabbit (NA934V) HRP-conjugated secondary antibodies were purchased from GE Healthcare Life Science and used at 1:3000.

### Statistics

All values are expressed as mean  $\pm$  SEM and the significance level was set as  $p < 0.05$ . Differences between the two groups were evaluated for significance using the unpaired Student t-test or the Mann-Whitney test when data deviated from a normal distribution. When more than two simultaneous comparisons were made, a two-way ANOVA was employed to compare data (unloading and glycerol-injection factors). When a significant effect was indicated, a Fisher significant difference post hoc test was performed.

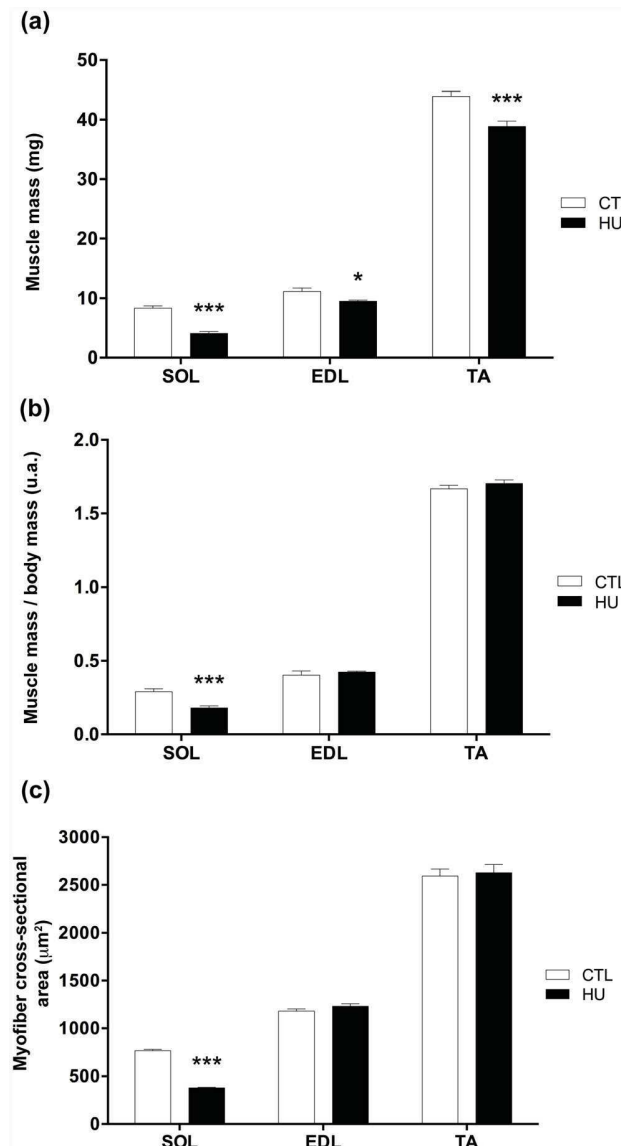
### Results

#### Body mass, muscle mass, and fiber cross-sectional areas following 21 days of hindlimb unloading

Body mass was significantly changed after 21 days of HU (24.57g at day 0 and 22.81g at day 21,  $p < 0.001$ ), whereas no difference was found in control conditions (26.13g at day 0 and 26.47g at day 21). SOL muscle mass, expressed in either absolute values or values normalized by body mass, decreased significantly after HU (-50.8% and -38% respectively,  $p < 0.001$ , [Fig 1a and 1b](#)) compared with CTL. EDL and TA muscle mass decreased significantly after HU, but only when expressed in absolute values (-15.1%,  $p < 0.05$ , and -11.5%,  $p < 0.001$ , respectively, [Fig 1a and 1b](#)). The mean CSA of SOL fibers was significantly reduced after HU compared with CTL (-50.6%,  $p < 0.001$ , [Fig 1c](#)). However, no significant differences were detected for the mean CSA of EDL and TA muscle fibers ([Fig 1c](#)). Thus, our results are in agreement with the literature, which has shown major atrophy in SOL, a postural muscle, following 21 days of HU [[6](#), [36](#)].

#### Hindlimb unloading disturbs regeneration in glycerol-injected tibialis anterior

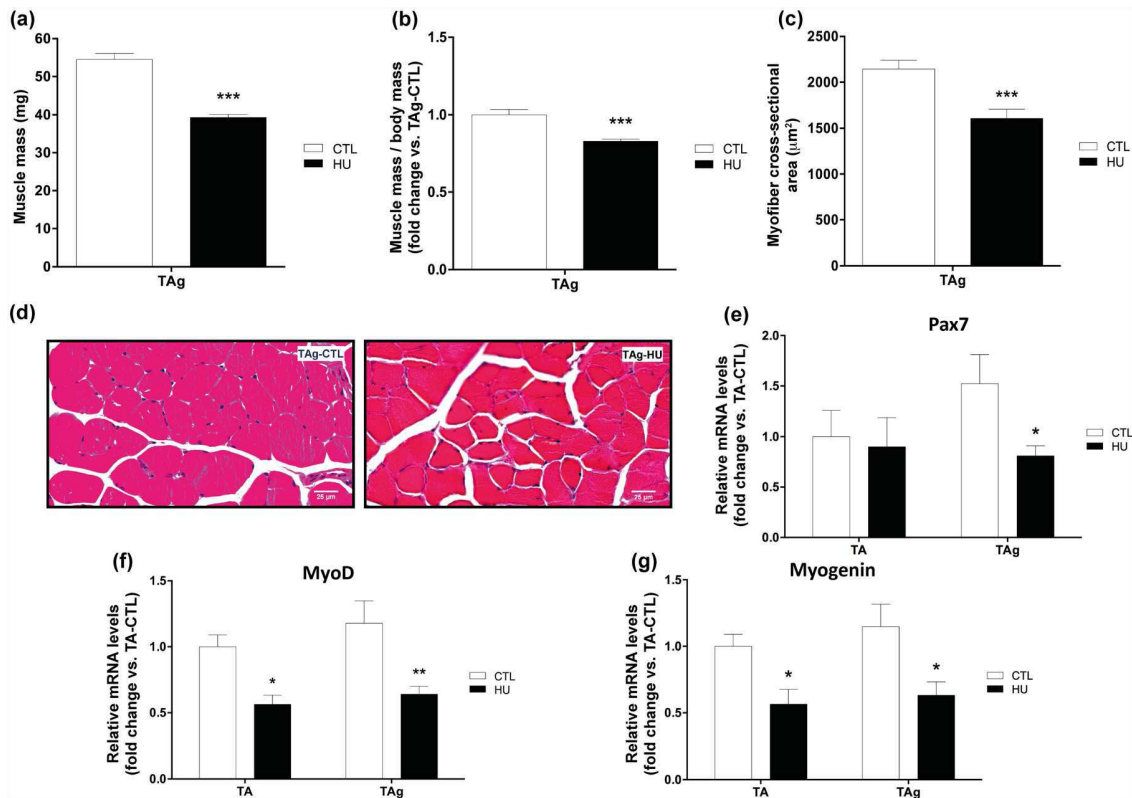
Glycerol-injected tibialis anterior (TA<sub>g</sub>) muscle mass, expressed in either absolute values or values normalized by body mass, decreased significantly after HU (-27.87% and -17.1% respectively,  $p < 0.001$ , [Fig 2a and 2b](#)) compared with TA<sub>g</sub>-CTL. In parallel, the mean CSA of TA<sub>g</sub>-HU fibers *versus* TA<sub>g</sub>-CTL was significantly reduced (-25.1%,  $p < 0.001$ , [Fig 2c and 2d](#)). This suggests that hindlimb unloading affects skeletal muscle regeneration and thereby reduces myofiber size and mass recovery. We further examined the mRNA induction of key genes implicated in the muscle regeneration processes and myogenesis in TA and TA<sub>g</sub> of the CTL



**Fig 1. Effects of hindlimb unloading on muscle mass and fiber cross-sectional areas.** Soleus (SOL), extensor digitorum longus (EDL), tibialis anterior (TA) skeletal muscle mass from control (CTL) and hindlimb unloading (HU) mice (a). SOL, EDL, and TA mass normalized by body mass from CTL and HU mice (b). Myofiber cross-sectional area (CSA) in  $\mu\text{m}^2$  of SOL, EDL, and TA skeletal muscles from CTL and HU mice (c). \*  $p < 0.05$  vs control group, and \*\*\*  $p < 0.001$  vs control group.

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and HU groups. mRNA induction of Pax7, a marker of quiescent and proliferative SCs in adult mice, decreased only in TAg-HU compared with TAg-CTL (-46.9%,  $p < 0.05$ , Fig 2e). Levels of MyoD and myogenin mRNA, two markers of activated and differentiation-engaged myoblasts, respectively, were reduced significantly in TA-HU versus TA-CTL (-43.7% and -43.5% respectively,  $p < 0.05$ , Fig 2f and 2g). A similar decrease in magnitude was also observed in TAg-HU versus TAg-CTL (-45.7%,  $p < 0.01$  and -44.85%,  $p < 0.05$ , respectively, for MyoD and myogenin, Fig 2f and 2g). Taken together, these results based on mRNA analysis could indicate that hindlimb unloading alone did not alter the number of SCs but did decrease their mitotic activity.



**Fig 2. Effects of hindlimb unloading and glycerol injection on tibialis anterior muscle.** Muscle mass (a), muscle mass/body mass (b) and cross-sectional area (CSA) (c) of glycerol-injected tibialis anterior (TA<sub>g</sub>) from control (CTL) and hindlimb unloading (HU) mice. Representative histological transverse paraffin-embedded muscle sections, stained with hematoxylin-eosin-saffron from TA<sub>g</sub> of each experimental group (CTL and HU) (d). Pax7 (e), MyoD (f) and myogenin (g) mRNA levels of TA and TA<sub>g</sub> from CTL and HU mice. \*  $p < 0.05$  vs control group, \*\*  $p < 0.01$  vs control group, \*\*\*  $p < 0.001$  vs control group (unloading effect).

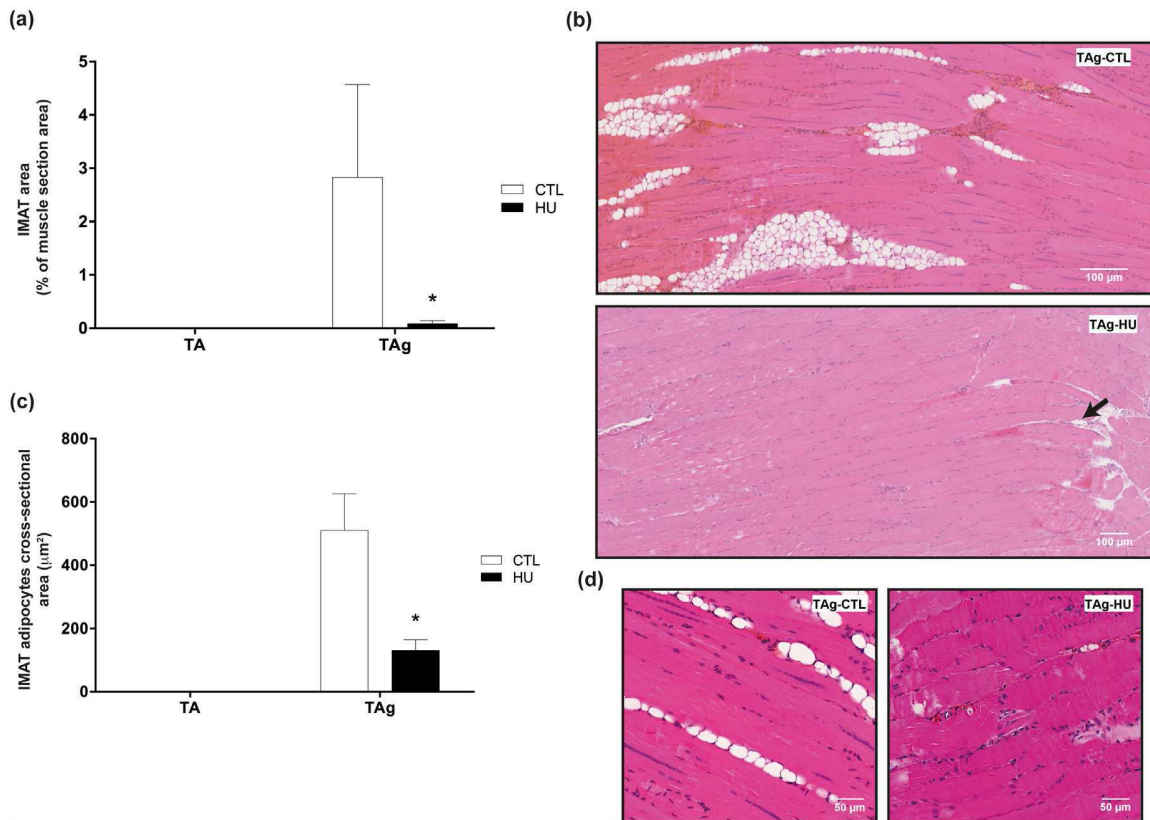
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However, when HU was associated with a regenerating context, we found a decrease in the SC pool and mitotic activity, which thus decreased the subsequent regrowth and myofiber size recovery.

### Hypoactivity inhibits IMAT accumulation in regenerating TA muscle

It is well known that the glycerol model of muscle regeneration induces IMAT development during muscle regeneration. In our study, we confirmed the presence of IMAT in the TA injected with glycerol of normal-loaded animals (TA<sub>g</sub>-CTL). The area occupied by IMAT 21 days after glycerol injection reached a mean 2.83% of the total muscle CSA, whereas TA-CTL showed no histological sign of IMAT (Fig 3a and 3b). Thus, as previously described in the study of Dani's group [21], it seems that the muscle microenvironment created by glycerol injection may favor adipogenesis from resident and/or recruited precursors, unlike what occurs in other regeneration models.

Conversely, unloading almost completely prevented IMAT development in TA<sub>g</sub>: the area occupied by IMAT was almost nil (0.09%,  $p < 0.05$ ) in TA<sub>g</sub>-HU (Fig 3a and 3b). Moreover, CSA morphometric analysis of the adipocytes from IMAT showed a decrease in TA<sub>g</sub>-HU compared with TA<sub>g</sub>-CTL ( $130\mu\text{m}^2$  and  $510\mu\text{m}^2$ , respectively,  $p < 0.05$ , Fig 3c and 3d).



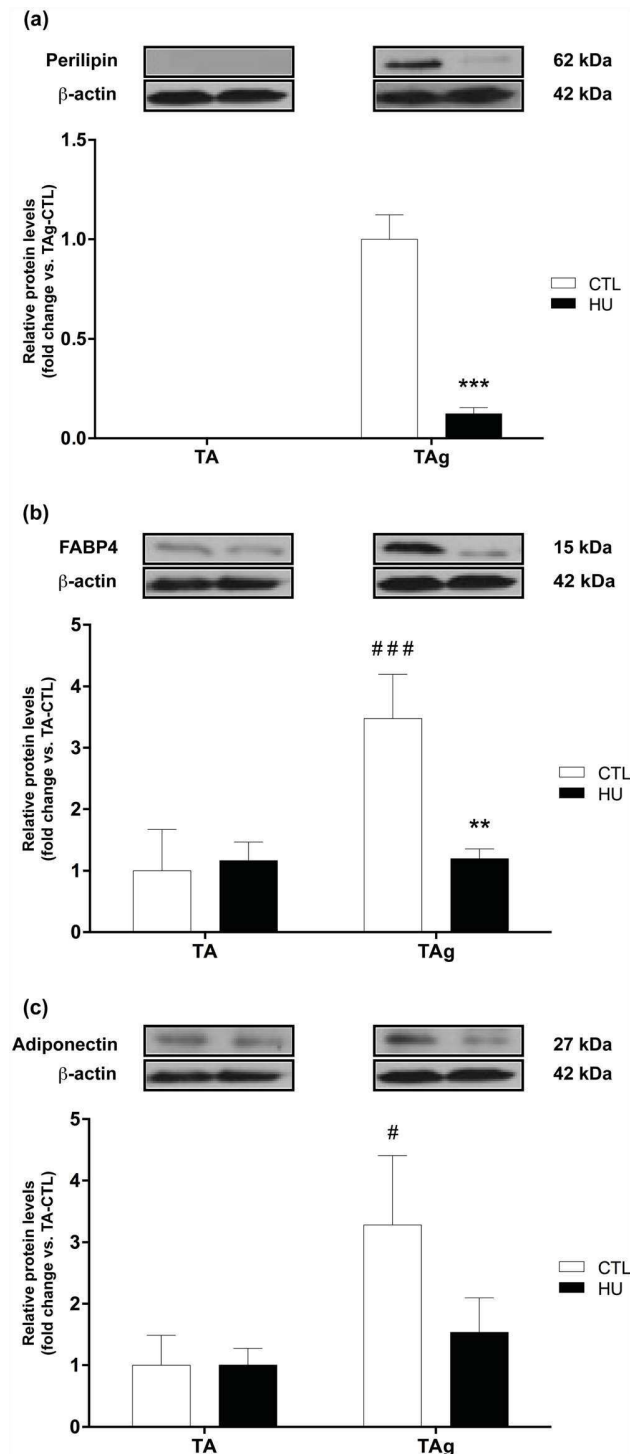
**Fig 3. Effects of hindlimb unloading on IMAT development and accumulation.** Intermuscular adipose tissue (IMAT) area in percentage of total muscle section area **(a)** and IMAT adipocyte cross-sectional area (CSA) in  $\mu\text{m}^2$  **(c)** of saline-injected (TA) and glycerol-injected (TAg) tibialis anterior from control (CTL) and hindlimb unloading (HU) mice. \*  $p < 0.05$  vs control group. Representative histological longitudinal paraffin-embedded muscle sections, stained with hematoxylin-eosin-saffron from TAg of each experimental group (CTL and HU) **(b, d)**.

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To further confirm the effects of hypoactivity on IMAT development, we quantified the expression of perilipin, fatty acid binding protein 4 (FABP4) and adiponectin, three markers of mature adipocytes. Concerning perilipin, the adipocyte lipid droplet protein marker, no signal was detected for the saline-injected TA, whereas its expression was clear in TAg, as expected (Fig 4a). In line with our previous results, perilipin expression in TAg was considerably decreased by HU (-87.7%,  $p < 0.001$ , Fig 4a). The expression level of FABP4, a cytoplasmic fatty acid chaperone, increased in TAg-CTL compared with TA-CTL (+246.7%,  $p < 0.001$ ) and returned to baseline level in TAg-HU ( $p < 0.01$ , Fig 4b). The expression level of adiponectin, an adipokine, increased in TAg-CTL compared with TA-CTL (+228.2%,  $p < 0.05$ ), whereas this difference was not observed between TAg-HU and TA-HU (Fig 4c). All together, these results were in agreement with our histological findings and confirmed that HU reduces IMAT accumulation in this model of muscle regeneration.

### Hypoactivity decreases the adipogenesis processes in regenerating muscles

After determining that unloading was able to inhibit IMAT development in the glycerol regeneration model, we investigated the underlying mechanisms that led to the decrease in IMAT development in HU conditions. We first analyzed the mRNA induction and protein expression



**Fig 4. Protein expression levels of mature adipocyte markers.** Perilipin (a), FABP4 (b) and adiponectin (c) protein content of saline-injected (TA) and glycerol-injected (TAg) tibialis anterior from control (CTL) and hindlimb unloading (HU) mice. \*\*  $p < 0.01$  vs control group, \*\*\*  $p < 0.001$  vs control group (unloading effect). #  $p < 0.05$  vs TA-CTL, ###  $p < 0.001$  vs TA-CTL (glycerol-injection effect).

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of PDGFR $\alpha$ , the cell surface marker of FAPs, and then we investigated the induction and/or expression of different markers involved in adipogenesis.

Firstly, we found no differences in PDGFR $\alpha$  protein expression or mRNA induction for saline-injected TA or TAg-CTL conditions (Fig 5a and 5b). The last confirming the return to basal values for PDGFR $\alpha$  marker 21 days after injury reported in the study of Lukjanenko et al. [22]. However, the protein content of PDGFR $\alpha$  was drastically lowered in TAg-HU *versus* TAg-CTL (-71.1%,  $p < 0.05$ , Fig 5b) and this result was confirmed by our immunohistochemical analysis (-77%,  $p < 0.001$ , Fig 5d), whereas the respective results for mRNA induction were not significantly different (Fig 5a). Antibody specificity was checked using mouse colon section as a positive control [37] (Fig 5c). As hypothesized, no change was observed in the induction of CCAAT/enhancer-binding protein  $\beta$  (C/EBP $\beta$ ) mRNA, a transcription factor implicated in an early step in adipogenesis (Fig 6a). However, mRNA induction of peroxisome proliferator-activated receptor- $\gamma$  (PPAR $\gamma$ ) and CCAAT/enhancer binding protein  $\alpha$  (C/EBP $\alpha$ ), two transcriptional factors implicated in the later steps of adipogenesis, was lower in TAg-HU *versus* TAg-CTL (-51.6% and -53.2%, respectively,  $p < 0.05$ , Fig 6b and 6c). Furthermore, we observed an approximately 7-fold higher induction of C/EBP $\alpha$  mRNA in TAg-CTL compared with TA-CTL ( $p < 0.01$ , Fig 6c).

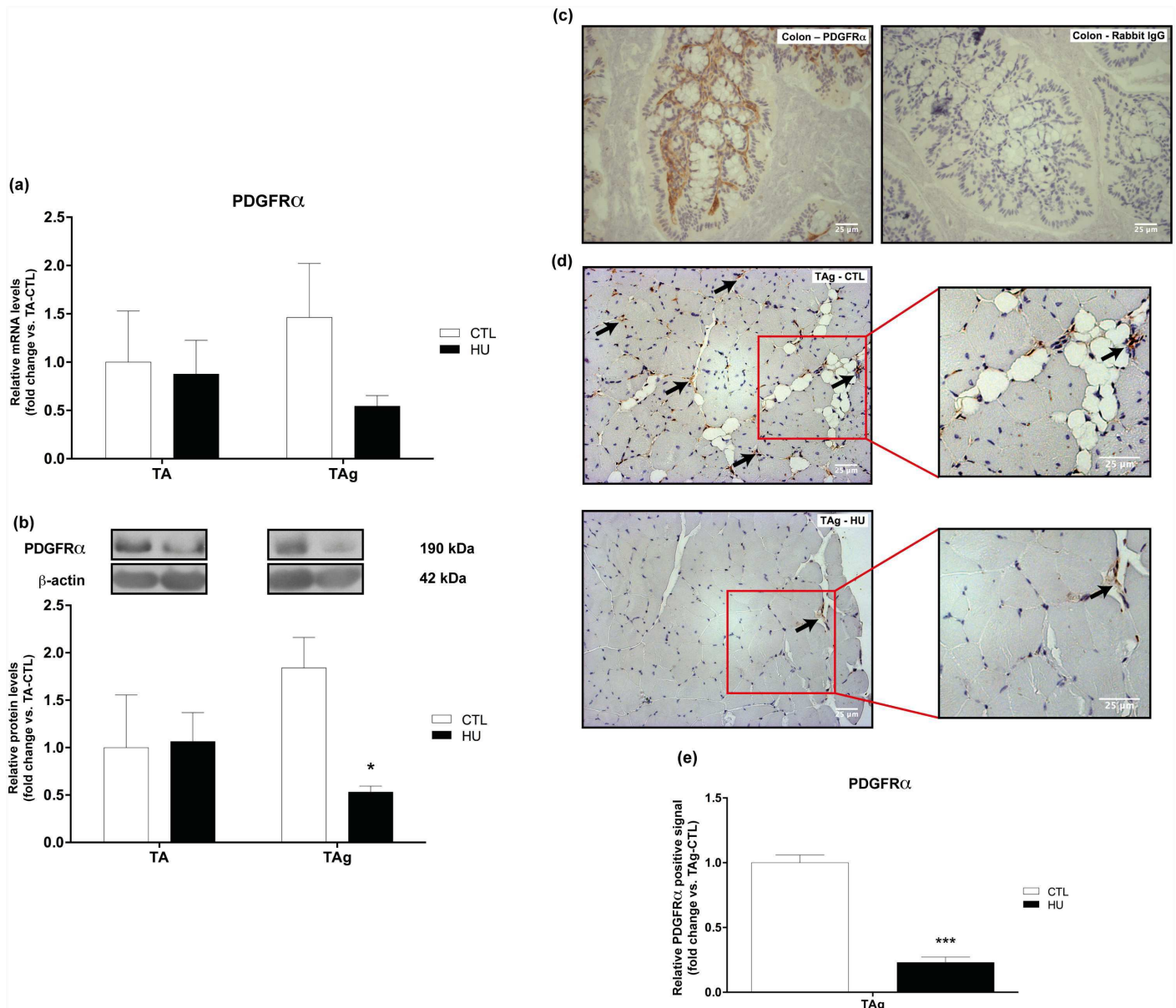
## Discussion

The main objective of this study was to characterize the effects of prolonged hypoactivity on IMAT development in regenerating muscles. For that purpose, we used the well-described skeletal muscle regeneration model of glycerol injection, known to lead to IMAT formation. Our study shows for the first time that hypoactivity almost completely prevents IMAT accumulation.

Although IMAT may be a variable part of healthy human skeletal muscles, its increase and accumulation are synonymous with muscle dysfunction, deconditioning, and even perturbed regeneration [27, 30, 38]. The fat infiltration of muscle remains understudied and little is known about whether hypoactivity can lower or conversely enhance IMAT accumulation in pathological or regenerating skeletal muscle.

In our experiment, TAg muscles exhibited adiposity 21 days after glycerol injection and this result was strengthened by the increase in protein expression of several mature adipocyte markers: perilipin, FABP4 and adiponectin. Moreover, we analyzed the expression of the major transcriptional factors implicated in adipogenesis and found a significant induction of C/EBP $\alpha$  mRNA in TAg-CTL compared with TA-CTL. However, no difference in C/EBP $\beta$  and PPAR $\gamma$  mRNA induction was observed, although these two transcriptional factors are known to participate in adipogenesis in a phase earlier than C/EBP $\alpha$ , which appears to be up regulated at the end of the adipogenesis process [39]. This is in accordance with our present study: 21 days after glycerol injection adipogenesis was largely accomplished and the early adipogenic transcription factors had returned to basal values [22].

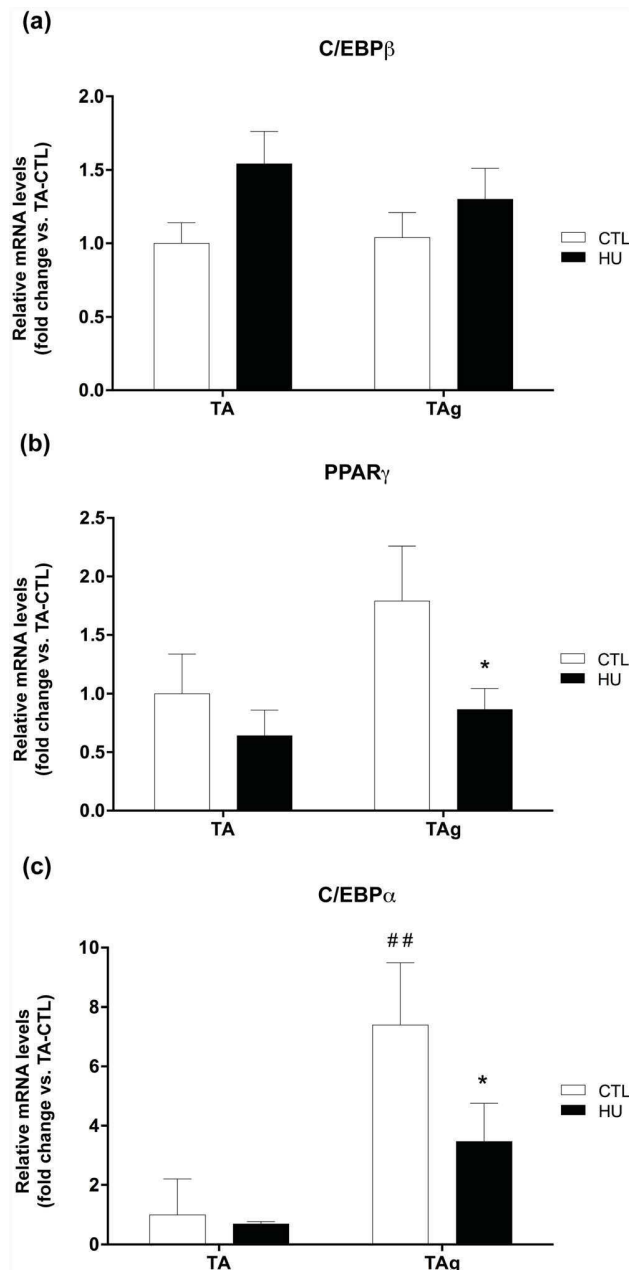
We further characterized the effects of prolonged hypoactivity on the confirmed IMAT accumulation. For this purpose, we used the well-characterized hindlimb suspension model and our study revealed for the first time an almost complete inhibition of adipogenesis in regenerating muscles induced by the glycerol model submitted to hypoactivity over the entire length of the regeneration period. Our histological examination of TAg-CTL and TAg-HU was performed over the entire depth of the muscle and we clearly observed the inhibition of adipogenesis in unloading conditions. This histological result was further supported by the quantification of perilipin, FABP4 and adiponectin expression, which considerably decreased following HU.



**Fig 5. Expression levels of the FAP cell surface marker PDGFRα.** PDGFRα mRNA (a) and protein (b) content of saline-injected (TA) and glycerol-injected (TAg) tibialis anterior from control (CTL) and hindlimb unloading (HU) mice. \*  $p < 0.05$  vs control group (unloading effect). Representative images (c, d) and quantification (e) of immunohistochemical analysis of PDGFRα-positive cells in mouse colon positive control (c) and glycerol-injected (TAg) tibialis anterior from control (CTL) and hindlimb unloading (HU) mice (d). \*\*\*  $p < 0.001$  vs control group.

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Even though the precise mechanisms leading to IMAT formation in the glycerol model are still unclear, previous studies have described the respective roles of multipotent stem cells such as muscle-derived FAPs, pericytes, and side population cells giving rise to adipogenic precursors [26, 40–43]. Recently, a few groups have identified muscle mesenchymal progenitors with the immunophenotype CD31-CD45-SM/C-2.6-PDGFRα+, which contribute to fat cell formation in skeletal muscle [20, 43, 44]. PDGFRα has also been used very recently in human to isolate muscle mesenchymal progenitors, which are equivalent to the mouse FAPs [27, 45]. We



**Fig 6. Changes in mRNA induction of adipogenesis markers.** C/EBP $\beta$  (a), PPAR $\gamma$  (b) and C/EBP $\alpha$  (c) mRNA levels of saline-injected (TA) and glycerol-injected (TAg) tibialis anterior from control (CTL) and hindlimb unloading (HU) mice. \*  $p < 0.05$  vs control group (unloading effect). ##  $p < 0.01$  vs TA-CTL (glycerol-injection effect).

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used this FAP cell surface marker in the present study and found a marked decrease in its protein expression following HU. Our study thus indicates that hypoactivity is able to decrease PDGFR $\alpha$ -positive FAPs, which represent 98% of PDGFR $\alpha$ -positive cells in regenerating muscle [19]. Moreover, as reported by Uezumi et al. [20], only the PDGFR $\alpha$ -positive cells can differentiate into adipocytes in glycerol-injected muscles. We further observed reduced levels of



the later adipogenic transcription factors PPAR $\gamma$  and C/EBP $\alpha$ . We did not detect any differences for C/EBP $\beta$ , certainly due to its earlier implication in adipogenesis [39].

Interestingly, FAPs are also known to promote skeletal muscle regeneration after injury in healthy muscle [19, 25]. Heredia et al. [25] showed that FAP proliferation and macrophage-like activity are essential to the regeneration process. In this context, decreased FAP proliferation could result in a decreased number of adipocytes, as well as impair the regenerative kinetics [25]. Skeletal muscle regenerative capacity is mainly dependent on the activation of SCs, which are finely controlled by the myogenic regulatory factors. Quiescent and proliferative SCs express the paired box transcription factor (Pax7) and its inactivation leads to a severe depletion of these muscle myogenic stem cells [46]. In regenerating muscle, the proliferating process is triggered by the expression of myoblast determination protein 1 (MyoD) and myogenic factor 5 (Myf5) [47, 48]. Once differentiation is initiated, myogenin appears to be implicated first, and then muscle-specific regulatory factor 4 (Mrf4) is activated during the maturation phase [49, 50]. In our study, we first described a decrease in MyoD and myogenin mRNA induction in TA-HU concomitant with no significant effect on Pax7. These results are in accordance with studies demonstrating that HU does not necessarily alter the number of SCs in fast-twitch skeletal muscle, probably indicating no SC loss by apoptosis [51], but that it does alter SC mitotic activity [7, 9, 51, 52]. We then observed a decrease in Pax7, MyoD, and myogenin mRNA induction, parallel to the decrease in muscle mass and CSA in TA<sub>g</sub>-HU. Taken together, these results confirm that reducing mechanical constraints throughout muscle regeneration disturbs SC-mediated regeneration and thus delays myofiber size recovery, as indicated in the study of Matsuba et al. [53]. Nevertheless, the study of Mozdziaik et al. [8] showed that the hindlimb unloading condition does not decrease but could even enhance the mitotic activity of SCs and probably that of non-muscle cells in the earlier stages of regeneration. However, once new myofibers are formed, their growth capacity is altered. Our results suggest that HU may alter regrowth after regeneration, but we cannot exclude the hypothesis that HU may enhance early regeneration processes, thereby inhibiting IMAT occurrence and further disturbing muscle regrowth. Clearly, additional studies are warranted to elucidate the early events related to mesenchymal non-muscle cells and their implication in both the regeneration process and adipogenesis during unloading conditions.

Currently, we do not know the exact underlying mechanisms leading to the inhibition of muscle adipogenesis in regenerating muscles under unloading conditions. As highlighted in the literature, hindlimb unloading appears to be a proinflammatory situation with macrophage infiltration [54–58]. Interestingly, recent studies have reported the critical role of inflammation and the immune system in muscle regeneration [59–62], and it appears that both macrophage shift and activity are essentials in this process. In addition, the study of Lukjanenko et al. [22] revealed that the glycerol injury model exhibits a disrupted inflammatory response compared with the cardiotoxin-induced injury model. Further studies are needed to elucidate the underlying mechanisms of unloading-induced inhibition of skeletal muscle IMAT development and accumulation and especially the effects of an inflammatory response on mesenchymal stem cells.

In conclusion, our study reports for the first time an almost complete inhibition of IMAT development in regenerating muscles under hypoactivity conditions. We found a decreased response of mesenchymal-derived precursor FAPs (PDGFR $\alpha^+$ ), which could explain the decrease in IMAT development in the present model. Hypoactivity seems to locally create a favorable environment leading to a decrease in PDGFR $\alpha$  positive cells.

These observations shed new light on the mechanisms that regulate IMAT development in skeletal muscle and highlight the importance of taking into account the level of mechanical constraint imposed on skeletal muscle during regeneration processes. Our findings point in the

same direction as those reported by Jarvinen and Lehto [31] concerning immobilization, which was found to mediate a decrease in fibrotic area after gastrocnemius injury in rats.

In the one hand, our results suggest that a rest period with reduced mechanical constraints might be needed immediately after injury to prevent IMAT accumulation. However, our study also shows that the regrowth of skeletal muscle fibers is impaired under hypoactivity, which highlights the importance of applying mechanical constraints as soon as possible after the rest period for the recovery of fiber size. Our present and future studies should contribute to a fuller understanding of IMAT accumulation and the establishment of rehabilitation guidelines for human muscle injuries.

## Supporting Information

**S1 Checklist. ARRIVE Checklist.**  
(PDF)

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## Author Contributions

Conceived and designed the experiments: AFP RD AC TB GP CD CAD. Performed the experiments: AFP RD GP TB. Analyzed the data: AFP RD GP AC AB. Contributed reagents/materials/analysis tools: AFP RD AC GP EJ CBG. Wrote the paper: AFP GP AC. Manuscript revision and feedback: AFP AC GP CD CAD RC AB.

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## Discussion de l'étude 1

L'objectif de cette étude était de caractériser l'effet d'une diminution des contraintes mécaniques, par le modèle de suspension du train arrière (HU), sur le développement des IMAT induit par le modèle glycérol de régénération musculaire.

Tout d'abord, nous avons retrouvé, chez nos souris contrôles, une accumulation d'IMAT de l'ordre de 3% de la surface globale du muscle 21 jours après l'injection intramusculaire de glycérol (**Fig.3**). Cette observation est en accord avec les résultats de l'étude de Pisani et al. (2010a), qui ont été les premiers à caractériser précisément ce modèle spécifique de régénération. Cette augmentation du contenu en IMAT a ensuite été vérifiée et validée au travers de marqueurs clés d'adipocytes matures (FABP4, périlipine et adiponectine, **Fig.4**) et de l'adipogenèse (C/EBP $\alpha$ , **Fig.6**). Le modèle glycérol utilisé dans cette étude a donc bien engendré le développement et l'accumulation d'infiltrations graisseuses attendus au sein du muscle injecté.

Notre étude a ensuite clairement mis en évidence une inhibition quasi-complète du développement des IMAT grâce à la réduction de l'activité contractile (modèle HU) (**Fig.3**). Ainsi, de manière cohérente, diminuer immédiatement les contraintes mécaniques imposées au tissu musculaire en régénération permet d'inhiber l'apparition d'infiltrations graisseuses, la présence de ces dernières caractérisant une régénération perturbée. Cette expérimentation est le reflet de la réduction d'activité d'un sportif par exemple, à la suite d'une blessure musculaire : réduire les contraintes mécaniques et donc réduire l'activité musculaire du muscle lésé à l'aide de béquilles s'il s'agit d'une blessure du membre inférieur, ou encore immobiliser l'articulation spécifique au muscle lésé. Notre étude complète également les travaux menés par Jarvinen & Lehto (1993) qui ont montré une diminution de l'apparition de fibrose au niveau du site de la lésion musculaire à la suite d'une immobilisation chez le rat.

Par ailleurs, notre étude souligne une diminution importante de l'expression de PDGFR $\alpha$  dans le groupe HU (**Fig.5**). Cette diminution d'expression du marqueur de surface spécifique des FAPs représente donc une diminution de leur présence. Ainsi la seule population de cellules souches permettant la formation d'adipocytes dans le modèle glycérol (Uezumi et al., 2010) se trouve diminuée dans la condition d'hypoactivité et corrèle avec l'inhibition d'IMAT reportée. De nombreuses études ont déjà démontré que les processus inflammatoires dans les premiers jours de la régénération, notamment l'infiltration des macrophages, étaient nécessaires pour une régénération efficace. La perturbation de ces mécanismes mène, dans de

nombreux modèles différents de régénération, à la survie des FAPs et au développement des IMAT et de fibrose (Arnold et al., 2007, Contreras-Shannon et al., 2007, Lemos et al., 2015, Segawa et al., 2008, Shireman et al., 2007, Summan et al., 2006). De manière intéressante, l'étude de Lukjanenko et al. (2013) a révélé une perturbation des processus inflammatoires dans le modèle glycérol de régénération comparé à celui de la CTX. De plus, la littérature a démontré que le modèle HU utilisé dans notre étude est une situation pro-inflammatoire associée avec une infiltration de macrophages (Andrianjafiniony et al., 2010, Hirose et al., 2008, Nguyen & Tidball, 2003a). Notre étude pose ainsi la question d'un possible rôle du système inflammatoire dans les processus menant à l'apparition des IMAT dans le modèle glycérol mais également dans l'inhibition observée avec le modèle HU.

Par ailleurs, la situation d'hypoactivité engendrée par le modèle HU est appliquée durant toute la durée du protocole et donc de régénération (21 jours). De nombreuses études avaient observé une diminution de la régénération musculaire dans cette situation de déconditionnement musculaire (appréciée par une diminution du recouvrement de la masse musculaire et de la CSA des fibres) (Darr & Schultz, 1989, Mozdziak et al., 1998, Wang et al., 2006). Notre étude a également souligné une diminution de la CSA des fibres musculaires pour notre groupe HU comparé au groupe CTL (**Fig.2C**), mettant ainsi en lumière une diminution de la croissance musculaire des nouvelles fibres musculaires. Le modèle HU est en effet un modèle de déconditionnement musculaire connu pour engendrer une balance protéique négative, c'est à dire en faveur de la protéolyse (Baldwin et al., 2013). Nous avons donc émis l'hypothèse que l'hypoactivité induite par le HU était nécessaire pour une régénération efficace mais que si cette situation perdure tout au long des processus de régénération, elle ne permet pas la croissance des nouvelles fibres musculaires. Cette hypothèse est ainsi soutenue par les résultats de l'étude de Mozdziak et al. (1998) qui soulignent que les mécanismes de prolifération des cellules satellites (en tant qu'acteurs majeurs des processus précoces de la régénération) ne sont pas altérés par le HU, 3 et 4 jours après injection de NTX.

En conclusion, cette première étude a permis de mettre en évidence l'importance d'une période de repos nécessaire après blessure, représentée par une diminution des contraintes mécaniques appliquées au muscle lésé, pour une régénération musculaire efficiente sans apparition d'IMAT. Cependant, notre étude souligne également une altération de la croissance du tissu musculaire dans cette condition d'hypoactivité, soulevant ainsi l'importance d'appliquer de nouvelles contraintes mécaniques le plus rapidement possible après la période

de repos nécessaire. Cette étude a donc contribué à une meilleure compréhension des mécanismes régissant le développement des IMAT lors de la régénération et d'autres études sont nécessaires afin de préciser et d'établir de manière plus précise les consignes de réhabilitation après blessure sportive chez l'homme.



**ETUDE 2 : LA PERTURBATION DE L'AXE TNF $\alpha$ /TGF- $\beta$ 1 DURANT LA  
REGENERATION MUSCULAIRE FAVORISE L'APPARITION D'IMAT**

Article 2

**TNF $\alpha$ /TGF- $\beta$ 1 axis disruption during skeletal muscle regeneration  
promotes fatty infiltrations development**

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## Contexte de l'étude 2

Au regard des résultats obtenus dans notre première étude, nous avons émis l'hypothèse que les processus inflammatoires pouvaient jouer un rôle dans l'inhibition du développement des IMAT observé dans notre condition d'hypoactivité.

Pour rappel, de nombreuses études ont démontré le rôle primordial des FAPs, qui sont positifs pour le marqueur de surface PDGFR $\alpha$ , dans le processus d'une régénération musculaire efficiente. Les FAPs sont capables de proliférer rapidement après blessure et participent à la phagocytose des débris nécrotiques tout en favorisant le processus myogénique des SCs (Heredia et al., 2013, Joe et al., 2010, Lemos et al., 2015). Par contre, dans le modèle glycérol de régénération, les FAPs contribuent au développement de fibrose et d'IMAT qui altèrent la fonction musculaire (Ieronimakis et al., 2016, Lukjanenko et al., 2013, Uezumi et al., 2010, Uezumi et al., 2011).

L'étude conduite par Lemos et al. (2015) a mis en lumière les acteurs régulant l'apoptose des FAPs. Leurs expérimentations ont révélé, de manière cohérente avec les travaux de Joe et al. (2010), que les FAPs proliféraient et atteignaient un pic d'expression entre le 3ème et le 4ème jour après blessure avant de rapidement rentrer dans une phase de mort programmée (d'apoptose) pour revenir à des valeurs basales entre 7 et 9 jours. Cette étude a ensuite montré que le TNF $\alpha$ , sécrété par les macrophages de type M1, était essentiel pour induire l'apoptose des FAPs. Après avoir proliféré et aidé à la prolifération des SCs, les FAPs rentrent donc, à partir du 3ème jour de la régénération, dans une phase d'apoptose qui permet alors de prévenir l'excès de fibrose et hypothétiquement d'IMAT. Cette étude a également montré que TGF- $\beta$ 1, sécrété par les macrophages de type M2, permettait au contraire de promouvoir la survie cellulaire des FAPs et inhibait donc l'action du TNF $\alpha$ . Un traitement avec du TGF- $\beta$ 1 pendant le pic d'expression du TNF $\alpha$  réduit l'apoptose des FAPs alors que l'inhibition de TGF- $\beta$ 1 permet l'effet inverse. Cette étude permet donc d'affirmer que le recrutement et la temporalité du shift phénotypique des macrophages durant la régénération musculaire permet de passer d'un environnement riche en TNF $\alpha$  à un environnement riche en TGF- $\beta$ 1, permettant ainsi de moduler la réponse et le devenir des FAPs.

Comme annoncé dans la discussion de l'étude 1, le modèle glycérol de régénération permettant le développement d'IMAT présente une réponse inflammatoire perturbée. En effet, l'étude de Lukjanenko et al. (2013) a notamment démontré que, comparé au modèle de régénération utilisant de la cardiotoxine, les muscles injectés de glycérol présentent une

réponse anti-inflammatoire plus prononcée et plus précoce. Une induction 2 fois supérieure des ARN messagers codant pour TGF- $\beta$ 1 et IL-10 a par exemple été retrouvée. Les auteurs ont donc conclu que le modèle glycérol de régénération était caractérisé par une puissante réponse anti-inflammatoire, suggérant ainsi, en faisant le lien avec l'étude de Lemos et al. (2015), un shift phénotypique des macrophages trop précoce et un développement d'IMAT lié à une déficience de l'apoptose des FAPs.

Dans ce contexte, notre 2<sup>ème</sup> étude a pour objectif d'élucider les mécanismes permettant l'inhibition du développement des IMAT observé avec le modèle HU de notre précédente étude. Nous avons également pour objectif de clarifier les mécanismes, notamment liés aux processus inflammatoires, à l'origine du développement des IMAT dans ce modèle glycérol de régénération musculaire.

**TNF $\alpha$ /TGF- $\beta$ 1 axis disruption during skeletal muscle regeneration  
promotes fatty infiltrations development**

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**Running title:** TNF $\alpha$ /TGF- $\beta$ 1 axis alteration drives IMAT development

## **Abstract**

Skeletal muscle regeneration ability appears to be a complex and precisely orchestrated process implying multiple cell types. Abnormal development of fibrosis or intermuscular adipose tissue (IMAT) is a strong evidence of regeneration failure and it's now well characterized that IMAT development impairs muscle function. While many stem cells in the muscle environment can adopt the capacity to differentiate into adipocytes, fibro-adipogenic progenitors (FAPs) represent the current population that appears to play a major role in IMAT development. Regulating FAPs fate is therefore a major objective to promote optimal muscle regeneration without IMAT development. In our study, using the glycerol model of muscle regeneration, we demonstrated that: i) decreasing mechanical constraints just after injury leads to an inhibition of IMAT development in the glycerol model of muscle injury, ii) TNF- $\alpha$  mediating FAPs apoptosis response may be disturbed in the glycerol model, leading to IMAT development, and iii) a treatment with decorin, a TGF- $\beta$ 1 inhibitor, restores FAPs apoptosis and decreases IMAT development in this specific model of muscle injury.

## **Introduction**

Skeletal muscle regeneration ability appears to be a complex and precisely orchestrated process implying multiple cell types. Satellite Cells (SCs), localized between the sarcolemma and the basal lamina of myofibers (Mauro, 1961), are the most largely studied and known to vastly participate to the regeneration processes after injury. Nevertheless, a growing number of studies, describing the crucial role of the interaction between inflammatory/immune systems and muscle resident stem cells, gradually reveal the complexity of muscle regeneration (Aurora & Olson, 2014, Madaro & Bouche, 2014, Maffioletti et al., 2014, Sciorati et al., 2015).

In a healthy but damaged muscle, a regeneration process leads to the establishment of new myofibers and to an efficient repair of damaged tissue thus restoring the original integrity (Charge & Rudnicki, 2004). Abnormal development of fibrosis or intermuscular adipose tissue (IMAT) is a strong evidence of regeneration failure. It's now well characterized that the degree of IMAT accumulation is a good predictor of both muscle function and physical capacities in older adults (Brioché et al., 2016, Goodpaster et al., 2001, Marcus et al., 2012, Visser et al., 2002) as well as an accurate measure of Duchenne muscular dystrophy (Wren et al., 2008). Many studies have also demonstrated a development of fibrosis and IMAT in case of an altered immune system response, especially related to macrophage function impairment (Arnold et al., 2007, Contreras-Shannon et al., 2007, Saclier et al., 2013, Segawa et al., 2008, Shireman et al., 2007, Summan et al., 2006).

Numerous studies have highlighted the importance of fibro/adipogenic progenitors (FAPs), which are positive for the cell surface marker platelet-derived growth factor receptor alpha (PDGFR $\alpha$  or CD140a), for an efficient regeneration. FAPs are able to proliferate quickly after injury, participate in the phagocytosis of necrotic muscle fibers and support SCs-mediated efficient muscle regeneration (Heredia et al., 2013, Joe et al., 2010, Lemos et al., 2015). In contrast, in muscle disuse and pathological conditions, or even in the glycerol-induced muscle degeneration, FAPs contribute to IMAT and fibrosis development (Ieronimakis et al., 2016, Lukjanenko et al., 2013, Uezumi et al., 2010, Uezumi et al., 2011). Moreover, Uezumi et al. (2010) have demonstrated that only PDGFR $\alpha$  positive cells can differentiate into adipocytes in the glycerol-injected muscles thereby demonstrating the major implication of FAPs, which represent 98% of PDGFR $\alpha$  positive cells in a regenerating muscle.

The glycerol model, originally developed in rabbit (Kawai et al., 1990), is now a muscle regeneration model often used to investigate IMAT development and its related adipogenic processes (Joe et al., 2010, Lukjanenko et al., 2013, Mahdy et al., 2015, Pagano et al., 2015, Pisani et al., 2010, Uezumi et al., 2010). The study of Lukjanenko et al. (2013) has clearly shown an increase in the adipogenic response amplitude compared to a cardiotoxin injury, leading the glycerol model to be associated with IMAT development and accumulation. Another important finding of this study is that the glycerol-injected muscles showed a higher anti-inflammatory cytokine mRNA induction, as demonstrated by an approximately 2-fold higher induction of TGF- $\beta$ 1 and IL-10. The authors concluded that glycerol-injected muscles were characterized by a stronger anti-inflammatory response, suggesting a too early M1 to M2 macrophage transition in comparison to cardiotoxin-induced muscle regeneration, thus affecting IMAT development. In light of a recent study demonstrating that TNF $\alpha$ , released by M1 macrophages, induces FAPs apoptosis whereas TGF- $\beta$ 1, released by M2 macrophages, promotes their survival (Lemos et al., 2015), we can hypothesize that pro-inflammatory processes could be deregulated in the glycerol model. Thereby, this specific regeneration model leads to a major increase in IMAT development by FAPs compared to others classical skeletal muscle regeneration models.

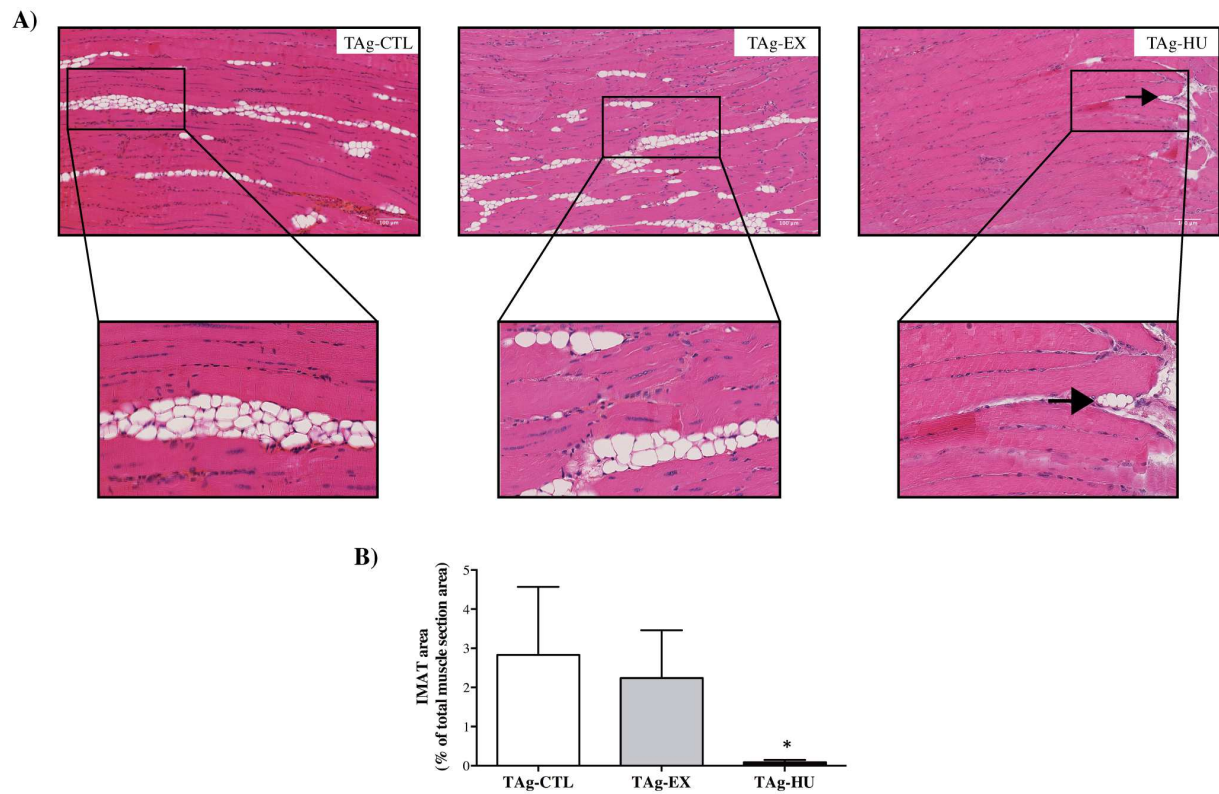
In a previous study, we have demonstrated for the first time an almost complete inhibition of IMAT development in glycerol-regenerating muscles under hindlimb unloading conditions (Pagano et al., 2015). This study raises the importance to take into account the level of mechanical constraints imposed on skeletal muscle during regeneration processes. Interestingly, several studies reported that hindlimb unloading appears to be a pro-inflammatory situation with macrophage infiltration (Andrianjafiniony et al., 2010, Gratas-Delamarche et al., 2014, Hirose et al., 2008, Kohno et al., 2012, Nguyen & Tidball, 2003), and lead us to wonder about the impact of inflammatory processes on the IMAT development inhibition observed in this situation. In this context, our study was designed i) in order to elucidate the underlying mechanisms related to the IMAT inhibition observed in our previous study and ii) to investigate the cellular processes at the origin of the IMAT development observed in this glycerol model of skeletal muscle regeneration.

## Results

### **Reduce mechanical constraints after injury decrease IMAT development and accumulation**

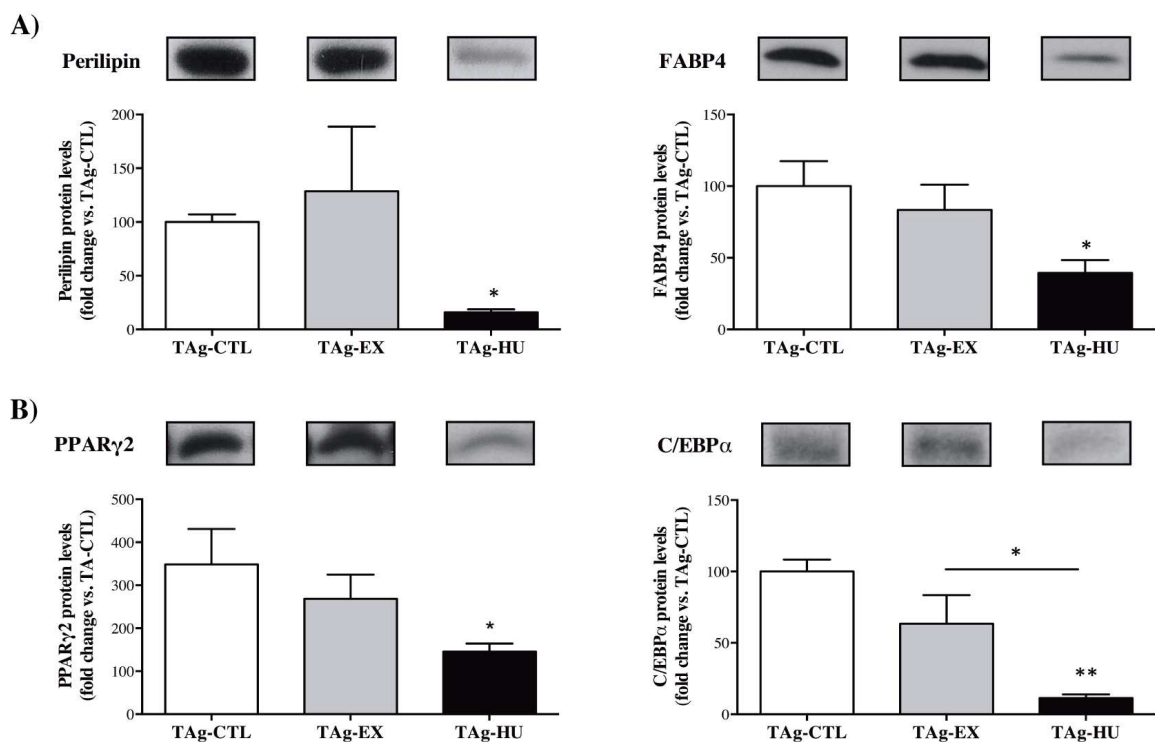
In our previous study, we have demonstrated that the area occupied by IMAT 21 days after a glycerol injection reached 2.83% of the total muscle CSA in the CTL group, and that HU condition almost completely inhibit IMAT development and the area occupied by IMAT (0.08%). In the present study, we also performed a trained group (EX) and we have not detected differences related to IMAT accumulation between the TAg-EX and TAg-CTL group. More precisely, the area occupied by IMAT 21 days after glycerol injection reached 2.24% of the total muscle CSA in the trained group (**Fig.1**). We further took the advantage measuring mature adipocyte markers by Western Blot on total muscle lysates to confirm and reinforce the presence/absence of IMAT. As expected, we observed no differences for perilipin and FABP4 markers in the TAg-EX group compared to the TAg-CTL group, but we found a clear decrease of these markers in the TAg-HU condition (-84% and -61% respectively, **Fig.2A**). We next investigated PPAR $\gamma$ 2 and C/EBP $\alpha$ , two important transcription factors implicated in adipogenesis, and we found a similar decrease in their protein expression in the TAg-HU condition only (-58% and -89% respectively, **Fig.2B**). All together, there results highlight an important role for reducing mechanical constraints immediately after injury to inhibit IMAT development and accumulation in glycerol-induced skeletal muscle injury.





**Figure 1. Effects of different mechanical constraint levels on IMAT accumulation**

**A)** Representative longitudinal paraffin-embedded muscle sections, stained with hematoxylin-eosin-saffron, from TAG of each experimental group (CTL, EX and HU), 21 days after glycerol muscle injury. **B)** Quantification of intermuscular adipose tissue (IMAT) area in percentage of total muscle section area from TAG of each experimental group (CTL, EX and HU), 21 days after glycerol muscle injury. \*  $p < 0.05$

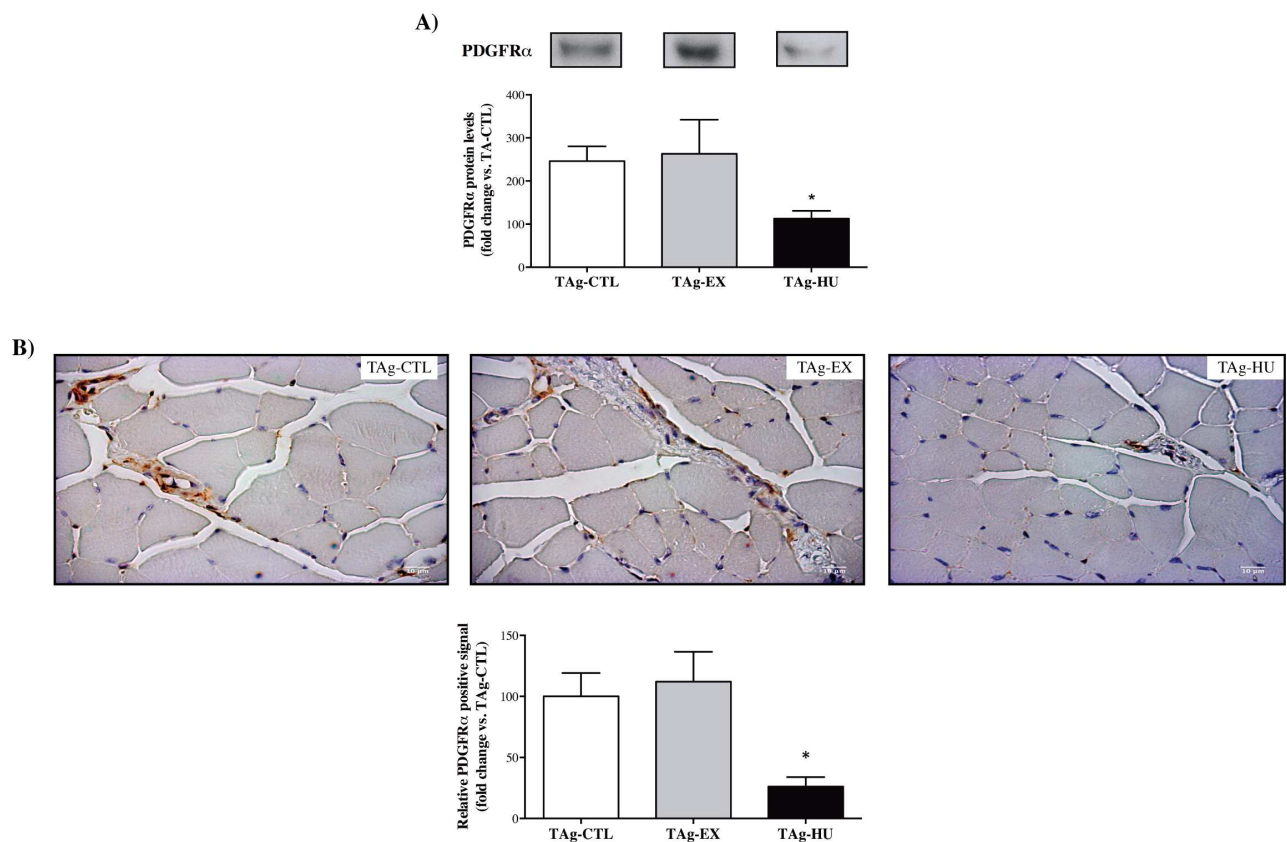


**Figure 2. Effects of different mechanical constraint levels on IMAT accumulation markers**

**A)** Perilipin and FABP4 protein levels from TAG of each experimental group (CTL, EX and HU), 21 days after glycerol muscle injury. **B)** PPAR $\gamma$ 2 and C/EBP $\alpha$  protein levels from TAG of each experimental group (CTL, EX and HU), 21 days after glycerol muscle injury. \*  $p < 0.05$  and \*\*  $p < 0.01$

### Glycerol-induced injury model disturbs FAPs apoptosis, which is restored by HU

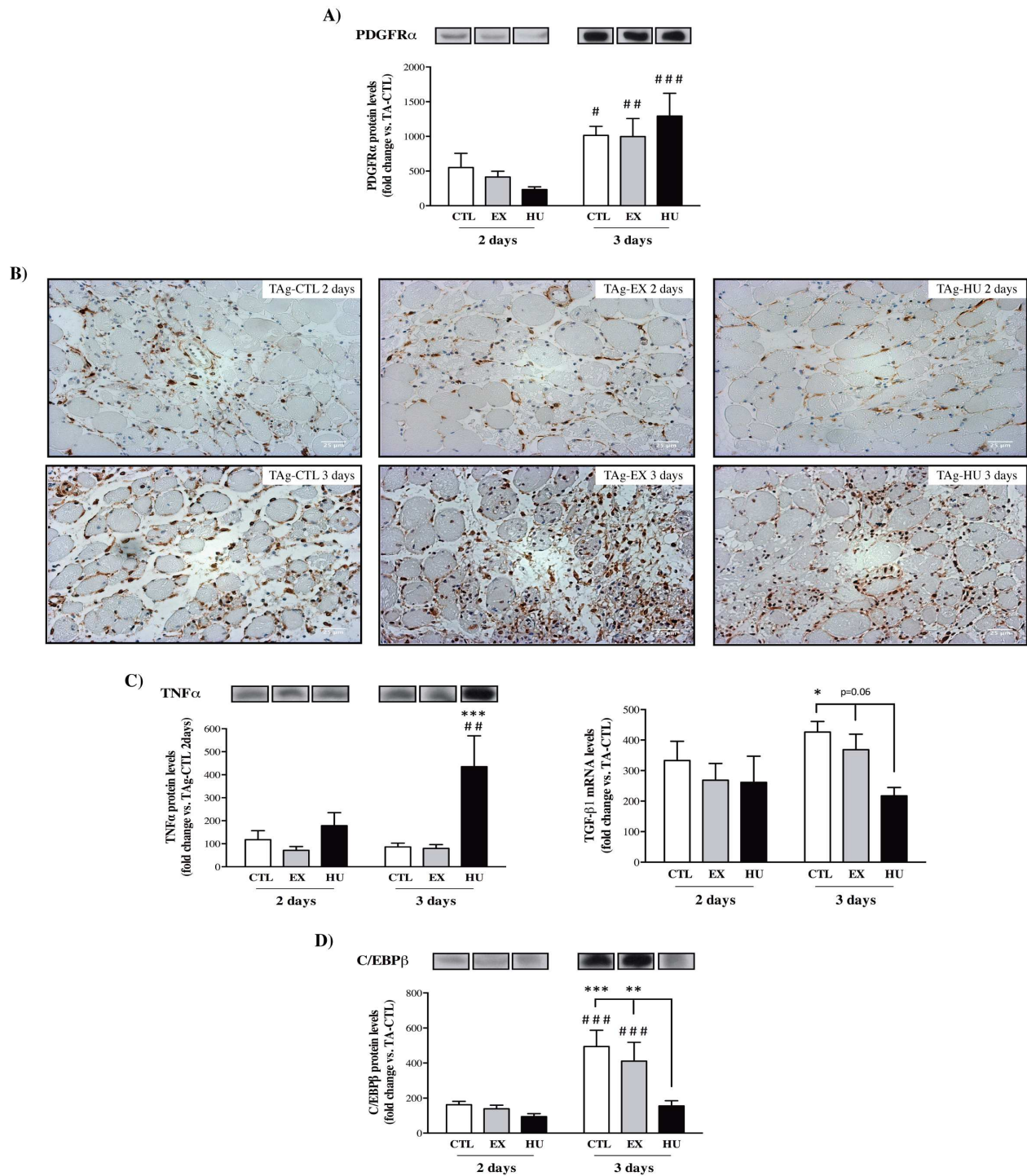
FAPs are known to rapidly proliferate after injury and to reach a peak of expression around 3 days after injury. After this key event, FAPs enter in an apoptosis phase and return to basal values observed in an uninjured muscle. In our experiments, we found a clear decrease in FAPs quantity, represented by the reliable expression of their specific cell surface marker PDGFR $\alpha$ , 21 days after glycerol-injection in our HU group compared to the CTL and EX groups (-54% and -57% respectively, **Fig.3A**). This result was next confirmed by our quantification of the number of PDGFR $\alpha$  positive cells by immunohistochemistry (-74% compared to the TAG-CTL group, **Fig.3B**). We thus observed that, unlike in the HU group, FAPs expression did not return to basal values in CTL and EX groups, and these results all matches with the degree of IMAT development.



**Figure 3. Effects of different mechanical constraint levels on FAPs cell surface marker PDGFR $\alpha$  expression**

*A) PDGFR $\alpha$  protein levels from TAg of each experimental group (CTL, EX and HU), 21 days after glycerol muscle injury. B) Representative PDGFR $\alpha$  immunostained histological transversal paraffin-embedded from TAg of each experimental group (CTL, EX and HU), 21 days after glycerol muscle injury and quantification of the PDGFR $\alpha$ -positive cells. \*  $p < 0.05$*

We next evaluated if the reported increase in PDGFR $\alpha$  expression was due to an enhancement in FAPs proliferation. We performed sacrifices 2 and 3 days after glycerol intra-muscular injection and found a massive and expected proliferation of FAPs in all groups, without any differences between them (**Fig.4A** and **Fig.4B**). Therefore, this result indicates strongly a defective FAPs apoptosis step in our CTL and EX groups, which is, on the contrary, restored by the HU condition. TNF $\alpha$  is a major cytokine, secreted by M1 pro-inflammatory macrophages, implicated in the promotion of FAPs apoptosis whereas TGF- $\beta$ 1, released by M2 macrophages, promotes their survival. We found a massive increase in the expression of TNF $\alpha$  only in our HU group, 3 days after injury (+405% compared to the TAg-CTL, **Fig.4C**) and this result was concomitant with a decreased induction in TGF- $\beta$ 1 mRNA (-49% compared to the TAg-CTL, **Fig.4C**). These results strengthen once again that FAPs apoptosis may be inhibited in our CTL and EX group, certainly leading to further IMAT development and accumulation. In parallel, and in order to consolidate the results obtained about IMAT development, we also quantify C/EBP $\beta$  protein expression in these early time points. This early key marker of adipogenesis is strongly inhibited 3 days after injury in our HU condition while its expression rises significantly in the two other groups (approximately a 3-fold increase, **Fig.4.D**), highlighting once again the important role of the HU condition in the inhibition of adipogenesis after glycerol injury.

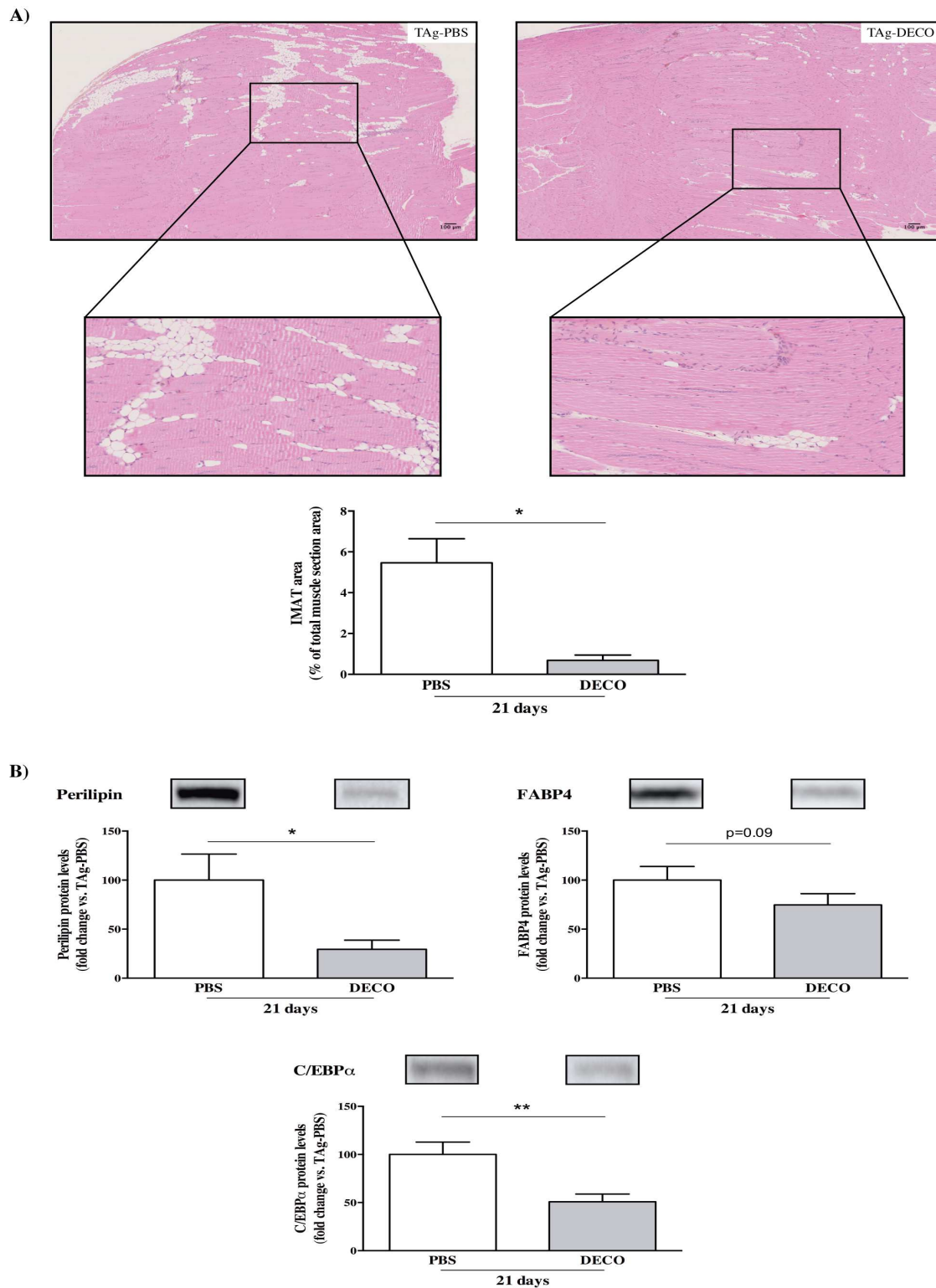


**Figure 4. Effects of different mechanical constraint levels, 2 and 3 days after glycerol muscle injury**

**A)** PDGFR $\alpha$  protein levels from TAG of each experimental group (CTL, EX and HU), 2 and 3 days after glycerol muscle injury. **B)** Representative PDGFR $\alpha$  immunostained histological transversal paraffin-embedded from TAG of each experimental group (CTL, EX and HU) 2 and 3 days after glycerol muscle injury. **C)** TNF $\alpha$  protein and TGF- $\beta$ 1 mRNA levels from TAG of each experimental group (CTL, EX and HU), 2 and 3 days after glycerol muscle injury. **D)** C/EBP $\beta$  protein level from TAG of each experimental group (CTL, EX and HU), 2 and 3 days after glycerol muscle injury. \*  $p < 0.05$ , \*\*  $p < 0.01$  and \*\*\*  $p < 0.001$  vs the TAG-CTL group at the same time after glycerol injury (effect of mechanical constraints variations). #  $p < 0.05$ , ##  $p < 0.01$  and ###  $p < 0.001$  vs the same group at 2 days after injury (time effect).

### **Decorin treatment inhibits IMAT development in the glycerol model of injury**

In order to confirm that IMAT development in the glycerol model of regeneration is due to an altered macrophages shift, and thus to an unbalanced cytokine responses, we further treated injured animals with decorin. Decorin, a small leucine-rich proteoglycan, is an extracellular matrix component of all collagen-containing tissues, and is known to be a strong inhibitor of the TGF- $\beta$  superfamily activity. In our experiments, decorin treatment strongly inhibited IMAT development and accumulation 21 days after glycerol injection. More precisely, IMAT reached around 6% of total muscle section area in the PBS-treated group, while the IMAT accumulation was largely inhibited with decorin treatment (about 1%, **Fig.5A**). Moreover, protein quantification of perilipin (-71%), C/EBP $\alpha$  (-49%) and, to a lesser extent, FABP4 (-25%) in glycerol-injured muscle treated with decorin also confirmed the decreased adipocyte differentiation compared to the PBS-treated one (**Fig.5B**). Finally, we also achieved sacrifices 5 and 9 days after glycerol intra-muscular injection and we reported a decrease, at day 5, in the expression of the major early adipogenesis factor C/EBP $\beta$  (-32%, **Fig.6A**).



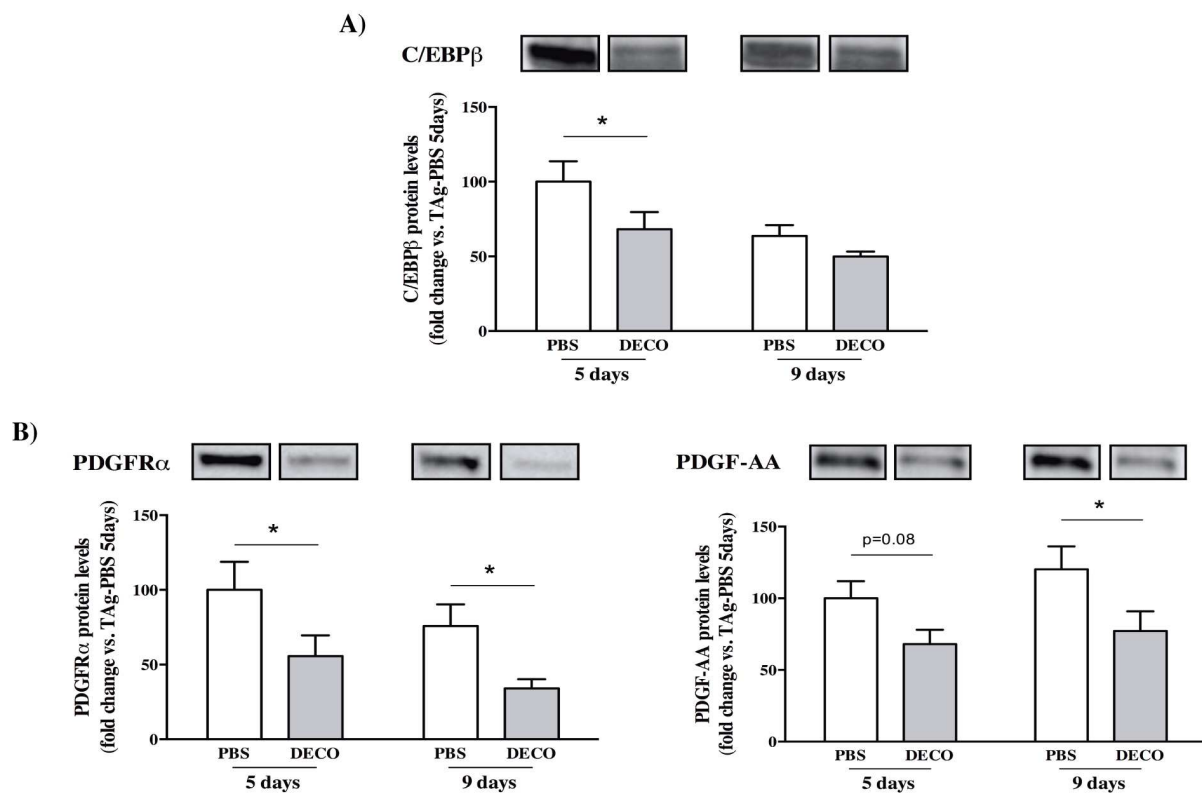
**Figure 5. Effects of decorin treatment on IMAT accumulation**

**A)** Representative histological longitudinal paraffin-embedded muscle sections, stained with hematoxylin-eosin-saffron from TAG of each experimental group (PBS and decorin-treated TAG) and quantification of intermuscular adipose tissue area in percentage of total muscle section area at 5 and 9 days after glycerol

muscle injury. **B)** Perilipin, FABP4 and C/EBP $\alpha$  protein levels from PBS-treated and decorin-treated TAG at 5 and 9 days after glycerol muscle injury. \*  $p < 0.05$  and \*\*  $p < 0.01$

### Decorin treatment prevents adipogenesis by restoring FAPs apoptosis

Our first experiments have highlighted a defective loss of FAPs, resulting in a higher expression of PDGFR $\alpha$  at 21 days after injury. Our results related to PDGFR $\alpha$  protein expression during the presumed FAPs apoptosis stage confirmed that decorin treatment would be able to restore FAPs apoptosis processes. Indeed, PDGFR $\alpha$  expression strongly decreased at day 5 and 9 in our decorin-treated injured muscles (-44% and -55% respectively, **Fig.6B**). Furthermore, we quantified the expression of PDGF-AA, implicated in FAPs proliferation, and also found a decreased expression of this growth factor at day 9 (almost significant at day 5) compared to the PBS-treated injured muscles (-36%, **Fig.6B**).



**Figure 5. Effects of decorin treatment, 5 and 9 days after glycerol muscle injury**

**A)** C/EBP $\beta$  protein levels from PBS-treated and decorin-treated TAG at 5 and 9 days after glycerol muscle injury. **B)** PDGFR $\alpha$  and PDGF-AA protein levels from PBS-treated and decorin-treated TAG at 5 and 9 days after glycerol muscle injury. \*  $p < 0.05$

## Discussion

Using the glycerol model of muscle regeneration, we highlighted the important impact of reducing mechanical constraints immediately after injury and emphasize right role of FAPs apoptosis stage for restricting IMAT development. Firstly, our previous study demonstrated the IMAT development inhibition, in the HU condition, 21 days after glycerol injury (Pagano et al., 2015). In the present study, we therefore performed, on the contrary, a trained group in order to increase mechanical constraints applied to the regenerating muscle. The IMAT percentage measured in this group, 21 days after injury, was almost the same that in the CTL group, and protein quantification of several key mature adipocyte and adipogenesis markers confirmed this result. These results reflect once again the importance to remove, or at least decrease, the mechanical constraints after injury for reaching efficient muscle regeneration. However, we and others also revealed a perturbed muscle regrowth after regeneration processes in HU condition (Matsuba et al., 2009, Mozdziak et al., 1998, Pagano et al., 2015), which highlights the importance of re-applying mechanical constraints as soon as possible for the recovery of fiber size after the needed rest period to regenerate optimally.

While many stem cells in the muscle environment can adopt the capacity to differentiate into adipocytes (Boppart et al., 2013, Judson et al., 2013, Sciorati et al., 2015, Uezumi et al., 2014), FAPs represent currently the population that appears to play a major role in IMAT development. The study of Uezumi et al. (2010) clearly showed that only progenitors expressing the cell surface receptor PDGFR $\alpha$  were able to differentiate into adipocytes in the glycerol model of muscle injury. Importantly, the study of Heredia et al. (2013) subsequently confirmed that PDGFR $\alpha$  was exclusively expressed by the FAPs after muscle injury. Monitoring FAPs fate is therefore a major objective to promote optimal muscle regeneration stages, preventing IMAT development and accumulation. FAPs are known to rapidly proliferate and reach a peak of expression around 3 days after injury (Joe et al., 2010, Lemos et al., 2015). In a healthy muscle, this population of muscle-resident stem cells help regeneration by improving muscle SCs myogenesis and also through their important phagocytic activity (Heredia et al., 2013, Joe et al., 2010). After this key event, FAPs enter in an apoptosis stage and return to basal values observed in an uninjured muscle, reflecting a required process for an efficient regeneration without fibrosis accumulation (Lemos et al., 2015). In our study, we highlighted an approximately 2-fold increase of PDGFR $\alpha$  protein expression 21 days after injury in the CTL and EX groups compared to the HU group.



Therefore, this last results can be interpreted either from an increase in FAPs proliferation or either from a deficiency in their apoptotic process, leading ultimately to IMAT development. Our study revealed no differences on FAPs proliferation at days 2 and 3 after glycerol injury between all experimental groups, thereby implicitly indicating a disturbed FAPs apoptosis in these groups. In accordance with the fact that  $TNF\alpha$ , released by M1 macrophages, contribute to FAPs apoptosis (Lemos et al., 2015), we also detected a strong increase in  $TNF\alpha$  protein expression 3 days after injury in the HU group. Thus, these results strongly suggest that altered FAPs apoptosis stage is a critical point, explaining, at least in part, the IMAT development in the glycerol model of muscle regeneration. Interestingly, the study of Lukjanenko et al. (2013) already raised a too early anti-inflammatory response in the glycerol model compared to the cardiotoxin model of muscle injury. These authors especially revealed an elevated response of TGF- $\beta$ 1 three days after glycerol injury and, in line with the study conducted by Lemos et al. (2015), this strong response of TGF- $\beta$ 1 promotes FAPs survival thought an inhibition of  $TNF\alpha$ -mediated FAPs apoptosis. Interestingly, we also highlighted a reduction in mRNA levels of TGF- $\beta$ 1 3 days after injury in our HU group. Thus, decreasing mechanical constraints during glycerol injury may participate to the recovery of FAPs apoptosis, thought an increased activity of  $TNF\alpha$ , and inhibits IMAT development.

In order to confirm or evaluate the involvement of the  $TNF\alpha$ /TGF- $\beta$ 1 axis in the IMAT development observed in the glycerol model, we treated injured mice with decorin. Decorin is a small leucine-rich proteoglycan localised in the extracellular matrix of all collagen-containing tissues. This myokine (Kanzleiter et al., 2014) is a major inhibitor of the TGF- $\beta$  superfamily activity, including TGF- $\beta$ 1 and myostatin (Acharjee et al., 2014, Hildebrand et al., 1994, Isaka et al., 1996, Kishioka et al., 2008, Li et al., 2007) and its overexpression leads to major improvements in muscle regeneration. In addition to improving muscle regeneration, decorin also limits the occurrence of fibrosis (Fukushima et al., 2001, Giri et al., 1997, Li et al., 2007, Sato et al., 2003, Zhu et al., 2007). Interestingly, decorin treatment was also found to improve macrophages activity (Comalada et al., 2003, Xaus et al., 2001). We therefore hypothesized that decorin treatment could inhibit IMAT development in the glycerol model by restoring  $TNF\alpha$ -mediated FAPs apoptosis thought TGF- $\beta$ 1 inhibition. Indeed, we firstly observed an inhibition of IMAT development in the decorin-treated compared to a PBS-treated and injured muscle and we confirmed this result by demonstrating a decrease in key mature adipocyte and adipogenesis markers expression. Then, analyse of PDGFR $\alpha$  marker at day 5 and 9 after injury, the critical time in which FAPs are in an apoptosis stage, revealed a

decreased expression with decorin treatment, together with a decrease in the expression of the growth factor PDGF-AA. These results highlight a decreased FAPs presence and may correspond to a restoration of FAPs apoptosis process, therefore leading to IMAT inhibition observed in this study with decorin treatment.

To conclude, this study raised three major points: i) decreasing mechanical constraints just after injury leads to a prevention of IMAT development in the glycerol model of muscle injury, ii) TNF- $\alpha$  mediated FAPs apoptosis response may be perturbed in this glycerol model, leading to IMAT development, and iii) decorin treatment, a TGF- $\beta$ 1 inhibitor, restores FAPs apoptosis stage and decreases IMAT development in this specific model of muscle injury. Finally, in addition to the potential clinical relevance of decorin treatment in all situations involving muscle plasticity and regeneration, this study also raised questions about the timing to resume muscle mechanical constraints after injuries in sports. To better precise this timing, further studies should contribute to define the time of the end of pro-inflammatory mechanisms, when the macrophages shift occurs and the FAPs enter in an apoptosis phase, leading to a muscle environment ready to achieve pro-regenerative and muscle fibers regrowth functions.

## **Materials and Methods**

### **Ethics statement**

This study was approved by the Committee on the Ethics of Animal Experiments of Languedoc Roussillon in accordance with the guidelines from the French National Research Council for the Care and Use of Laboratory Animals (CEEA-LR-14002). All efforts were made to minimize animal suffering.

### **Animals**

Experiments were carried out on at least 6-month-old C57BL6J female mice from our own stock. Animals were maintained on a 12h/12h light–dark cycle and provided with food and water ad libitum. Experiments were performed at 22°C.

### **Experimental groups and muscle sampling**

Experimental procedures were performed under anesthesia using isoflurane inhalation. In a first set of experiments, mice were injected with 25µl of 50% v/v glycerol in both tibialis anterior (TA<sub>g</sub>) and three experimental groups were formed: hindlimb-unloaded (HU), trained (EX) and control (CTL) groups for three different time points (2, 3 and 21 days). Tail-suspended experiments were conducted with suspension cages and the protocol used in other studies (Amblard et al., 2003, Malaval et al., 2008, Pagano et al., 2015). In a second set of experiments, concerning decorin treatment experiments, mice were also injected with 25µl of 50% v/v glycerol in both tibialis anterior (TA<sub>g</sub>) and two experimental groups were formed: a decorin-treated group (DECO) and a PBS-treated group (PBS) for three different time points (5, 9 and 21 days). Decorin treatments were administered 3 and 6 days after injury with intramuscular injections in the right TA<sub>g</sub> (50µg of decorin diluted in 25µl of PBS), the left TA<sub>g</sub> were injected with 25µl of PBS. At the end of the protocol, for each mouse, one TA<sub>g</sub> was rapidly dissected out and immediately fixed overnight in 4% paraformaldehyde solution at room temperature and then paraffin-embedded. The second TA<sub>g</sub> was dissected out and rapidly frozen in liquid nitrogen for quantification of protein content, as described elsewhere.

### **Training sessions**

Training sessions were conducted on a motor-driven treadmill (Exer-6M Treadmill; Columbus Instruments). In our training protocol, 2 days after glycerol injection mice

rigorously performed the same training consisting in 8 exercise sessions distributed along the remaining 19 days of protocol. At each exercise session, mice run at 10m/min during 10min, and running speed was increased by 1m/min every 2min during 16min (thus until 18m/min speed). The speed was maintained at 18m/min until 45min of exercise. Concerning the early time points sacrifices of our study (at 2 and 3 days after glycerol injection), in order to distance mice from the acute effects of training, the TAg-EX 2 days group performed an exercise session 1 day after the glycerol injection and was euthanized 1 day after the end of the exercise. The TAg-EX 3 days group performed two sessions, 1 and 2 days after glycerol injection, and was euthanized 1 day after the last session.

### **Histology**

TAg muscles were fixed in 4% neutral-buffered formalin (24h) and paraffin-embedded. The paraffin-embedded tissues were sectioned (3 $\mu$ m thick) every 50 $\mu$ m, and sections were processed by hematoxylin/eosin/saffron (H/E/S) staining. Stained slides were digitalized with the NanoZoomer slide scanner with an x40 objective (Hamamatsu) for subsequent IMAT area evaluation. Images were analysed with ImageJ (1.46r version) software.

### **Immunohistochemistry**

The immunohistochemistry protocol was globally performed as previously described (Pirot et al., 2014). Briefly, TAg muscle sections were deparaffinised, rehydrated, and incubated for antigen retrieval in EDTA buffer at 100°C for 10 min. Sections were incubated in 0.3% H<sub>2</sub>O<sub>2</sub> for 20 min and endogenous biotins were blocked using the Avidin-Biotin Blocking kit (Vector Laboratories, CliniSciences). Nonspecific antibody binding was blocked by incubation with TBS containing 20% normal goat serum for 30 min at RT. Sections were then incubated ON at 4°C with anti-PDGFR $\alpha$  antibody diluted at 1:250 or non-specific rabbit IgG (Vector Laboratories, CliniSciences) at the same concentration. Antibody binding was revealed by the streptavidin/biotin-peroxydase complex method using ABC Vectastain kit and the peroxidase substrate DAB (Vector Laboratories, Clinisciences).

### **mRNA extraction and real-time polymerase chain reaction (qPCR)**

Total RNAs were isolated from homogenate muscle samples using the RNeasy Fibrous Tissue Mini Kit following the manufacturer's instructions (Qiagen). RNA concentration was determined by spectrophotometric analysis (Eppendorf AG, Hamburg, Germany), and integrity was checked by the OD<sub>260nm</sub>/OD<sub>280nm</sub> absorption ratio (>1.7). Reverse transcription reaction was performed with 2 $\mu$ g of total RNA using the RevertAid First Strand

cDNA Synthesis kit (Thermo Scientific) according to the manufacturer's instructions. qPCR analysis was performed in a MiniOpticon detection system (Bio-Rad, Hercules, CA) with 10 $\mu$ L of KAPA SYBR Fast Universal Readymix (CliniSciences), 300nM of both forward and reverse primers, 2 $\mu$ L of diluted cDNA template and water to a final volume of 20 $\mu$ L. PCRs were performed in duplicate using the following cycle parameters: 30s at 98°C, 40 cycles of 1s at 95°C and 15s at 60°C. Relative mRNA levels were normalized to rp-S9 and tubulin housekeeping gene levels, which were unaffected by the experiment. Results are expressed using the comparative cycle threshold (CT). The relative changes in the level of a specific gene were calculated with the  $\Delta\Delta$ CT formula. Tgfb1 (NM\_011577.1) primer sequences are GCAACATGTGGAAGCTCTACCAG for the forward primer and CAGCCACTCAGGCGTATCA for the reverse primer.

### **Proteins isolation and Western Blotting**

Muscle samples were homogenized in 10 volumes of lysis buffer (50mM Tris-HCl pH7.5, 150mM NaCl, 1mM EGTA, 100mM NaF, 5mM Na<sub>3</sub>VO<sub>4</sub>, 1% Triton X-100, 1% SDS, 40mM  $\beta$ -glycerophosphate and protease inhibitor mixture (P8340; Sigma-Aldrich)) and centrifuged at 10.000g for 10 min (4°C). 60 $\mu$ g of protein extracts were loaded on home made SDS-polyacrylamide gels or on Stain Free™ 4-20% precast gels (4568095; Bio-Rad) before electrophoretic transfer onto a nitrocellulose membrane (Bio-Rad; Trans-Blot® Turbo™ Blotting System). After transfer, membranes were blocked with 50mM Tris-HCl pH7.5, 150mM NaCl, and 0.1% Tween 20 (TBS-T) containing 5% skimmed milk or BSA and incubated overnight at 4°C with primary antibodies. Membranes were incubated for 1 h with a peroxidase-conjugated secondary antibody. Immunoblots were revealed using a Pierce ECL kit (32106; Thermo Scientific) and proteins were visualized by enhanced chemiluminescence using the ChemiDoc™ Touch Imaging System and quantified with Image Lab™ Touch Software (version 5.2.1).  $\beta$ -actin was used as a loading control for home made gels whereas Stain Free technology was used with Bio-Rad precast gels. A great number of methodological studies already validated this technology and explained in detail its functioning (Colella et al., 2012, Faden et al., 2016, Gilda & Gomes, 2013, Gilda & Gomes, 2015, Gurtler et al., 2013, Rivero-Gutierrez et al., 2014, Vigelso et al., 2014, Zeitler et al., 2016).

### **Antibodies**

Anti-PDGFR $\alpha$  (#3174), anti-perilipin (#9349), anti-FABP4 (#3544) and anti-C/EBP $\alpha$  (#8178) primary antibodies were purchased from Cell Signaling and used at 1:500. Anti-PPAR $\gamma$  (sc-

7273; 1:200), anti-C/EBP $\beta$  (sc-150; 1:200) anti-TNF $\alpha$  (sc-52746; 1:200), anti-PDGF-A (sc-128; 1:200) and anti- $\beta$ -actin (sc-81178; 1:4000) primary antibodies were purchased from Santa Cruz. Anti-mouse (sc-2005) and anti-rabbit (sc-2004) HRP-conjugated secondary antibodies were purchased from Santa Cruz and used at 1:4000.

### **Statistics**

All values are expressed as mean  $\pm$  SEM and the significance level was set as  $p < 0.05$ . Differences between the two groups were evaluated for significance using the unpaired Student t-test or the Mann-Whitney test when data deviated from a normal distribution. When more than two simultaneous comparisons were made, one-way or two-way ANOVA was employed to compare data (level of mechanical constraints or treatment and time factors). When a significant effect was indicated, a Fisher significant difference post hoc test was performed.

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## **Conflict of Interest**

The authors have no conflicts of interest to disclose.

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## Discussion de l'étude 2

Le but de cet étude était d'élucider les mécanismes permettant l'inhibition du développement des IMAT en condition d'hypoactivité (modèle HU) par rapport à la condition contrôle de notre précédente étude. L'autre objectif était celui de comprendre les mécanismes intimes à l'origine du développement des IMAT dans ce modèle glycérol de régénération musculaire.

Tout d'abord, notre étude a une nouvelle fois souligné l'importance de diminuer les contraintes mécaniques afin d'inhiber l'apparition d'IMAT. De plus, nous avons rajouté dans cette étude un groupe d'animaux subissant une augmentation des contraintes mécaniques (via l'exercice physique) dès 2 jours après injection de glycérol et jusqu'à 21 jours (groupe EX). Les résultats concernant l'accumulation d'IMAT du groupe EX ne sont pas différents de notre groupe contrôle et la quantification de plusieurs marqueurs clés d'adipocytes mature et des processus adipogéniques ont confirmé ce résultat (**Fig.1 et 2**).

Si la littérature a détecté de nombreuses populations de cellules souches pouvant s'engager dans un lignage adipogénique (Boppart et al., 2013, Judson et al., 2013, Sciorati et al., 2015, Uezumi et al., 2014b), les FAPs représentent la population qui joue un rôle majeur dans le développement d'IMAT (Heredia et al., 2013, Uezumi et al., 2010). Les FAPs sont connus pour proliférer et atteindre un pic d'expression aux alentours de 3 jours après blessure et, dans un muscle sain, aident à la régénération musculaire (Joe et al., 2010, Lemos et al., 2015). Après cette étape clé, les FAPs entrent dans une phase d'apoptose et leur expression retourne à des valeurs basales observée dans un tissu musculaire intact (Lemos et al., 2015). Dans notre étude, nous avons mis en lumière une augmentation de l'expression de PDGFR $\alpha$ , soulignant une présence accrue des FAPs, 21 jours après blessure dans nos groupes CTL et EX comparé au groupe HU (**Fig.3**). Par conséquent, cette situation résulte, soit d'une augmentation de la prolifération des FAPs, soit d'une déficience de leur processus apoptotique, amenant ainsi au développement d'IMAT dans le modèle glycérol de régénération.

Nous avons ensuite réalisé des sacrifices à 2 et 3 jours après injection de glycérol dans nos trois groupes expérimentaux et nous n'avons trouvé aucune différence d'expression de PDGFR $\alpha$ , suggérant une prolifération des FAPs équivalente (**Fig.4A et 4B**). Au regard des résultats soulignant une présence accrue des FAPs, 21 jours après blessure dans nos groupes

CTL et EX comparé au groupe HU, une prolifération équivalente des FAPs 2 et 3 jours après injection suggère alors implicitement une diminution du processus d'apoptose des FAPs.

L'étude de Lemos et al. (2015) a récemment mis en évidence le rôle clé du système immunitaire dans la régulation des FAPs par apoptose. En effet, les macrophages de type M1 (pro-inflammatoires) recrutés, dès les premiers jours, au niveau du site de la lésion sécrètent du TNF $\alpha$ , une cytokine induisant la mort cellulaire des FAPs. Cette étude a également montré que TGF- $\beta$ 1, sécrété par les macrophages de type M2 (anti-inflammatoires), permettait au contraire de promouvoir la survie cellulaire des FAPs et inhibait donc l'action du TNF $\alpha$ . De manière intéressante, nous avons également souligné une forte augmentation significative de l'expression de TNF $\alpha$  3 jours après blessure dans le groupe HU comparé aux groupes CTL et EX en parallèle d'une diminution significative des ARN messagers codant pour TGF- $\beta$ 1 (**Fig.4C**). Ainsi, le modèle de suspension du train arrière utilisé pour diminuer les contraintes mécaniques permettrait de participer à la récupération du processus apoptotique des FAPs, perturbé dans le modèle glycérol de régénération, et ainsi à une inhibition du développement des IMAT.

Afin de confirmer l'implication de l'axe TNF $\alpha$ /TGF- $\beta$ 1 dans l'apparition d'IMAT par le modèle glycérol, nous avons ensuite traité nos souris avec de la décorine. La décorine est un protéoglycan riche en leucine localisé dans la matrice extra-cellulaire de tous les tissus contenant du collagène. Cette myokine (Kanzleiter et al., 2014), est un inhibiteur majeur de l'activité de la superfamille des TGF- $\beta$ , incluant notamment TGF- $\beta$ 1 et la myostatine (Acharjee et al., 2014, Hildebrand et al., 1994, Isaka et al., 1996, Kishioka et al., 2008, Li et al., 2007), et sa surexpression améliore la régénération musculaire. Par ailleurs, en plus d'améliorer les processus de régénération, la décorine permet également de limiter l'apparition de fibrose (Fukushima et al., 2001, Giri et al., 1997, Li et al., 2007, Sato et al., 2003, Zhu et al., 2007) et d'augmenter l'activité des macrophages (Comalada et al., 2003, Xaus et al., 2001). Nous avons donc émis l'hypothèse qu'un traitement avec de la décorine permettrait d'inhiber le développement d'IMAT dans le modèle glycérol de régénération en restaurant l'apoptose des FAPs. Nous avons choisi de traiter nos animaux à la décorine à 3 et 6 jours après injection de glycérol, phase durant laquelle s'opère normalement les processus apoptotiques des FAPs, médiés par la sécrétion de TNF $\alpha$  par les macrophages de type M1, qui semblent perturbés dans le modèle glycérol de régénération. Cette hypothèse a été vérifiée puisque nous avons reporté un effet inhibiteur du traitement avec la décorine sur le développement des IMAT 21 jours après injection de glycérol (**Fig.5**). Nous avons ensuite

réalisé des sacrifices 5 et 9 jours après blessure, correspondant à la phase dans laquelle les FAPs sont en phase apoptotique et reviennent à des valeurs basales. Notre étude a alors mis en évidence une diminution de l'expression de PDGFR $\alpha$  et de PDGF-AA (**Fig.6B**), un facteur essentiel pour la prolifération des FAPs. Ce résultat semble démontrer une restauration de l'apoptose des FAPs avec le traitement avec la décorine. Ainsi, cette augmentation des processus apoptotiques des FAPs permet une inhibition du développement des IMAT dans le modèle glycérol.

Pour conclure, cette étude a souligné 3 points majeurs concernant le développement des IMAT : i) nous avons confirmé qu'une diminution des contraintes mécaniques immédiatement après blessure musculaire était nécessaire pour une régénération efficace sans développement d'IMAT, ii) la réponse pro-inflammatoire, représentée par l'expression de TNF $\alpha$ , est certainement perturbée dans le modèle glycérol, menant ainsi au développement d'IMAT et iii) un traitement avec de la décorine, un inhibiteur de TGF- $\beta$ 1, permet de restaurer l'apoptose des FAPs et diminue le développement des IMAT dans ce modèle spécifique de régénération.

**ETUDE 3 : 3 JOURS DE REDUCTION D'ACTIVITE CHEZ L'HOMME  
INDUIT LE DEVELOPEMENT D'IMAT**

Article 3

**3 days of human skeletal muscle disuse promote fatty infiltrations  
development**

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### Contexte de l'étude 3

Comme décrit dans le premier chapitre de la revue de littérature, de nombreuses situations physiologiques ou pathologiques impliquant une diminution des sollicitations fonctionnelles du muscle strié squelettique engendrent un déconditionnement musculaire. L'environnement en microgravité, réel ou simulé, sont des situations bien décrites dans la littérature pour engendrer ce phénomène qui se caractérise principalement par une perte de masse et de force musculaire mais également un shift phénotypique vers des fibres rapides, augmentant de ce fait la fatigabilité musculaire (Baldwin et al., 2013). Cependant, bien que la masse musculaire soit intimement liée à la force musculaire dans un muscle sain, de nombreuses études ont également soulevé que la perte de force excédait largement les pertes de masse dans des situations de déconditionnement musculaire (Berg et al., 1997, Delmonico et al., 2009, Di Prampero & Narici, 2003, Jubrias et al., 1997). Ainsi, ces études mettent en évidence le rôle potentiel d'autres facteurs participant à la perte de force.

Parmi ces facteurs, le phénomène de développement et d'accumulation d'infiltrations graisseuses (IMAT) joue certainement un rôle majeur. Pourtant naturellement présents mais de manière variable chez l'homme, de nombreuses études rapportent que le développement et l'accumulation d'IMAT apparaît dans de nombreuses situations (pathologiques ou non) toutes intimement liées au phénomène de déconditionnement musculaire : sarcopénie (Brioche et al., 2016, Marcus et al., 2010), inactivité (Manini et al., 2007), obésité et diabète de type II (Gallagher et al., 2014, Goodpaster et al., 1999, Karampinos et al., 2012), dénervation (Ceylan et al., 2014, Dolor et al., 1998), rupture tendineuse (Farshad et al., 2011, Kuzel et al., 2013), régénération perturbée (Contreras-Shannon et al., 2007, Pagano et al., 2015, Shireman et al., 2007) ou encore Dystrophie Musculaire de Duchenne (Uezumi et al., 2014a, Wren et al., 2008). Les IMAT ont majoritairement été étudiés dans un contexte de vieillissement et d'obésité, et leur degré d'accumulation a été corrélé avec une diminution de la fonction musculaire et l'apparition d'une insulino-résistance (Delmonico et al., 2009, Goodpaster et al., 2000a, Miljkovic-Gacic et al., 2008, Visser et al., 2005).

Cependant, si les IMAT sont connus pour être intimement liés à de nombreuses situations pathologiques ou physiologiques, il semble que leur développement soit largement dépendant d'une inactivité et/ou d'une hypoactivité musculaire (Brioche et al., 2016, Farr et al., 2012, Leskinen et al., 2009, Manini et al., 2007, Tuttle et al., 2011). L'étude de Manini et al. (2007) a par exemple démontré, chez des jeunes adultes sains, une augmentation de l'accumulation d'IMAT de 15 et 20% sur différents groupes musculaires à la suite de 4 semaines d'inactivité.



De manière intéressante, une étude longitudinale menée sur 32 années impliquant des jumeaux, a également démontré que le jumeau inactif avait une augmentation de 54% du volume occupé par les IMAT en comparaison avec son jumeau physiquement actif (Leskinen et al., 2009).

Même si de nombreuses études réalisées chez l'homme sont disponibles dans la littérature concernant l'accumulation d'infiltrations graisseuses dans de nombreuses situations impliquant une diminution de l'activité musculaire, aucune ne présente les mécanismes à son origine. De plus, aucune étude n'est pour l'instant disponible sur l'effet d'une courte période d'inactivité, ni sur l'effet de la microgravité simulée, sur le développement d'IMAT. L'objectif de cette étude était donc d'investiguer les effets d'une courte période de microgravité simulée sur le développement des infiltrations graisseuses. Nous avons donc saisi l'avantage d'avoir pu utiliser le modèle humain de dry immersion (DI) afin d'étudier les effets de 3 jours d'inactivité sévère sur le développement des IMAT. Cette étude s'est inscrite dans le contexte des expérimentations humaines menées par l'agence spatiale française (Clinique spatiale, MEDES, Toulouse). Le modèle d'immersion sèche (Dry Immersion, DI), détaillé dans la partie 1.3.2. de la revue de littérature, a été utilisé afin d'induire un déconditionnement musculaire rapide et de courte durée chez l'homme (3 jours).

**3 days of human skeletal muscle disuse promote fatty infiltrations  
development**

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**Running title:** Dry immersion increases IMAT

## **Abstract**

Skeletal muscle is in constant adaptation due to many environmental and intrinsic fluctuations, which is defined as its plasticity. A decrease in mechanical constraints lead to muscle deconditioning, a well-described phenomenon defined by a loss of muscle mass, together with a loss of strength and muscle power. Fatty infiltrations, or IMAT for intermuscular adipose tissue, are now well-recognized component of muscle deconditioning. Although IMAT may be a variable part of healthy human skeletal muscle, its increase and accumulation are linked to muscle dysfunction and it seems that their development is largely attributable to inactivity. While several human studies have already reported that inactivity leads to IMAT development, none has been conducted during a short-time period of inactivity. In our study, we therefore took the advantage of using the dry immersion (DI) model of simulated microgravity to induce severe short-time period of muscle inactivity. Skeletal muscle biopsies were obtained from the vastus lateralis (VL) of healthy males (n=12; age: 32 ± 6), before and after 3 days of DI. We firstly showed that only 3 days of DI were able to induce a decrease in VL myofibers cross-sectional areas, predominantly in slow-twitch myofibers. We then highlighted a major increase in the protein expression of perilipin and FABP4, two key markers commonly used to assess IMAT quantification. We also observed an induction of C/EBP $\alpha$  mRNA, indicating a raise in the late adipogenic processes, driving to IMAT development. While many stem cells in muscle environment can adopt the capacity to differentiate into adipocytes, Fibro-adipogenic progenitors (FAPs) represent currently the population that appears to play a major role in IMAT development. In our study, we highlight a raise in PDGFR $\alpha$  expression, the specific cell surface marker of FAPs, in response to 3 days of DI, and it is well recognized that an unfavorable muscle environment drive FAPs to ectopic adiposity and fibrosis. This study is therefore the first one to emphasize that a short-time period of severe inactivity is able to induce muscle deconditioning with IMAT development and accumulation. Our study also reveals that FAPs could be the main implicated resident muscle stem cells population.

**Keywords:** Skeletal muscle, disuse, inactivity, microgravity, dry immersion, fat infiltration, adipogenesis, FAPs

## **Introduction**

Skeletal muscle is the most abundant tissue in the human body representing about 40% of the body weight and about 30% of the basal energy expenditure (Reid & Fielding, 2012). This tissue is in constant adaptation due to many environmental and intrinsic fluctuations, which is defined as its plasticity. An increase in stimulation, exercise, or nutrition can cause a positive protein balance and involve muscle hypertrophy and reinforcement (Egan & Zierath, 2013, Sartorelli & Fulco, 2004). Conversely, a decrease in mechanical constraints leads to muscle deconditioning and atrophy (Baldwin et al., 2013, Cros et al., 1999, Hanson et al., 2013, Kawashima et al., 2004, Manini et al., 2007, Pagano et al., 2015).

Muscle deconditioning is now a well-described phenomenon, defined by a loss of muscle mass, together with accompanied by a loss of strength and muscle power (Cruz-Jentoft et al., 2010, Fielding et al., 2011). However, there is now a growing body of evidence that the loss of strength and power mostly exceeds the loss of muscle mass observed after inactivity (Berg et al., 1997, Delmonico et al., 2009, Di Prampero & Narici, 2003, Jubrias et al., 1997), suggesting that other factors are involved. Among these factors, the phenomenon of fatty infiltration accumulation (or IMAT for Intermuscular Adipose Tissue) may play a critical role. These fat cells, located under the epimysium, between muscle fibers and bundles fibers, represent real adipocytes clusters, that is ectopic fat depot, localized outside muscle cells, and do not be confused with intramyocellular triglyceride accumulation (Addison et al., 2014, Vettor et al., 2009). Although IMAT may be a variable part of healthy human skeletal muscle, its increase and accumulation are linked to muscle dysfunction, deconditioning, and even disrupted regeneration processes (Addison et al., 2014, Brioche et al., 2016, Marcus et al., 2010, Pagano et al., 2015, Sciorati et al., 2015, Uezumi et al., 2014b). An accumulation of these fatty infiltrations was observed in many conditions closely linked to muscle deconditioning: inactivity (Leskinen et al., 2009, Manini et al., 2007, Tuttle et al., 2011), denervation (Ceylan et al., 2014, Dulor et al., 1998), diabetes (Gallagher et al., 2014, Goodpaster et al., 1999, Karampinos et al., 2012), tenotomy (Kuzel et al., 2013, Laron et al., 2012) or even sarcopenia (Brioche et al., 2016, Marcus et al., 2010, Song et al., 2004). Even though IMAT accumulation is strongly linked to many different physiological and/or pathological situations, it seems that fatty development in muscles is more related to inactivity. Indeed, several studies demonstrated a negative correlation between physical activity and amount of fatty infiltrations (Brioche et al., 2016, Farr et al., 2012, Leskinen et al., 2009, Manini et al., 2007, Tuttle et al., 2011). The study of Manini et al. (2007)

highlighted in healthy young adults, an increase in IMAT content of 15% in the thigh and 20% in the calf, after 4 weeks of unilateral lower limb suspension. Interestingly, the longitudinal study of Leskinen et al. (2009) also revealed that IMAT accumulation was greater in inactive co-twins compared to their active counterparts.

While many stem cells in muscle environment can adopt the capacity to differentiate into adipocytes, fibro-adipogenic progenitors (FAPs) represent for the moment the main population to play a major role in IMAT development. The study of Uezumi et al. (2010) clearly showed that only progenitor cells expressing the cell surface receptor PDGFR $\alpha$ , representing FAPs in this study, were able to differentiate into adipocytes after muscle injury induced by the glycerol model of regeneration in mice. Importantly, the study of Heredia et al. (2013) subsequently confirmed, after muscle injury, that PDGFR $\alpha$  was exclusively expressed by the FAPs. Other studies have also confirmed the adipogenic potential of FAPs, *in vivo* (Heredia et al., 2013, Uezumi et al., 2011).

In the past decades, the scientific community validated several selected cell, animal, and human experimental models for studying and deciphering the processes of muscle wasting. Nowadays, ground-based models of simulated microgravity protocols contribute largely to scientific studies exploring muscle disuse and aging (Baldwin et al., 2013, Briocche et al., 2016). Continuous exposure to bed rest in head-down tilt position have been one of the most used model of simulated microgravity. Studies using this model have produced and continue to generate a lot of data concerning the effects of inactivity on healthy individuals (Arentson-Lantz et al., 2016, Berg et al., 1997, Buehlmeier et al., 2013, Chopard et al., 2005, Chopard et al., 2009, Mutin-Carnino et al., 2014). However, the dry immersion (DI) model of simulated microgravity appears to induce much more quickly the muscle deconditioning changes observed with head-down bed rest model (Navasiolava et al., 2011). Indeed, DI involves immersing a subject in thermoneutral water and, for a relatively short period, this model can faithfully reproduce most of physiological effects of microgravity, including centralization of body fluids, support unloading, and hypokinesia. Originally used and described by Shulzhenko et al. (1976), muscle atrophy following DI was observed by several authors, and predominantly for slow fibers (Litvinova et al., 2004, Moukhina et al., 2004, Shenkman et al., 1997, Shenkman et al., 2004), associated with a decrease in sarcomere protein content (Shenkman et al., 2004).

To date, only the study of Manini et al. (2007) investigated the effects of a human ground-based model of muscle disuse on IMAT development, and no study has been conducted within a short-time period of inactivity. We therefore took the advantage of using the DI model to investigate the effects of 3 days of DI on muscle IMAT development and accumulation markers.

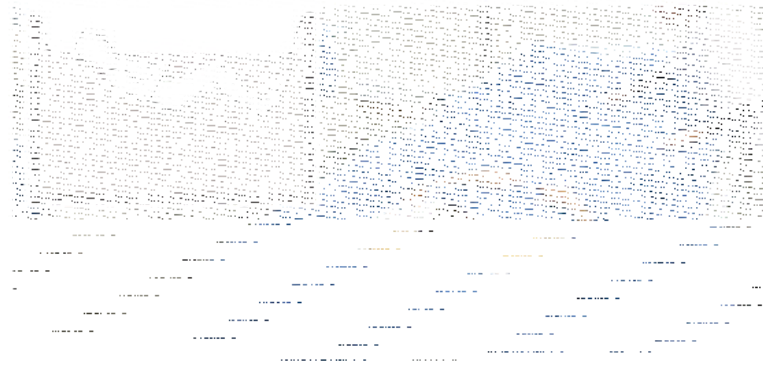
## **Material and Methods**

### **Subjects and ethics statement**

Twelve healthy male subjects were selected for the experiment. (age:  $31.8 \pm 4.1$  ; size:  $179 \pm 6$  cm; weight:  $74.8 \pm 5.6$  kg; BMI:  $23.6 \pm 1.2$ ). The subjects had no medical history or physical signs of neuromuscular disorder, they were non-smokers, taking no drugs and medication. All subjects gave informed consent to the procedures of the experimentation, which was approved by the local Ethics Committee (CPP Sud-Ouest outre-Mer I, France) in accordance with the Declaration of Helsinki. All experiments were conducted at the space clinic of the Institute of Space Medicine and Physiology (Medes-IMPS, Rangueil hospital) in Toulouse (France) and was sponsored by the French National Space Agency (CNES).

### **Overall study design**

This experiment consisted in a period of dry immersion (DI) for 3 days with a 3-day ambulatory control period before DI and a 2-day recovery period after DI. In the ambulatory and recovery periods, all subjects remained active and ambulatory and subjects were asked to not exercise during the 8 days of the study. During the DI period, subjects remained immersed in a supine position in a controlled thermo-neutral bath ( $33 \pm 0.5^{\circ}\text{C}$ ) continuously, (**Fig.1**) except for a daily 20-minute extraction for toilet procedure and weighting (in bed rest position), and were instructed to not produce any unnecessary movements with their limbs. The study was conducted in a quiet room at a stable temperature of  $\sim 25^{\circ}\text{C}$ . Each subject had a daily medical examination and the MEDES team took several standardized measurements. For example, discomfort and psychological assessments were made via questionnaires and body temperature was taken twice daily with a tympanic thermometer. Subjects received three solid meals/day during the study with the requirement to finish all meals. The individual energy intake was calculated by multiplying resting metabolic rate with a physical activity level of 1.6 during pre and post DI and 1.3 during DI. Coffee, tea, alcohol, smoking, and drugs were prohibited throughout the experiment. Only paracetamol was allowed if needed.



*Figure 1. Dry immersion experimental model (taken from Treffel et al., (2016))*

### **Muscle biopsy**

Skeletal muscle biopsy was performed before DI (Pre-DI, 8 to 13 days before DI) and during the final day of DI (Post-DI, before reambulation) from the vastus lateralis (VL) according to a well-established Bergstrom method (Bergstrom, 1975). Pre- and post-DI biopsies were obtained from the same leg, in a location as close as possible and, for each biopsy, two pieces were selected with an optic microscope for histological analyzes. One piece was immediately embedded in small silicone casts filled with a cryoprotector OCT, immediately frozen in liquid nitrogen, and stored at  $-80^{\circ}\text{C}$  until further analysis. The other piece was immediately fixed overnight in 4% paraformaldehyde solution at room temperature for 24h and then paraffin-embedded. The remaining biopsy was rapidly frozen in liquid nitrogen, and stored at  $-80^{\circ}\text{C}$ , for mRNA and protein content quantification.

### **Cryosection immunohistochemistry**

Transverse serial cross sections ( $10\mu\text{m}$  thick) of VL samples were obtained using a cryostat maintained at  $-25^{\circ}\text{C}$ . Before labeling, sections were dried and fixed 10 minutes into acetone. Sections were then washed in PBS, blocked and permeabilized with 0.1% Triton-X100 and 20% horse serum. Sections were incubated with primary antibodies for 1h at  $37^{\circ}\text{C}$ , followed by washes in PBS and incubation with the secondary antibody for 1h at  $37^{\circ}\text{C}$ . Fiber typing was manually determined, and the fiber sizes were analyzed with ImageJ (1.46r version) software.



### **Paraffin-embedded histological and immunochemical analyzes**

Muscle biopsies were fixed in 4% neutral-buffered formalin (24h) and paraffin-embedded. The paraffin-embedded tissues were sectioned (3µm thick), and sections were processed by hematoxylin/eosin/saffron (H/E/S) staining. Stained slides were digitalized with the NanoZoomer slide scanner with an x40 objective (Hamamatsu). Others slides were reserved for subsequent immunochemistry analyzes.

The immunohistochemistry protocol was globally performed as previously described (Pirrot et al., 2014). Briefly, skeletal muscle sections were deparaffinised, rehydrated, and incubated for antigen retrieval in EDTA buffer at 100°C for 10 min. Sections were incubated in 0.3% H<sub>2</sub>O<sub>2</sub> for 20 min and endogenous biotins were blocked using the Avidin-Biotin Blocking kit (Vector Laboratories, CliniSciences). Nonspecific antibody binding was blocked by incubation with TBS containing 20% normal goat serum for 30 min at RT. Sections were then incubated ON at 4°C with anti-PDGFR $\alpha$  antibody diluted at 1:250 or non-specific rabbit IgG (Vector Laboratories, CliniSciences) at the same concentration. Antibody binding was revealed by the streptavidin/biotin-peroxydase complex method using ABC Vectastain kit and the peroxidase substrate DAB (Vector Laboratories, Clinisciences). Images were analyzed with ImageJ (1.46r version) software.

### **mRNA extraction and real-time polymerase chain reaction (qPCR)**

Total RNAs were isolated from homogenate muscle samples using the miRNeasy Mini Kit following the manufacturer's instructions (Qiagen). RNA concentration was determined by spectrophotometric analysis (Eppendorf AG, Hamburg, Germany), and integrity was checked with the Agilent 2100 bioanalyzer (Agilent Technologies) using the RNA 6000 Nano kit according to the manufacturer's instructions (Imbeaud et al., 2005). Reverse transcription reaction was performed with 2µg of total RNA using the RevertAid First Strand cDNA Synthesis kit (Thermo Scientific) according to the manufacturer's instructions. qPCR analysis was performed in a StepOnePlus Real-Time PCR System (Applied Biosystems) with 10µL of KAPA SYBR Fast Universal Readymix (CliniSciences), 300nM of both forward and reverse primers, 2µL of diluted cDNA template and water to a final volume of 20µL. The forward and reverse primers used to amplify genes are listed in **Table 1**. All PCRs were performed in duplicate using the following cycle parameters: 20s at 95°C, 40 cycles of 3s at 95°C and 30s at 60°C. Relative mRNA levels were normalized to  $\beta$ 2-microglobulin and cyclophilin A housekeeping gene levels, which were unaffected by the experiment. Results are expressed

using the comparative cycle threshold (CT). The relative changes in the level of a specific gene were calculated with the  $\Delta\Delta CT$  formula.

**Table 1.** Real-time PCR primers

Gene	Forward	Reverse	Amplicon size
C/EBP $\alpha$	GACCAGAAAGCTGAGTTGTGAG	CCACAAAGCCCAGAAACCTA	69 bp
C/EBP $\beta$	CTCCAGGTAGGGGCTGAAGT	TTTAGACCCATGGAAGTGCC	150 bp
cyclophilin A	TTCCTCCTTTCACAGAATTATTCCA	CCGCCAGTGCCATTATGG	75 bp
PDGFR $\alpha$	AAGACCTGGGCAAGAGGAAC	GAACCTGTCTCGATGGCACT	67 bp
PPAR $\gamma$	GTGCCAGTTTCGATCCGTAGA	GGCCAGCATCGTGTAGATGA	142 bp
rpS9	CGGCCCGGGAGCTGTTGACG	CTGCTTGC GGACCCTAATGT	247 bp

### Proteins isolation and Western Blotting

Muscle samples were homogenized in 10 volumes of lysis buffer (50mM Tris-HCl pH7.5, 150mM NaCl, 1mM EGTA, 100mM NaF, 5mM Na<sub>3</sub>VO<sub>4</sub>, 1% Triton X-100, 1% SDS 40mM  $\beta$ -glycerophosphate and protease inhibitor mixture (P8340; Sigma-Aldrich)) and centrifuged at 10.000g for 10 min (4°C). 60 $\mu$ g of protein extracts were loaded on Stain Free™ 4-20% precast gels (4568095; Bio-Rad) before electrophoretic transfer onto a nitrocellulose membrane (Bio-Rad; Trans-Blot® Turbo™ Blotting System). After transfer, membranes were blocked with 50mM Tris-HCl pH7.5, 150mM NaCl, and 0.1% Tween 20 (TBS-T) containing 5% skimmed milk or BSA and incubated overnight at 4°C with primary antibodies. Membranes were then incubated for 1 h with a peroxidase-conjugated secondary antibody. Immunoblots were revealed using a Pierce ECL kit (32106; Thermo Scientific) and proteins were visualized by enhanced chemiluminescence using the ChemiDoc™ Touch Imaging System and quantified with Image Lab™ Touch Software (version 5.2.1). Stain Free technology was used as a loading control. A great number of methodological studies already validated this technology and explained in detail its functioning (Colella et al., 2012, Faden et al., 2016, Gilda & Gomes, 2013, Gilda & Gomes, 2015, Gurtler et al., 2013, Rivero-Gutierrez et al., 2014, Vigelso et al., 2014, Zeitler et al., 2016).

### Antibodies

Anti-laminin (L9393; 1:400) and anti-type II MHC (M4276; 1:200) antibodies were purchased from Sigma-Aldrich. Anti-type I MHC (BA-D5) was purchased from DSHB and used at 1:10. Secondary antibodies Alexa Fluor 488 (A11029) and Alexa Fluor 568 (A11031) were purchased from Invitrogen and used at 1:800. Anti-PDGFR $\alpha$  (#3174), anti-perilipin

(#9349), anti-FABP4 (#3544) and anti-C/EBP $\alpha$  (#8178) primary antibodies were purchased from Cell Signaling and used at 1:500. Anti-PPAR $\gamma$  (sc-7273) and anti-C/EBP $\beta$  (sc-150) primary antibodies were purchased from Santa Cruz and used at 1:200. Anti-mouse (sc-2005) and anti-rabbit (sc-2004) HRP-conjugated secondary antibodies were purchased from Santa Cruz and used at 1:4000.

### **Statistics**

All values are expressed as mean  $\pm$  SEM and the significance level was set as  $p < 0.05$ . Differences between PreDI and postDI were evaluated for significance using the paired Student t-test or the Wilcoxon matched-pairs signed rank test when data deviated from a normal distribution (Shapiro-Wilk normality test).

## Results

### Dry immersion induces muscle atrophy

In order to validate the efficiency of the DI model, we firstly investigated the effects of the protocol on muscle cross-sectional area (CSA). We observed a significant decrease in the global *vastus lateralis* (VL) fibers CSA, reaching 10.6% ( $p < 0.05$  - **Fig.2A**). The CSA before DI (pre-DI) was  $6283\mu\text{m}^2$  against  $5617\mu\text{m}^2$  at the end of the DI protocol (post-DI). We next performed immunocytochemistry experiments with the objective to characterize this decrease in CSA per fiber type. We showed that, 3 days after DI, the decrease in CSA was completely attributable to MyHCI myofibers (-10,6%;  $p < 0.05$ ), while MyHCII CSA has not changed (**Fig.2B**). These results highlight that DI rapidly promotes muscle deconditioning in VL.

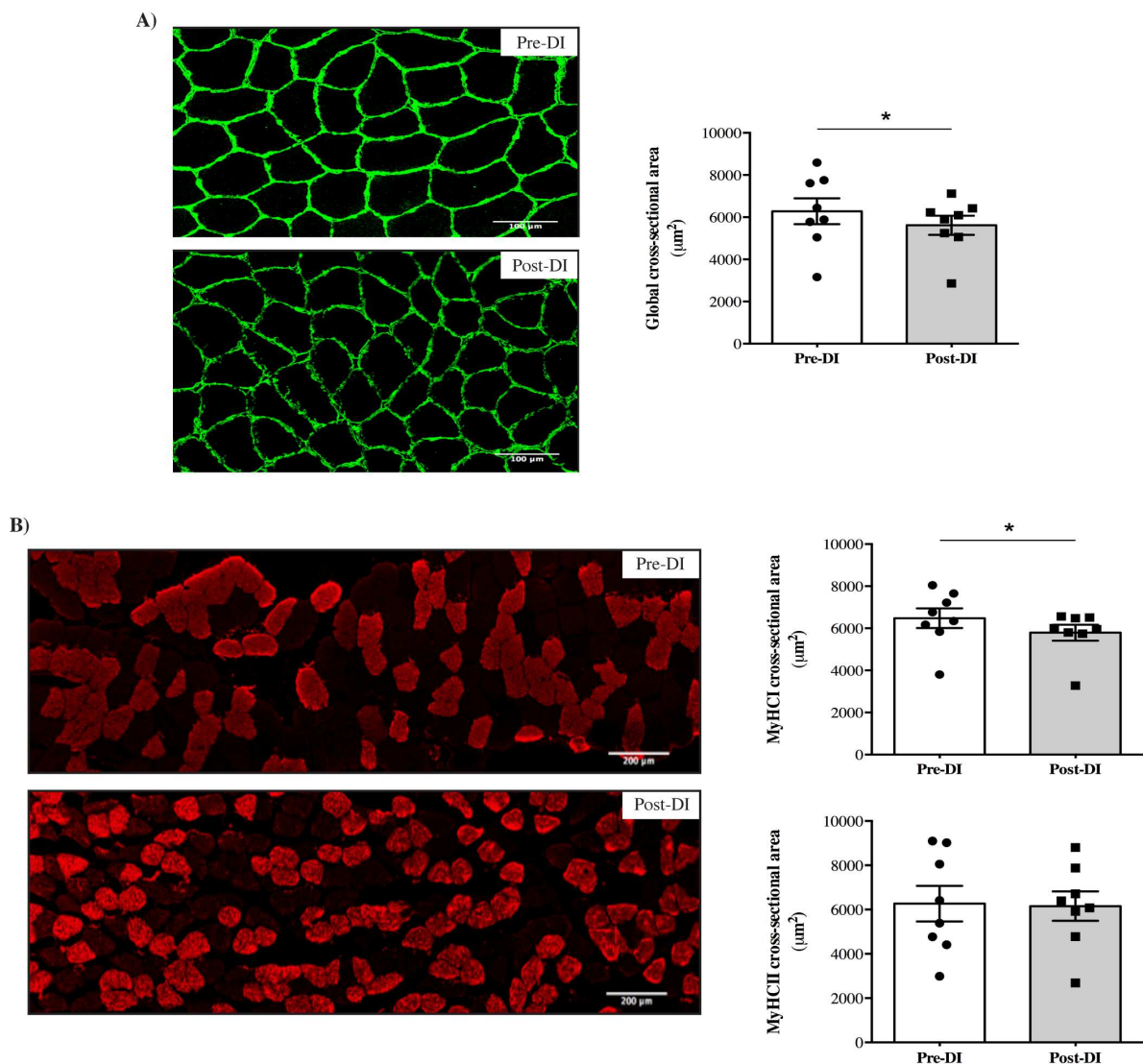
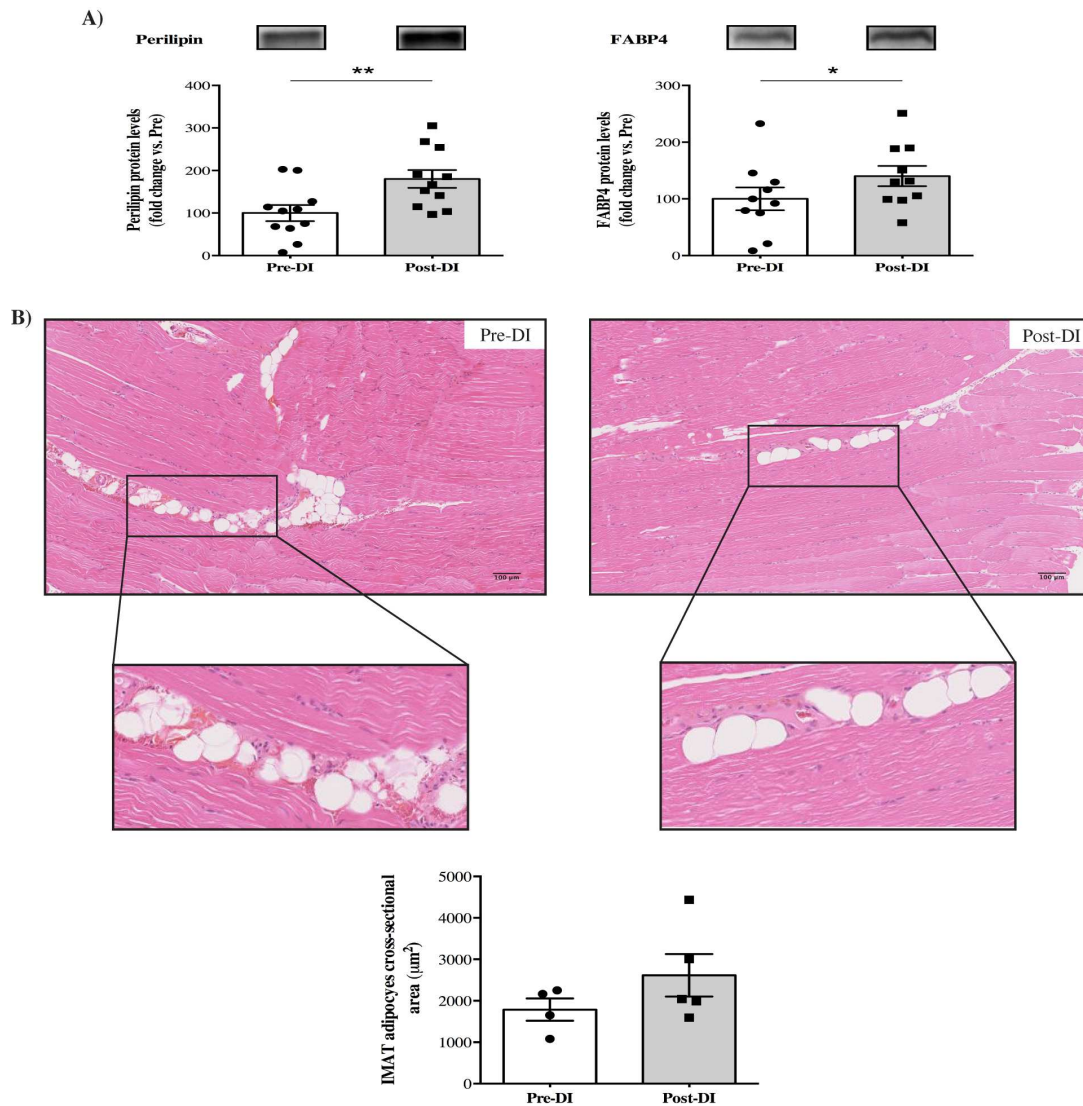


Figure 2. Changes in cross-sectional area measurements after 3 days of DI

*A) Cross-sectional area (CSA) measurement of all myofibers from vastus lateralis muscle biopsies taken before (Pre-DI) and after (Post-DI) 3 days of dry immersion with representative transversal muscle sections. B) Cross-sectional area (CSA) measurement of type I and type II myofibers from Pre-DI and Post-DI muscle biopsies with representative transversal muscle sections. \*  $p < 0.05$*

### **Fatty infiltrations markers are upregulated after dry immersion**

In order to evaluate the effects of 3 days of simulated microgravity on IMAT development, we quantified the expression of perilipin and fatty acid binding protein 4 (FABP4), two major markers of mature adipocytes commonly used in the literature. We observed a marked increase in the protein expression of perilipin and FABP4 after the dry immersion protocol (+80%;  $p < 0.01$  and +40%  $p < 0.05$  respectively - **Fig.3A**). In addition, we also analyzed IMAT adipocytes CSA, in the few sections that they appear, and found an increase in the post-DI condition (**Fig.3B**). Unfortunately, the number of subjects on which we could quantify IMAT was too low to apply powerful statistics tools ( $n=4$  for Pre-DI and  $n=5$  for Post-DI) and this difference did not reach statistical significance (**Fig.3B**).



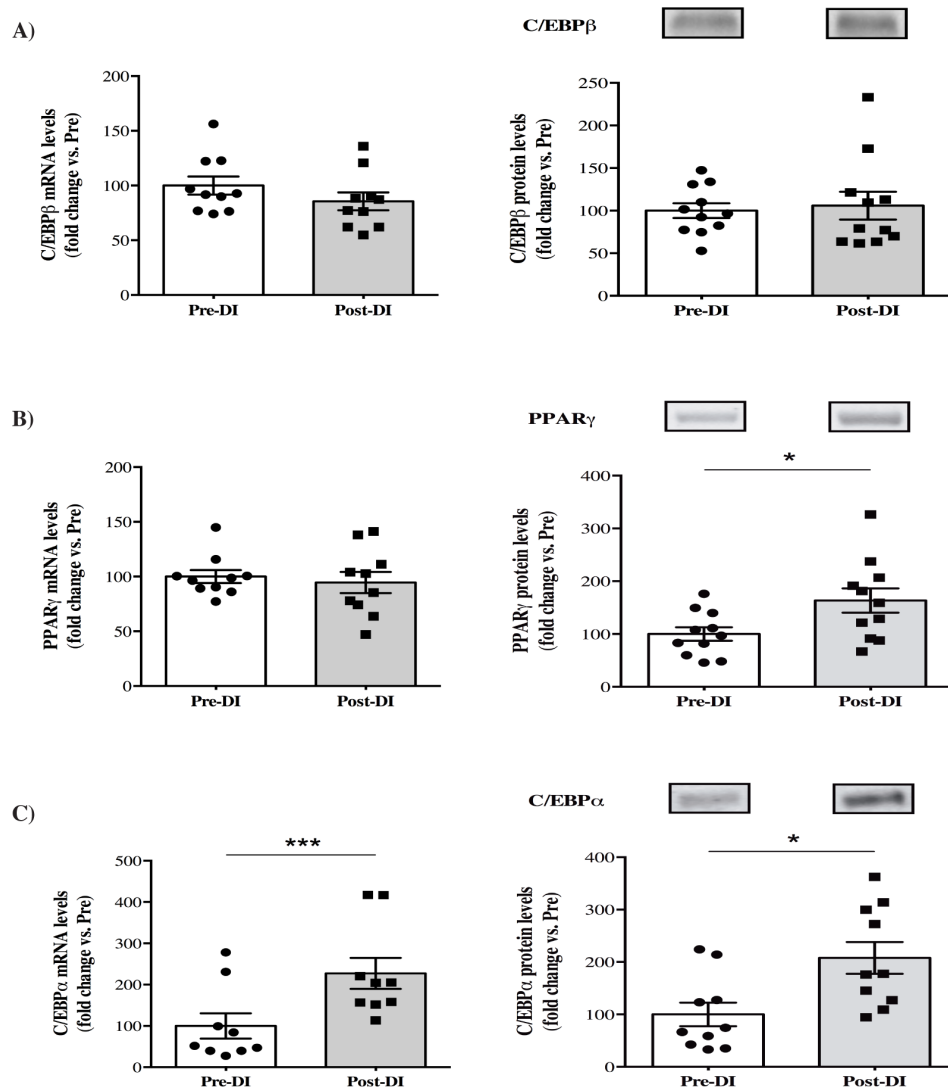
**Figure 3. Changes in IMAT accumulation after 3 days of DI**

**A)** FABP4 and perilipin protein levels from vastus lateralis muscle biopsies taken before (Pre-DI) and after (Post-DI) 3 days of dry immersion. **B)** Representative histological longitudinal paraffin-embedded vastus lateralis muscle sections, stained with hematoxylin-eosin-saffron, from Pre-DI and Post-DI muscle biopsies and IMAT adipocyte cross-sectional area (CSA) in  $\mu\text{m}^2$ . \*  $p < 0.05$  and \*\*  $p < 0.01$

### Dry immersion enhances the expression of key adipogenic transcription factors

C/EBP $\beta$  plays an important role during the first hours after adipogenic differentiation initiation. Interestingly we did not detect any differences in mRNA or protein levels of C/EBP $\beta$  after our simulated-microgravity protocol (**Fig.4A**). In the light of our previous results on mature adipocyte markers, indicating that adipogenic process is already greatly engaged, we hypothesized that later markers of adipogenesis would be differentially expressed. We therefore performed mRNA and protein levels analysis of PPAR $\gamma$  and C/EBP $\alpha$ . Surprisingly, no differences were detected for PPAR $\gamma$  mRNA induction whereas an increase was observed

for its protein expression (+64%;  $p < 0.05$  - **Fig.4B**). Moreover, C/EBP $\alpha$  protein expression increased after dry immersion (+108%;  $p < 0.05$ ) and we observed an almost similar increase in its mRNA induction (+127%;  $p < 0.001$  - **Fig.4C**). Consistently with the results on mature adipocyte markers, the elevated expression of C/EBP $\alpha$  and PPAR $\gamma$  after 3 days of DI may also indicate a raise in the late adipogenic processes, certainly driving to IMAT development.



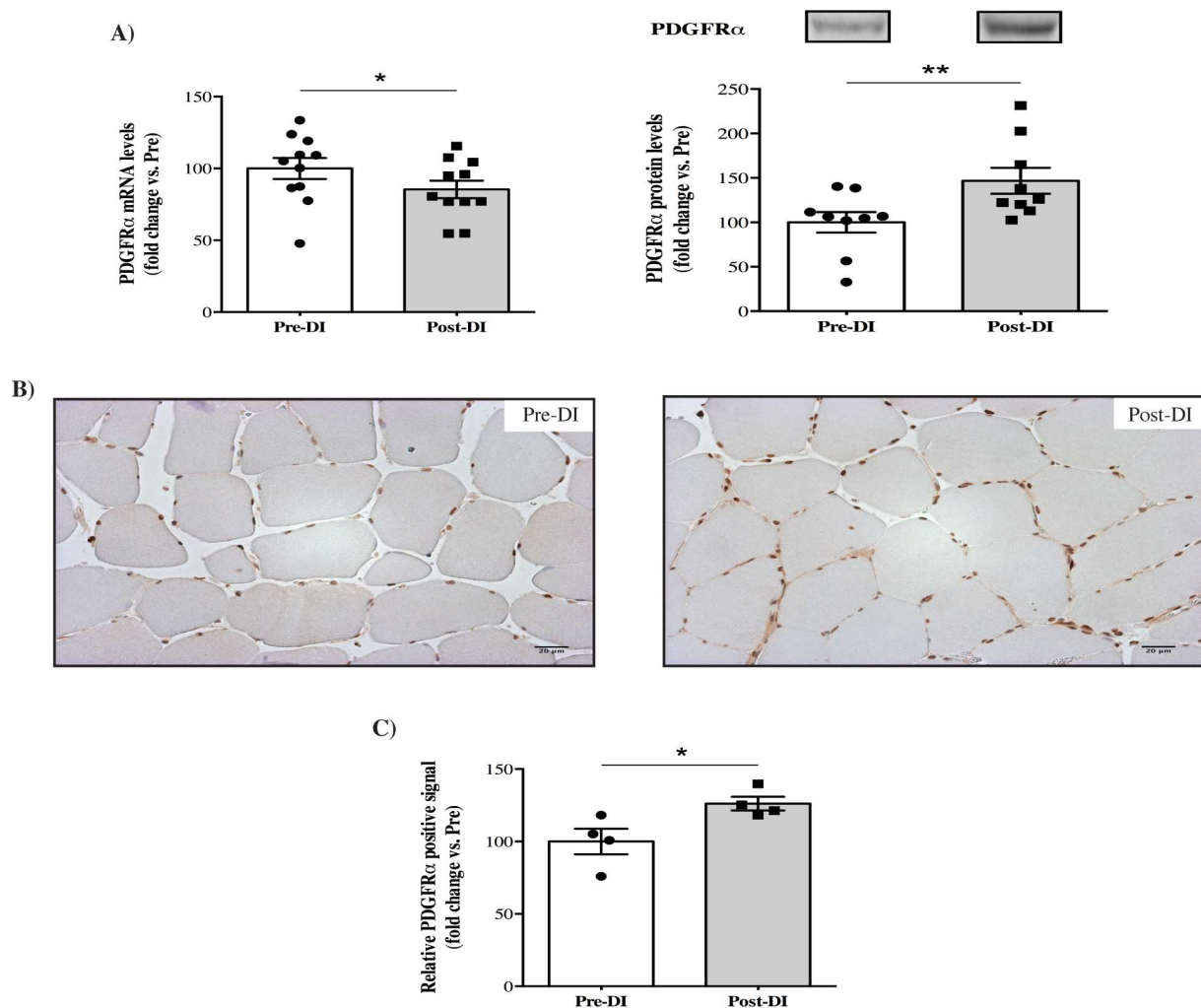
**Figure 4. Changes in key adipogenic markers after 3 days of DI**

**A)** Changes in C/EBP $\beta$  mRNA and protein levels from vastus lateralis muscle biopsies taken before (Pre-DI) and after (Post-DI) 3 days of dry immersion. **B)** Changes in PPAR $\gamma$  mRNA and protein levels from Pre-DI and Post-DI muscle biopsies. **C)** Changes in C/EBP $\alpha$  mRNA and protein levels from Pre-DI and Post-DI muscle biopsies. \*\*\*  $p < 0.001$

## Dry immersion promotes FAPs proliferation

FAPs are well known to be, at least in animals, the major muscle-resident stem cells implicated in the apparition of IMAT. We hypothesized that these mesenchymal stem cells,

which are positive and identified in many studies with the cell surface marker PDGFR $\alpha$ , were implicated in the IMAT development observed in our study. We therefore quantified mRNA induction and protein levels of PDGFR $\alpha$  after 3 days of DI. We curiously found a minor but significant decrease in mRNA induction of PDGFR $\alpha$  whereas protein levels were clearly enhanced (Fig.5A). The result on protein levels was then confirmed by our immunohistochemical analysis (Fig.5B). Therefore, in parallel to an increase in mature adipocyte markers and C/EBP $\alpha$ , the raise in PDGFR $\alpha$  expression probably highlights a FAPs proliferation in response to 3 days of DI. These FAPs, in an unfavorable environment, are well known to drive IMAT development.



**Figure 5. Changes in FAPs cell surface marker PDGFR $\alpha$  after 3 days of DI**

**A)** Changes in PDGFR $\alpha$  mRNA and protein levels from vastus lateralis muscle biopsies taken before (Pre-DI) and after (Post-DI) 3 days of dry immersion. **B)** Representative histological transversal paraffin-embedded vastus lateralis muscle sections, immunostained with PDGFR $\alpha$  antibody, from Pre-DI and Post-DI muscle biopsies. **C)** Quantification of the PDGFR $\alpha$ -positive signals \*  $p < 0.05$  and \*\*  $p < 0.01$



## Discussion

The main objective of this study was to characterize the effects of 3 days of simulated microgravity on IMAT development and accumulation. For that purpose, we used the innovative dry immersion model (DI), firstly use in Europe in this experiment. Our study shows for the first time that only 3 days of severe inactivity were able to increase key markers of IMAT development and accumulation.

Skeletal muscle atrophy in humans was already observed in many studies related to microgravity but mainly using long-duration protocols (Baldwin et al., 2013, Berg et al., 1997, Fitts et al., 2010, Trappe et al., 2009, Trappe et al., 2004). Indeed, few studies have examined effects on microgravity on short periods. The study of Edgerton et al. (1995) has shown a significant decrease in VL CSA after 11 days of real space flight (-16% on MyHCI and -36% on MyHCII). In the specific context of DI, a decrease in CSA of both slow and fast myofibers was already reported after 3 days (5-9%) and after 7 days (15-18%) (Navasiolava et al., 2011, Shenkman et al., 2004). In our study, we observed a global decrease in VL myofibers CSA after only 3 days of DI (10,6%) and, more specifically, a 9,8% decrease in MyHCI. Our results are thus in accordance with the previous studies demonstrating that only 3 days of this protocol is able de induce muscle atrophy.

Our study was then dedicated to investigate IMAT development and accumulation. Fatty infiltrations development is an important characteristic of muscle deconditioning and was particularly studied in relation to aging and inactivity (Brioche et al., 2016). There is no study conducted in humans available concerning the effects of microgravity, or simulated microgravity, on IMAT development. In a closed context, Manini et al. (2007) have demonstrated that only 4 weeks of unilateral limb suspension on healthy young adults was sufficient to induce a major increase in IMAT content. In our study, we found a clear increase in two key mature adipocytes markers: FABP4 and perilipin. These two markers are commonly used in the literature to evaluate IMAT accumulation (Heredia et al., 2013, Joe et al., 2010, Lukjanenko et al., 2013, Pagano et al., 2015, Uezumi et al., 2010) and their increase highlight that only 3 days of dry immersion was able to induce an IMAT accumulation. These results were then supported by an increase in mRNA and protein expression of key adipogenic markers after DI. Indeed, we found a clear increase in the protein expression of PPAR $\gamma$  and C/EBP $\alpha$  while just C/EBP $\alpha$  mRNA were enhanced. PPAR $\gamma$  and C/EBP $\alpha$  are required transcription factors to induce adipocyte development and their deletion in mice

leads to adipocyte formation dysfunction and premature death (Barak et al., 1999, Linhart et al., 2001, Rosen et al., 1999, Wu et al., 1999).

Another point raised in our study was the expected increase expression of PDGFR $\alpha$ , a key cell surface marker of FAPs. According to the recent animal studies focused on IMAT development and muscle-resident stem cells, FAPs may be the main source of IMAT during an altered muscle homeostasis (Heredia et al., 2013, Joe et al., 2010, Mozzetta et al., 2013, Sohn et al., 2015, Uezumi et al., 2010, Uezumi et al., 2011). Some studies conducted in humans also suggest that FAPs could be the stem cells population leading to IMAT development (Arrighi et al., 2015, Laurens et al., 2015, Uezumi et al., 2014a). Our study is the first to highlight an increase expression of PDGFR $\alpha$ , reflecting the highly probable FAPs proliferation, after 3 days of DI-mediated simulated microgravity. In this unfavorable muscular environment, FAPs may be the main source of fatty infiltrations development. Indeed, numerous papers have already demonstrated that FAPs are highly influenced by their environment. For example, Uezumi et al. (2010) have shown that FAPs cells isolated from glycerol-injected muscle did not differentiate into adipocytes in CTX-injected muscle, and in contrast, those isolated from CTX-injected muscle accumulated in degenerated areas and differentiated into adipocytes in glycerol-injected muscle. Interestingly, Mozzetta et al. (2013) showed that in young mdx mice, FAPs were sensitive to adipogenesis inhibition mediated by HDAC inhibitors while, in old mdx mice, the same treatment was unable to be as effective as in young mdx mice. These studies clearly showed that the surrounding muscle environment largely regulates the fate of FAPs and we could therefore hypothesized that in our model of severe inactivity, FAPs are influenced to drive IMAT development.

To conclude, this study is therefore the first one to emphasize that a short-time period of severe inactivity, mediated by the innovative dry immersion model, is able to induce muscle deconditioning with IMAT development and accumulation. Our study also reveals that FAPs could be the resident muscle stem cells population implicated in IMAT development and further studies are needed to explore an efficient strategy to counteract this phenomenon that impairs muscle function.

## **Conflict of interest statement**

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## **Authors and Contributors**

Substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work; AP; TB; CA; RD; AC; GP

Drafting the work or revising it critically for important intellectual content; AP; TB; CA; AC; GP

Final approval of the version to be published; AP; TB; AC; GP

Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved; AP; TB; CA; RD; AC; GP

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### Discussion de l'étude 3

L'objectif de cette étude était de caractériser les effets de 3 jours de microgravité simulée sur le développement et l'accumulation d'IMAT. Pour ce faire, nous avons utilisé le modèle novateur et exploité pour la première fois, en Europe, de dry immersion (DI). Ce modèle « d'immersion sèche », a été décrit et validé pour la première fois par des spécialistes en biologie et médecine spatiale à Moscou (Shulzhenko et al., 1976). Il consiste à immerger un sujet dans de l'eau thermiquement neutre, et recouverte d'un tissu élastique étanche. Le sujet est alors immergé, et repose librement dans l'eau, tout en restant sec. L'avantage considérable de cette technique, par rapport à celle du traditionnel bed rest, est représenté par l'absence de structure de support sous le corps des sujets. Ainsi l'absence de support mécanique crée alors une situation plus proche de celle retrouvée lors d'un environnement en microgravité (Navasiolava et al., 2011). DI est un modèle de microgravité simulée largement utilisé en Russie, mais reste bien moins connu partout ailleurs, et plus particulièrement dans les régions occidentales pour lesquelles le modèle bed rest est encore privilégié.

Dans un premier temps, les premiers résultats de notre étude démontrent l'efficacité de notre modèle de déconditionnement musculaire. En effet, nous avons reporté, en seulement trois jours de DI, une diminution moyenne des sections transversales des fibres musculaires du *vastus lateralis* (VL) de 10,6% (**Fig.2A**). Cette diminution étant principalement portée sur les fibres de type I (-9,8%, **Fig.2B**). Ces résultats sont en accord avec ceux retrouvés dans la littérature (Navasiolava et al., 2011, Shenkman et al., 2004b) et valident donc notre modèle de microgravité simulée.

En ce qui concerne l'apparition d'IMAT, notre étude est donc la première à investiguer les effets de la microgravité simulée sur leur développement. Dans un contexte assez proche, l'étude de Manini et al. (2007) a démontré que seulement 4 semaines d'inactivité, induite par le modèle ULLS, étaient capables d'augmenter de manière importante le contenu en IMAT de plusieurs groupes musculaires (15 à 20%). Dans notre étude, nous avons observé une augmentation de l'expression protéique de FABP4 et périlipine, deux marqueurs clés de la présence d'adipocytes matures et communément utilisés dans la littérature (Heredia et al., 2013, Joe et al., 2010, Lukjanenko et al., 2013, Pagano et al., 2015, Uezumi et al., 2010) (**Fig.3**). Ces résultats sont également renforcés par une augmentation de l'expression de C/EBP $\alpha$  et PPAR $\gamma$ , deux facteurs de transcriptions clés pour les processus d'adipogenèse. La littérature a unanimement souligné le rôle primordial de ces deux facteurs de transcription dans le développement de l'adipocyte, et leur délétion entraîne une dysfonction de la

formation de tissu adipeux et une mort prématurée (Barak et al., 1999, Linhart et al., 2001, Rosen et al., 1999, Wu et al., 1999). Nos résultats soulignent donc que 3 jours de microgravité simulée engendrent une augmentation des processus d'adipogenèse ainsi que du contenu musculaire en IMAT (**Fig.4C**).

Un autre point important soulevé par notre étude concerne l'augmentation de l'expression de PDGFR $\alpha$  après 3 jours de DI (**Fig.5**). Cette augmentation, au niveau protéique et par analyse immunohistochimique, résulte très probablement d'une prolifération des FAPs dans ce modèle d'hypoactivité. Selon les études animales récentes qui ont porté sur l'étude du développement des IMAT et des cellules souches résidant dans l'environnement musculaire, les FAPs sont certainement les cellules souches à l'origine des IMAT (Heredia et al., 2013, Joe et al., 2010, Mozzetta et al., 2013, Sohn et al., 2015, Uezumi et al., 2010, Uezumi et al., 2011). Certaines études réalisées chez l'homme suggèrent également un rôle des FAPs dans le développement des IMAT (Arrighi et al., 2015, Laurens et al., 2015, Uezumi et al., 2014a). Notre étude est donc la première à mettre en évidence une probable prolifération des FAPs après 3 jours de microgravité simulée par le biais du modèle DI. Dans cet environnement musculaire défavorable, les FAPs sont certainement la source de l'augmentation du contenu en IMAT observé dans notre étude. En effet, la littérature scientifique a déjà démontré que les FAPs sont très largement influencés par leur environnement. Par exemple, l'étude de Uezumi et al. (2010) a montré que des FAPs isolés à partir d'un muscle blessé au travers du modèle glycérol ne se différenciaient pas en adipocytes une fois réinjectés dans un muscle lésé via une injection de cardiotoxine. De manière logique, avec la même méthode réalisée à l'inverse (de la cardiotoxine vers le glycérol), les FAPs ne se différencient pas en adipocytes. De manière intéressante, Mozzetta et al. (2013) ont observé, chez des jeunes souris *mdx*, que les FAPs étaient sensibles à un traitement à base d'inhibiteurs des HDAC (histone déacetylases) inhibant leur capacité adipogénique. Cette étude a ensuite montré que les FAPs des souris *mdx* âgées étaient insensibles à ce même traitement. Ainsi, ces deux études suggèrent fortement que l'environnement musculaire régule de manière importante le devenir des FAPs et nous pouvons ainsi émettre l'hypothèse que les FAPs sont influencés vers un lignage adipogénique par le modèle de microgravité simulée utilisé dans notre étude.

Pour conclure, cette étude est donc la première à mettre en évidence qu'une courte période d'hypoactivité, médiée par le modèle novateur de DI, est capable d'induire un déconditionnement musculaire accompagné d'un développement d'IMAT. Notre étude a également révélé que les FAPs pouvaient être la population de cellules souches résidant dans

le tissu musculaire à l'origine des IMAT. Enfin, cette étude soulève le besoin de développer des stratégies efficaces pour lutter contre l'apparition des phénomènes précoces du déconditionnement musculaire, incluant le développement des infiltrations graisseuses.

# DISCUSSION GENERALE ET PERSPECTIVES

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Les travaux réalisés au cours de ma thèse ont contribué à l'accroissement des connaissances scientifiques relatives à la thématique des infiltrations graisseuses et de leurs exacerbations dans des situations d'hypoactivité ou de régénération musculaire. Nous avons montré dans l'étude 3, chez des hommes sains, que seulement 3 jours d'inactivité induite par le modèle dry immersion (DI) entraînaient une augmentation du contenu musculaire en IMAT. Dans un contexte de régénération musculaire, induite par le modèle glycérol chez la souris, nous avons dans un premier temps, au travers de l'étude 1, démontré une inhibition quasi-complète de l'apparition des IMAT en diminuant les contraintes mécaniques appliquées au muscle lésé. Ensuite, dans l'étude 2, nous avons précisé le rôle de l'axe TNF $\alpha$ /TGF- $\beta$ 1 et donc souligné le rôle potentiel des processus inflammatoires nécessaires dans l'apoptose des FAPs afin de limiter le développement des IMAT. Ces trois études soulèvent ainsi de nombreuses questions et la nécessité de travaux supplémentaires afin de préciser le rôle des IMAT dans la dysfonction musculaire, ainsi que les processus et facteurs de régulation qui contrôlent le développement et l'accumulation d'IMAT.

### **Un déconditionnement musculaire et une apparition d'IMAT très rapide**

L'étude réalisée chez l'homme a donc permis d'identifier, pour la première fois, le caractère très précoce du développement des infiltrations graisseuses lors d'une situation de déconditionnement musculaire. En effet, en seulement 3 jours de DI, de nombreux marqueurs utilisés pour quantifier la présence d'IMAT augmentent de manière significative. Ces résultats sont toutefois à remettre dans le contexte du modèle utilisé. Le modèle DI est un modèle très sévère d'hypoactivité, simulant les effets de la microgravité de manière plus prononcée que le modèle bed rest. L'absence de support mécanique avec le modèle DI crée, en effet, un état plus proche de la situation retrouvée lors d'un environnement de microgravité (Navasiolava et al., 2011). Dans cette étude, un autre groupe a rapporté les mesures du volume musculaire global ainsi que le pourcentage d'IMAT (Demangel et al, J Physiol, soumis). Leurs mesures effectuées par IRM soulignent une perte de volume musculaire d'environ 4% et un pourcentage inchangé d'infiltrations graisseuses (6%). L'augmentation du niveau d'expression des différents marqueurs dans notre étude pourrait ainsi souligner un processus d'adipogenèse accrue mais non encore suffisant pour être discriminant en imagerie par résonance magnétique.

Si notre étude est la première à mettre en évidence une augmentation du développement des IMAT en lors des trois premiers jours d'inactivité, elle est aussi la seule à souligner une augmentation du nombre de FAPs présents dans l'environnement musculaire. Si l'on admet que les FAPs représentent la population majeure de cellules souches pouvant s'engager dans la lignée adipogénique (Heredia et al., 2013, Joe et al., 2010, Mozzetta et al., 2013, Sohn et al., 2015, Uezumi et al., 2010, Uezumi et al., 2011), il est assez intéressant de penser qu'en seulement 3 jours les FAPs ont pu proliférer, s'engager dans un lignage adipogénique et que les pré-adipocytes soient devenus des adipocytes matures en si peu de temps. Pourtant, si l'on se permet de faire le parallèle avec les SCs, qui sont de loin les cellules souches de l'environnement musculaire les plus étudiées, la littérature a déjà démontré que les SCs étaient une population très hétérogène avec des capacités de différenciation très différentes (Almeida et al., 2016, Motohashi & Asakura, 2014, Tierney & Sacco, 2016). L'étude de Rantanen et al. (1995) a par exemple mis en lumière une augmentation des ARN messagers codant pour la protéine myogénine dès 4h et 8h après blessure. Ainsi, dès les premières heures après une lésion, des SCs sont déjà capables de se différencier alors que dans cette même étude les premiers signes de prolifération de SCs n'apparaissent qu'à partir de 24h post-blessure. Drummond et al. (2010) ont également montré une augmentation de l'expression protéique de la myogénine dès 6h après un exercice en résistance (engendrant des microlésions), alors que l'expression de la protéine MyoD reste inchangée jusqu'à au moins 48h après l'exercice. Ainsi, ces études mettent en avant le fait qu'il puisse y avoir une sous-population de SCs directement prête à se différencier, sans même avoir proliféré, et diffère donc des populations capables de proliférer rapidement ou même lentement. Si l'on admet également que les FAPs puissent être une population hétérogène, on pourrait aussi penser qu'il existe une telle sous-population capable de se différencier très rapidement, sans même proliférer. Les FAPs étant très sensibles à l'environnement musculaire (Mozzetta et al., 2013, Uezumi et al., 2010), cette sous-population de FAPs, alors exposée à notre environnement de microgravité, pourrait donc être la première à mettre en place des processus adipogéniques amenant *in fine* à une augmentation du contenu en IMAT dès 3 jours d'inactivité.

Notre étude soulève ainsi de nombreuses perspectives concernant de futurs travaux. Par exemple, des publications scientifiques ont clairement détaillé des protocoles pouvant être utilisés afin d'isoler les différentes populations de cellules souches présentes dans l'environnement musculaire dont les FAPs et les SCs (Agle et al., 2015, Lau et al., 2014, Liu

et al., 2015, Pisani et al., 2010b, Uezumi et al., 2016). Ainsi, après mise en culture, il sera ainsi possible de répondre, *in vitro*, à de nombreuses questions scientifiques concernant les FAPs isolés à partir d'un environnement lié au déconditionnement musculaire :

- Est-ce que les adipocytes dérivés des FAPs isolés après une période d'inactivité sont plus insulino-résistants que ceux isolés chez le même sujet avant cette période d'inactivité ?

- Est-ce que les adipocytes dérivés des FAPs isolés après une période d'inactivité induisent une résistance à l'insuline des myotubes en co-culture, comme cela a été démontré à l'aide de FAPs issus de muscles de sujets obèses dans l'étude de Laurens et al. (2015) ?

- Ces FAPs permettront ils toujours de contribuer à l'augmentation des processus myogéniques des SCs comme observé dans l'étude de Joe et al. (2010) ?

- Ces FAPs sécrètent ils toujours autant de cytokines comme IL-6, IGF-1 ou la Fstn qui sont nécessaires pour l'augmentation des processus myogéniques ?

- Ces FAPs ont ils une augmentation de leur potentiel adipogénique dans un milieu de culture le favorisant, ou même dans un milieu de culture myogénique ?

- Quels pourraient être les myokines ou adipo/myokines impliquées dans la dialogue entre cellules musculaire/satellite et FAPs lors de l'exercice physique ?

- En faisant de nouveau le parallèle avec les SCs, qui présentent une hétérogénéité en fonction du type de muscle, les FAPs isolés à partir du VL ont ils les mêmes caractéristiques (potentiel adipogénique, sécrétion de cytokines, aide de la myogenèse) que ceux isolés à partir du soléaire ou des gastrocnémiens ?

### **Augmenter l'activité physique pour lutter contre l'apparition d'IMAT : un raisonnement logique mais toujours incomplètement décrit**

Si les études montrent que les IMAT sont intimement liés à l'inactivité musculaire, il apparaît aussi logique qu'une augmentation de l'activité contractile permette de limiter le développement des IMAT. La première étude à avoir montré une augmentation du contenu en IMAT, chez la personne âgée, a été publiée en 1983 par Borkan et al. (1983), et pourtant, il a fallu 25 ans pour voir apparaître la première étude détaillant l'effet d'une augmentation de l'activité physique sur les IMAT pour cette même population (Goodpaster et al., 2008). Si par

la suite, de nombreuses études ont détaillé, toujours chez les personnes âgées principalement, le rôle primordial de l'activité physique pour combattre l'apparition des IMAT avec l'avancée en âge (Brioché et al., 2016), aucune étude n'est cependant disponible dans la littérature concernant les mécanismes impliqués.

L'exercice physique est connu pour engendrer des adaptations majeures du métabolisme musculaire (Egan & Zierath, 2013), notamment par l'AMPK en tant que senseur énergétique de la cellule musculaire (Pagano et al., 2014, Sanchez et al., 2012a), et promouvoir l'utilisation des acides gras présents dans les gouttelettes lipidiques à proximité des mitochondries. Cependant, il n'y a pour l'instant aucune étude disponible dans la littérature qui traite d'une utilité des adipocytes matures présents à l'extérieur de la cellule musculaire pour le métabolisme musculaire à l'exercice. Au contraire, une étude a récemment montré que les adipocytes dérivés des FAPs chez l'homme possèdent une insulino-résistance *in vitro* (Arrighi et al., 2015). Ainsi, il est difficile d'émettre l'hypothèse que les IMAT contribuent aux adaptations métaboliques à l'exercice.

La revue de Gorgens et al. (2015) a permis d'identifier de nombreuses adipokines, myokines et adipomyokines régulées par l'exercice aigu ou l'entraînement, et qui possèdent un rôle sur les FAPs. Parmi elles, on retrouve par exemple une diminution de l'expression de la Mstn ainsi qu'une augmentation de l'expression de PDGF-AA, IL-6, TNF $\alpha$ , IL-4, IL-13, IL-15, Fstn ou encore CCR2 (Czarkowska-Paczek et al., 2010, Gorgens et al., 2015, Prokopchuk et al., 2007). Ainsi, toutes ces variations de l'expression de ces cytokines, liées à l'exercice, pourraient potentiellement moduler la prolifération et la différenciation des FAPs en adipocytes pendant l'avancée en âge par exemple. Des expérimentations de co-culture, en parallèle de l'utilisation d'un système de contractilité *in vitro*, permettraient d'explorer des pistes concernant les différents facteurs impliqués dans ce dialogue entre cellule musculaire/satellite et FAPs.

Dans la mesure où l'exercice physique est réalisable chez l'homme, il se révèle comme une contre-mesure clé pour lutter contre le développement d'IMAT (voir partie 3.6. de la revue de littérature). Cependant, dans certaines conditions pathologiques pour lesquelles l'activité physique est difficile voire impossible, il est nécessaire de conduire des travaux dans le but de trouver des thérapies efficaces contre la perte de masse mais aussi contre l'apparition d'infiltrations graisseuses dès les premiers jours d'inactivité. Par exemple, lors de séjours en hôpitaux pour des opérations programmées nécessitant un alitement de plusieurs jours voire



semaines, il serait nécessaire d'étudier et de mettre en place des contre-mesures afin de prévenir l'apparition du déconditionnement musculaire et le déclenchement du développement des IMAT.

### **L'importance de diminuer les contraintes mécaniques appliquées au muscle lésé**

Si l'homme possède naturellement des IMAT de manière variable, la souris, qui représente un des modèles animaux les plus étudiés en biologie et physiologie, ne présente pas ou très peu d'IMAT. Ainsi, afin de pouvoir étudier les mécanismes cellulaires et moléculaires du développement des IMAT, le modèle glycérol de régénération a été utilisé. Ce modèle, présenté dans la partie 3.3.2. de la revue de littérature, permet donc d'engendrer des mécanismes de dégénérescence puis régénération associés au développement d'IMAT, signe d'une régénération perturbée. Cependant, l'étude des IMAT dans le contexte du modèle glycérol implique donc d'étudier les mécanismes de régénération sous-jacents à l'apparition d'IMAT. Cela nous éloigne ainsi du développement des IMAT dans le contexte de l'avancée en âge par exemple. Le modèle glycérol avait été initialement appelé « experimental glycerol myopathy » en raison de la ressemblance des mécanismes de dégénérescence avec ceux de la DMD y compris en ce qui concerne l'apparition d'IMAT (Kawai et al., 1990), et l'étude des IMAT dans cet environnement s'apparente ainsi plus à un contexte de DMD ou de blessures musculaires sportives, s'éloignant ainsi de celui d'une simple inactivité musculaire.

La première étude présentée dans ce manuscrit de thèse avait pour objectif de caractériser l'effet d'une diminution des contraintes mécaniques, par le modèle de suspension du train arrière (HU), sur le développement des IMAT induit par le modèle glycérol de régénération musculaire. Si le fait de combiner un modèle de déconditionnement avec celui d'une régénération musculaire faisant apparaître des IMAT pouvait s'avérer contradictoire, nos résultats ont démontré que les IMAT ne se développaient pas dans cette condition d'hypoactivité, et se sont donc révélés inattendus de prime abord. Pourtant, ces résultats illustrent ainsi les recommandations d'immobiliser ou soulager un muscle fraîchement lésé à la suite d'une blessure musculaire.

D'autres études ont utilisé cette méthodologie afin de promouvoir le rôle bénéfique d'un modèle d'hypoactivité sur la régénération musculaire. En effet, l'étude de Mokhtarian et al. (1999) avait induit une immobilisation du soléaire et de l'EDL chez des souris *mdx* âgées de

14 jours seulement. Les auteurs avaient émis et confirmé leur hypothèse qui consistait à immobiliser et éviter les blessures induites par la contraction musculaire et ainsi protéger le tissu musculaire des cycles de dégénérescence/régénération perpétuels liés à cette maladie génétique (détaillé au 2.2.2.). Jarvinen & Lehto (1993) ont également démontré qu'une immobilisation, après une blessure par lacération des gastrocnémiens chez le rat, permettait de diminuer l'apparition de fibrose au niveau du site de lésion. Cependant, dans notre étude, la situation d'hypoactivité, engendrée par le modèle HU, est appliquée durant toute la durée du protocole et donc de la régénération (21 jours). De nombreuses études ont observé une diminution de la régénération musculaire dans cette situation de déconditionnement musculaire (appréciée par une diminution du recouvrement de la masse musculaire et de la CSA des fibres) (Darr & Schultz, 1989, Mozdziak et al., 1998, Wang et al., 2006). Notre étude a également souligné une diminution de la CSA des fibres musculaires pour notre groupe HU comparé au groupe CTL, mettant ainsi en lumière un retour à l'état normal perturbé. Nous avons donc émis l'hypothèse que l'hypoactivité induite par le HU était nécessaire pour une régénération efficace mais que si cette situation perdure tout au long des processus de régénération, elle altère alors la croissance des nouvelles fibres musculaires formées.

En parallèle d'une inhibition quasi-complète du développement des IMAT dans notre groupe HU, nos résultats ont également démontré une diminution de la présence des FAPs dans l'environnement musculaire. Ainsi la seule population de cellules souches permettant la formation d'adipocytes dans le modèle glycérol (Uezumi et al., 2010) se trouve diminuée dans la condition d'hypoactivité et corrèle avec l'inhibition d'IMAT retrouvée.

### **Un rôle primordial des processus inflammatoires : quid de la prise d'anti-inflammatoires à la suite d'une blessure musculaire ?**

Nous avons ensuite, au travers de l'étude 2, exploré les mécanismes impliqués dans l'inhibition du développement des IMAT avec le modèle glycérol observée avec l'hypoactivité mais également les mécanismes relatifs à leur développement par le modèle glycérol. Des publications récentes ont ensuite orienté nos travaux sur les mécanismes inflammatoires.

En effet, un grand nombre d'études relatives à la régénération musculaire soulignent clairement l'importance des mécanismes inflammatoires et anti-inflammatoires dans le bon déroulement des processus de régénération (détaillées dans la partie 2.5.). Le rôle des

macrophages, ainsi que le timing précis du passage d'un environnement pro-inflammatoire, riche en TNF $\alpha$ , à un environnement anti-inflammatoire, riche en TGF- $\beta$ 1, a particulièrement été mis en lumière par l'étude de Lemos et al. (2015). Le TNF $\alpha$  promeut l'apoptose des FAPs, qui après avoir proliféré doivent rentrer en apoptose, alors que TGF- $\beta$ 1 permet leur survie. Si cette fine balance et ce timing sont altérés, la littérature démontre alors l'apparition de fibrose au niveau du muscle lésé. De manière intéressante, l'étude de Lukjanenko et al. (2013) a démontré une réponse anti-inflammatoire trop précoce dans le modèle glycérol comparé au modèle CTX, reflétée par une expression augmentée de TGF- $\beta$ 1, et ainsi le modèle glycérol semble promouvoir la survie des FAPs et leur différenciation en adipocytes.

Nos travaux ont confirmé que l'apoptose des FAPs était certainement altérée dans le modèle glycérol. Nous avons aussi montré que le HU permettait de restaurer cette apoptose, tout comme un traitement avec de la décorine (un inhibiteur de TGF- $\beta$ 1). Cependant, nos expérimentations souffrent d'une limite essentielle : nous n'avons pas encore mesuré de manière quantitative l'apoptose des FAPs. Ces expérimentations sont programmées, nous allons réaliser une co-immunofluorescence TUNEL/PDGFR $\alpha$  afin de mettre en évidence la présence des processus apoptotiques spécifiques aux FAPs à 5 et 9 jours après injection de glycérol (dans nos groupes CTL/EX/HU mais également CTL/DECO). Ces résultats permettront ainsi de valider (ou d'invalider) les hypothèses soutenues dans l'étude 2.

Si ces hypothèses se vérifient, notre étude remettra largement en question l'utilisation d'anti-inflammatoires immédiatement après blessure musculaire. De nombreuses études avaient déjà démontré le rôle primordial des processus inflammatoires dans les premiers temps de la régénération musculaire pour une bonne restauration de l'intégrité originale, sans fibrose, du muscle lésé. Notre étude souligne à son tour la nécessité d'une phase pro-inflammatoire afin d'induire l'apoptose des FAPs et l'inhibition du développement des IMAT. La prise d'anti-inflammatoires est donc certainement à proscrire dans les premiers jours suivant une blessure musculaire mais d'autres études sont nécessaires, y compris chez l'homme, afin de préciser le timing précis du shift d'un environnement pro à anti-inflammatoire lors de la régénération. Permettant *in fine* d'affiner les recommandations relatives au timing de la prise d'anti-inflammatoires après blessure. Cependant, ce timing sera certainement complexe à définir car il sera fortement dépendant du type de blessure, de l'importance de la lésion, du type de muscle lésé, mais aussi de la variabilité inter-individuelle présente chez l'homme.

## **Inhiber TGF- $\beta$ 1 pour limiter l'apparition de fibrose et d'IMAT ?**

Lors de la régénération musculaire, une augmentation de l'expression de TGF- $\beta$ 1 est physiologique et promeut le processus de formation de tissu cicatriciel, nécessaire pour reconstruire le tissu musculaire lésé. Cependant, une expression maintenue et élevée de TGF- $\beta$ 1 est aussi connue pour engendrer une augmentation anormale de la synthèse de tissu cicatriciel, par la formation excessive de matrice extracellulaire, et donc l'apparition pathologique de fibrose (Garg et al., 2015). En effet, en plus de promouvoir la production de collagène via l'augmentation de l'activité transcriptionnelle de SMAD4, TGF- $\beta$ 1 diminue également la production de matrix metalloproteinases (MMPs), qui représentent des protéases endogènes permettant de dégrader les constituants de la matrice extracellulaire (Garg et al., 2015). De par le rôle majeur que possède TGF- $\beta$ 1 dans le développement de la fibrose, cette cytokine est donc devenue une cible de choix pour les thérapies anti-fibrotiques. Ainsi, de nombreuses études ont utilisé des traitements à base d'inhibiteurs, directs ou indirects, de TGF- $\beta$ 1 (lorsatan, suramin, INF $\gamma$  ou déocrine) et ont démontré une amélioration des processus de régénération, accompagnée d'une diminution de l'apparition de fibrose (Bedair et al., 2008, Chan et al., 2003, Foster et al., 2003, Fukushima et al., 2001, Giri et al., 1997, Kobayashi et al., 2013, Li et al., 2007, Park et al., 2012, Sato et al., 2003, Terada et al., 2013, Zhu et al., 2007).

Cependant, aucune de ces études n'avaient fait un lien entre l'inhibition de TGF- $\beta$ 1 et l'augmentation du processus d'apoptose des FAPs. En effet, l'étude de Lemos et al. (2015) a été la première à mettre en évidence le rôle de l'axe TNF $\alpha$ /TGF- $\beta$ 1 dans les processus apoptotiques de FAPs, mais également l'effet du nilotinib dans la modulation de cet axe. Le nilotinib est un inhibiteur de tyrosine kinases qui permet d'inhiber indirectement TGF- $\beta$ 1 (via une inhibition de la MAP kinase p38) induisant l'apoptose des FAPs, *in vitro* et *in vivo*, et ainsi une diminution de l'accumulation de fibrose. Cette étude a également montré cet effet bénéfique chez la souris *mdx*, qui représente un modèle de régénération perturbée. Cependant, les auteurs ont aussi souligné que le nilotinib n'était pas un inhibiteur spécifique de TGF- $\beta$ 1. Ce traitement est en effet capable d'inhiber directement PDGFR $\alpha$ , connu pour engendrer une augmentation des processus liés à la fibrose (voir partie 3.4.2 de la revue de littérature). Les auteurs ont ainsi conclu que la synergie des deux mécanismes était certainement responsable des résultats obtenus dans cette étude.

Concernant notre étude, nous avons fait le choix d'utiliser la décorine comme inhibiteur de TGF- $\beta$ 1 (présentée dans la discussion de l'étude 2), et qui est connue pour interagir de manière spécifique et directe avec les membres de la superfamille des TGF- $\beta$ , dont TGF- $\beta$ 1 et la Mstn. Nous avons démontré qu'un traitement avec de la décorine permettait également d'inhiber le développement d'IMAT induit par le modèle glycérol de régénération en restaurant l'apoptose des FAPs (paramètre prochainement quantifié par co-immunofluorescence TUNEL/PDGFR $\alpha$ ). Notre étude permet donc d'envisager de nombreuses autres études explorant les effets de la décorine dans des modèles favorisant le développement et l'accumulation d'IMAT.

***Figure 31. Illustration des différents inhibiteurs de la voie du TGF- $\beta$ 1***

*Représentation schématique de la voie du TGF- $\beta$ 1 et de ses différents inhibiteurs (en vert). Il est à noter l'absence du nilotinib, capable d'inhiber p38. Extrait de Garg et al. (2015).*

**Pourquoi l'entraînement en endurance n'a-t-il pas réduit le développement d'IMAT pendant les processus de régénération ?**

Notre 2ème étude apporte également un résultat important : notre entraînement en endurance n'a pas diminué le développement des IMAT dans un contexte de régénération lié au modèle glycérol. Pourtant, comme développé dans les parties 3.6. et 3.7.2., l'activité

contractile permet de limiter l'apparition d'IMAT chez la personne âgée dans un contexte dénué de régénération, mais surtout l'activité contractile permet également d'augmenter l'expression de nombreuses myokines ayant un rôle sur les FAPs.

Cependant, l'environnement musculaire lié au glycérol est connu pour diriger les FAPs vers un lignage adipogénique (Uezumi et al., 2010). Or, l'exercice physique permet aussi la sécrétion de PDGF-AA par la fibre musculaire (Czarkowska-Paczek et al., 2010) et ce facteur, en se liant à son récepteur PDGFR $\alpha$ , permet au FAPs de rentrer dans le cycle cellulaire. Les études présentées dans la partie 3.7.2. soulignent également la prolifération des FAPs en réponse à une augmentation de l'activité physique. Combiné à l'environnement néfaste du modèle glycérol pour les FAPs, nous pouvons émettre l'hypothèse que cette réponse des FAPs à l'entraînement dans notre étude est spécifique au modèle glycérol.

### **Modulation du lignage des FAPs, quels facteurs influencent le devenir des FAPs ?**

Si le modèle glycérol de régénération est maintenant bien connu pour orienter les FAPs vers l'adipogenèse, quels sont les facteurs impliqués ? Aucune étude n'a pour l'instant répondu à cette dernière question. Par contre une étude très récente de Dong et al. (2016) a démontré le rôle majeur que possédait la myostatine (Mstn) dans l'évolution des FAPs vers un lignage fibroblastique. Cette étude a démontré, *in vitro* et *in vivo*, que la Mstn engendrait un engagement des FAPs vers la fibrogenèse (dans un contexte de maladie rénale chronique). Ces auteurs ont également montré, *in vitro*, que la Mstn augmentait la prolifération des FAPs (de manière SMAD3 dépendante) mais permettait surtout aux FAPs de s'engager dans un lignage fibroblastique y compris dans un milieu de culture adipogénique. Leurs résultats montrent clairement que l'addition de 40nM de Mstn dans le milieu de culture inhibe complètement la différenciation adipogénique des FAPs, ainsi que l'expression de marqueurs clés comme périlipine, PPAR $\gamma$  et C/EBP $\alpha$ . Cette étude est donc la première à mettre en évidence un facteur clé décidant du potentiel de différenciation des FAPs et en appelle beaucoup d'autres. Ces expérimentations contribueront à optimiser les stratégies pour inhiber la différenciation des IMAT, dans des situations de déconditionnement musculaire liées à l'hypoactivité, à l'avancée en âge, ou encore au contexte pathologique de la DMD.

# TRAVAUX SUPPLEMENTAIRES

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Article 4

**Muscle wasting and aging: Experimental models, fatty infiltrations, and prevention**

Thomas Brioche, Allan F. Pagano, Guillaume Py, Angèle Chopard

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## Review

## Muscle wasting and aging: Experimental models, fatty infiltrations, and prevention

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## ABSTRACT

Identification of cost-effective interventions to maintain muscle mass, muscle strength, and physical performance during muscle wasting and aging is an important public health challenge. It requires understanding of the cellular and molecular mechanisms involved. Muscle-deconditioning processes have been deciphered by means of several experimental models, bringing together the opportunities to devise comprehensive analysis of muscle wasting. Studies have increasingly recognized the importance of fatty infiltrations or intermuscular adipose tissue for the age-mediated loss of skeletal-muscle function and emphasized that this new important factor is closely linked to inactivity. The present review aims to address three main points. We first mainly focus on available experimental models involving cell, animal, or human experiments on muscle wasting. We next point out the role of intermuscular adipose tissue in muscle wasting and aging and try to highlight new findings concerning aging and muscle-resident mesenchymal stem cells called fibro/adipogenic progenitors by linking some cellular players implicated in both FAP fate modulation and advancing age. In the last part, we review the main data on the efficiency and molecular and cellular mechanisms by which exercise, replacement hormone therapies, and  $\beta$ -hydroxy- $\beta$ -methylbutyrate prevent muscle wasting and sarcopenia. Finally, we will discuss a potential therapeutic target of sarcopenia: glucose 6-phosphate dehydrogenase.

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## Contents

1. Introduction .....	2
2. Translational approaches and experimental models for studies on skeletal muscle wasting and aging .....	3
2.1. Real microgravity experiments .....	4
2.2. Human ground-based models of muscle disuse .....	4
2.3. Animal ground-based models of muscle disuse .....	5
2.4. Cell models of muscle wasting .....	5
3. Characteristics of sarcopenia-related muscle deconditioning .....	6
4. Cellular and molecular alterations of skeletal muscle .....	7
5. IMAT: an important factor of sarcopenia .....	8
5.1. The fight against inactivity: the real purpose .....	9
5.2. How does fat infiltration occur in sarcopenia? .....	10

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6.	Strategies against sarcopenia .....	12
6.1.	Exercise as the most effective strategy against sarcopenia .....	12
6.1.1.	Exercise during aging improves protein balance .....	12
6.1.2.	Exercise during aging decreases myonuclear apoptosis .....	13
6.1.3.	Exercise during aging stimulates SCs .....	14
6.1.4.	Exercise during aging improves mitochondrial function and dynamics .....	14
6.2.	Hormone replacement therapies as a possible strategy .....	15
6.2.1.	Testosterone replacement therapy .....	15
6.2.2.	GH replacement therapy .....	15
6.3.	Nutritional interventions: the case of HMB .....	17
6.3.1.	HMB, who are you? .....	17
6.3.2.	HMB: a very potent strategy against various muscle-wasting conditions .....	17
6.3.3.	Efficiency of HMB in various muscle-wasting conditions in the elderly .....	17
6.3.4.	Molecular and cellular mechanisms by which HMB prevents muscle wasting .....	18
6.4.	A potential therapeutic target in sarcopenia: glucose 6-phosphate dehydrogenase .....	19
	Acknowledgments .....	21
	References .....	21

## 1. Introduction

Skeletal muscle is the most abundant tissue in the human body representing ~40% of the body weight and ~30% of the basal energy expenditure (Reid and Fielding, 2012). Skeletal muscle plays a central role in locomotion enabling a person to perform activities of daily living, posture maintenance, and balance (Reid and Fielding, 2012). Moreover, skeletal muscle performs a major function in thermogenesis processes, energy supply (this tissue contains the most important glucose and amino acids stocks), and insulin resistance protection (Brook et al., 2015). In order to ensure these essential functions, skeletal muscle must have sufficient mass and quality.

Skeletal muscle plasticity expresses itself at different levels. Substantial muscle adaptations are first observed during childhood and adolescent growth with an increase in muscle mass and strength. The opposite trend appears by the age of 30 and older: a natural decrease in muscle mass defined as sarcopenia (Giresi et al., 2005; Thomas, 2007). Skeletal muscle plasticity can also translate into muscle tissue adaptations to environmental constraints. Thus, an increase in stimulation, exercise, or nutrition can cause a positive protein balance and involve muscle hypertrophy and reinforcement (Cureton et al., 1988; Sartorelli and Fulco, 2004; Staron et al., 1990). Conversely, a decrease in mechanical constraints will lead to muscle deconditioning and atrophy (Bonaldo and Sandri, 2013; Glass, 2005; Jackman and Kandarian, 2004; Kandarian and Stevenson, 2002; Pagano et al., 2015; Ventadour and Attaix, 2006). Skeletal muscle deconditioning can be defined as primary deconditioning, in case of direct consequences of unfavorable environmental conditions, such as chronic disuse, immobilization, bed rest, a microgravity environment, sedentary lifestyle, and aging, or as secondary deconditioning, in case of indirect consequences of pathological changes like cancer (cachexia), diabetes, or chronic obstructive pulmonary disease (COPD).

Muscle deconditioning occurring with aging is characterized by a decrease in muscle mass, in muscle strength, and in physical performance and is considered a geriatric syndrome called sarcopenia (Cruz-Jentoft et al., 2010; Fielding et al., 2011; Morley et al., 2011; Muscaritoli et al., 2010). Multiple factors contribute to sarcopenia, including

diet, chronic diseases, physical inactivity, and the aging process itself (Derbre et al., 2014; Sayer et al., 2008; Thompson, 2007). Due to social, technological, and medical progress, life expectancy has been increasing since the 19th century in our modern western societies, leading to global aging of the world population. Currently, it is projected that the number of elderly people will double worldwide from 11% of the population to 22% by 2050 (UN, 2007). Inevitably, due to this aging population, prevalence of sarcopenia is growing, and it is currently estimated that one-quarter to one-half of men and women of age 65 and older are likely sarcopenic (Janssen et al., 2004). The increasing prevalence of sarcopenia is considered catastrophic for the public health costs, and, for example, the total cost of sarcopenia to the American healthcare system is approximately \$18.4 billion (Janssen et al., 2004). These healthcare costs are linked to general deterioration of the physical condition resulting in an increased risk of falls and fractures, a progressive inability to perform basic activities of daily living, loss of independence for the elderly, and ultimately, death (Cruz-Jentoft, 2012; Delmonico et al., 2007; Goodpaster et al., 2006). Identification of cost-effective interventions to maintain muscle mass, muscle strength, and physical performance in the elderly is a major public health challenge. It requires understanding the cellular, molecular, and systemic mechanisms as well as the underlying pathways involved in sarcopenia onset and development.

To identify these mechanisms, the gold standard of research is comparison of healthy young people with old healthy people. Such a project is hard to undertake due to the high cost, the difficulty with finding healthy old people, and the invasive method used (e.g., muscle biopsies). To overcome these problems, muscle deconditioning processes have been deciphered by means of several experimental models, bringing together the opportunities to devise comprehensive analysis of muscle wasting.

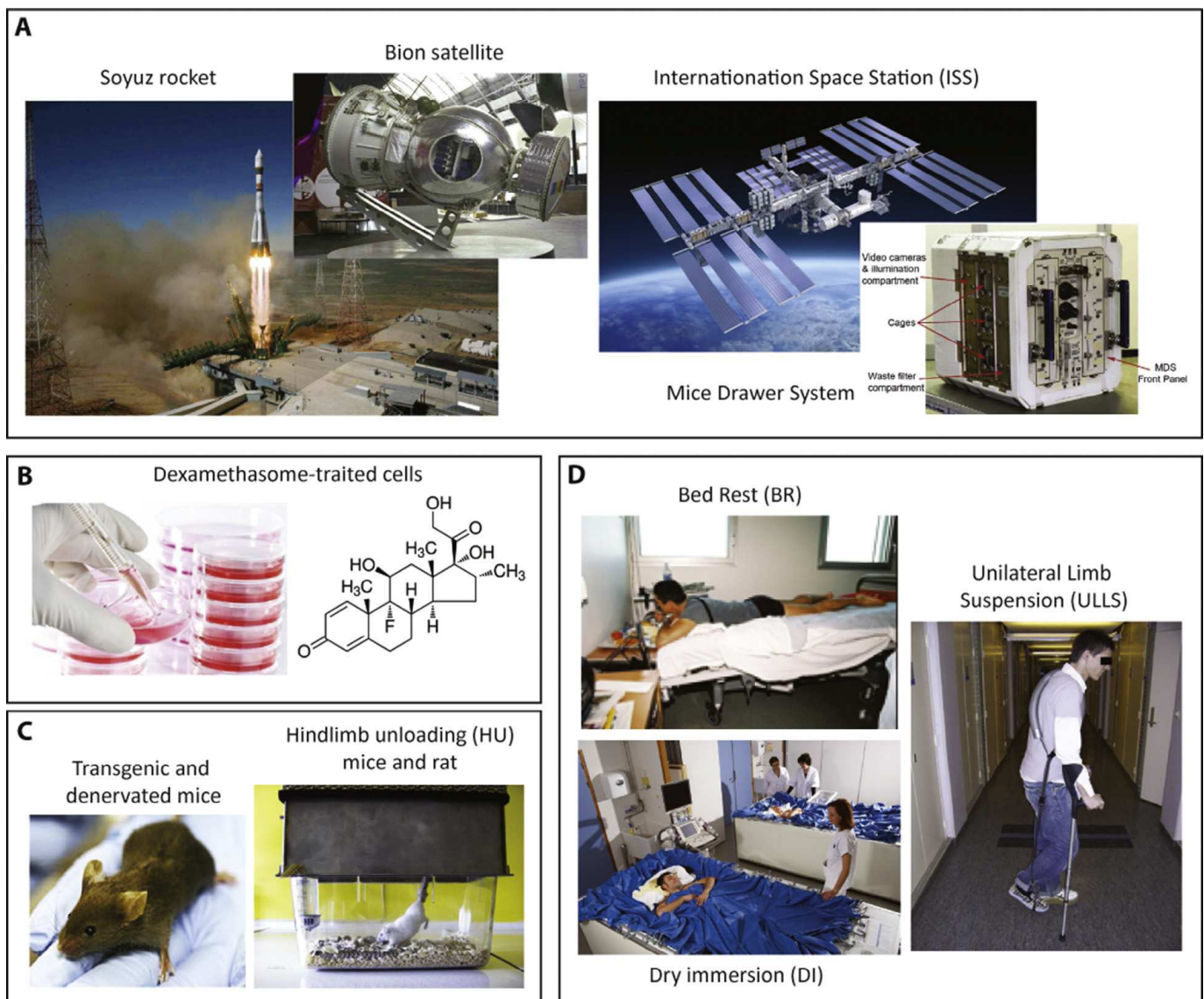
Abundant literature already describes a multitude of muscle adaptations affecting the expression of metabolic, structural, and contractile proteins during muscle deconditioning (Bonaldo and Sandri, 2013; Brioché and Lemoine-Morel, 2016; Chopard et al., 2001, 2005; Fitts et al., 2000; Schiaffino et al., 2007; Stein et al., 2002), and these data will be presented only briefly in this review. To date, our understanding of the effects of

hypokinesia and hypodynamia on skeletal muscle has mainly been derived from studies focused on elucidating the specific contributions of transcriptional mechanisms, intracellular signaling molecules, and the extent of crosstalk among these various pathways during muscle wasting. The present review is aimed at addressing three main points. First, we mainly focus on available experimental models involving cell, animal, or human experiments to study muscle wasting. The second part points out the role of fatty infiltrations, also called intermuscular adipose tissue (IMAT), in muscle wasting and aging, keeping in mind that the loss of strength and power mostly exceeds the loss of muscle mass during muscle deconditioning (Berg et al., 1997; di Prampero and Narici, 2003). The third part presents the main data on the efficiency and molecular and cellular mechanisms by which exercise, replacement

hormone therapies, and  $\beta$ -hydroxy- $\beta$ -methylbutyrate (HMB) prevent muscle wasting and sarcopenia.

## 2. Translational approaches and experimental models for studies on skeletal muscle wasting and aging

In the past decades, the scientific community validated several selected cell, animal, and human experimental models for studying and deciphering the processes of muscle wasting. Various ground-based models and spaceflight studies contribute at this time to the research into muscle weakness and appear to contribute substantially to studies on the aging process (Fig. 1). Musculoskeletal research in the spaceflight context contributes to the research on age-related osteoporosis and sarcopenia (Fitts et al., 2000; Trappe,



**Fig. 1.** Experimental models used to study skeletal muscle wasting. (A) Microgravity devices: the life sciences BION satellite launched on a Soyuz rocket, and the actual orbital International Space Station (ISS) including the Mice Drawer System (MDS). (B) Models of cell line experiments with induction of muscle wasting. (C) Ground-based animal models of muscle wasting and aging including transgenic mice, denervated, and hindlimb-unloaded rodents. (D) Human experimental protocols for studies on muscle deconditioning: bed rest with a  $-6^\circ$  head-down tilt position, dry immersion, and a model of unilateral lower-limb suspension, with a left shoe with a thick sole and the shoulder strap running around the foot and ankle to unload the right foot.

2009). Although aging and spaceflight may involve biological changes in the structure and function via different cellular and molecular pathways, they share the common feature of adaptation to changing levels of strain (Lecker et al., 2004a). Thus, the studies on adaptation of the musculoskeletal system, from microgravity to  $1 \times g$  conditions, mimic the age-related muscle atrophy. Furthermore, the types of countermeasures required to maintain muscle function in altered-gravity environments must take into account preservation of muscle strength, endurance, and postural balance. Similar approaches are relevant to the prevention of falls and fractures in elderly subjects. Moreover, the greatest difficulty with studying the aging process is the long time span, and microgravity experiments appear to involve physiological events mimicking aging-associated changes that take a lifetime to develop (Biolo et al., 2003). Indeed, muscle atrophy and weakness, reduced capacity for exercise, unbalanced immune responses, insulin resistance, and impaired oxidative metabolism contribute to the effects of both aging and space travel.

### 2.1. Real microgravity experiments

These experiments on skeletal muscle include mainly studies on rodents and humans. In the past decades, life science Spacelab and Spacehab programs included experiments involving human astronauts as well as animals and insects taken along on several missions (Fitts et al., 2010; Miu et al., 1990; Roffino et al., 1998). On-board experiments on rodents have also been conducted on the series of life science satellites called BION, and more recently BION-M. For this program, spacecraft are launched on a Soyuz rocket from the Plesetsk Cosmodrome in northern Russia (Ogneva et al., 2015; Roffino et al., 2006; Ulanova et al., 2015).

The actual orbital International Space Station flies 400 km above the ground at the speed of 28,800 km/h. It takes only 90 min for the weightless laboratory to make a complete circle around the Earth. Astronauts working and living aboard the Station experience 16 sunrises and sunsets each day. The International Space Station has provided the opportunity to study long-term microgravity. A hallmark of spaceflight is considerable variability in the extent of muscle atrophy and functional loss among crewmembers (Fitts et al., 2000, 2010; Trappe, 2009). The Mice Drawer System program actually gives an opportunity to accommodate mice aboard the International Space Station, and has been aimed, for example, at research on the consequences of long-term (91-day) exposure to microgravity in mice (Cancedda et al., 2012; Sandona et al., 2012).

### 2.2. Human ground-based models of muscle disuse

Experimental ground-based models of induced muscle atrophy and weakness contribute for the most part to scientific studies related to muscle disuse and aging. The goal of these types of studies is also to identify appropriate countermeasures for men and women to prevent or suppress disuse-induced muscle atrophy such as that encountered during short- and long-term microgravity and in the clinic (Blottner et al., 2006; Chopard et al., 2005, 2009a; Rudnick et al., 2004). Studies conducted on human healthy subjects

are mainly focused on bed rest (Pavy-Le Traon et al., 2007), dry immersion (Navasolava et al., 2011), and unilateral limb suspension (ULLS) experiments (Brocca et al., 2015; Gustafsson et al., 2010; Hackney and Ploutz-Snyder, 2012; Tesch et al., 2008) and allow researchers to distinguish the effects of disuse from those associated with comorbidities, both in the context of fracture healing and in atrophy due to a prolonged hospital stay.

In ULLS protocols, the treated limb is flexed and suspended above the ground by means of a shoulder harness (Fig. 1) (Horstman et al., 2012). This model has been successfully used for decades to induce disuse atrophy in one limb (Brocca et al., 2015; Tesch et al., 2008). Moreover, by confining disuse muscle atrophy to the unloaded limb, the model closely resembles joint unloading after muscle and skeletal injuries, a frequent and relevant condition in clinical practice. ULLS globally causes a loss of muscle mass (just as other disuse models) of approximately 0.44% per day in the vastus lateralis, which is among the most affected muscles (Hackney and Ploutz-Snyder, 2012). Like bed rest and spaceflight, ULLS primarily affects the soleus over gastrocnemius fibers. In contrast to the other models, slow soleus fibers obtained after ULLS show a decrease in unloaded shortening velocity and a greater reduction in specific force (Widrick et al., 2002).

Continuous exposure of healthy volunteers to a  $-6^\circ$  head-down tilt position while resting in a bed has been shown to be an excellent model of many of the physiological changes that take place in spaceflight. In addition to providing the opportunity to study physiological, cellular, and molecular events, the bed rest studies also allow for testing the effectiveness of countermeasures and related protocols. Bed rest studies have produced and continue to generate a wealth of data on the physiological effects of inactivity on healthy individuals, its clinical implications, and the importance of activity for health. These studies have collectively shown that the antigravity extensors of the knee and ankle are the most affected (Alkner and Tesch, 2004b; LeBlanc et al., 1992; Rittweger et al., 2005). The greater relative decrease in maximal strength than size (as demonstrated in these studies) suggests that atrophy alone cannot account for the strength loss (Alkner and Tesch, 2004a, 2004b). Altered motor control, changes in the properties of the contractile machinery, reduced efficiency of the electromechanical coupling, and fat infiltrations may contribute to the loss of strength (Addison et al., 2014; di Prampero and Narici, 2003). In the past 20 years, a more comprehensive understanding emerged regarding the complex nature and variety of ways in which the reduction in z-axis gravitational stimuli affects body sensors and responses when a person is lying continuously in the horizontal position or during head-down bed rest (Pavy-Le Traon et al., 2007). Removal of both z- and x-axis gravitational stimuli can be achieved only in outer space or possibly reduced in the dry-immersion bed rest model.

Dry immersion involves immersing a subject (covered with an elastic waterproof fabric) in thermoneutral water. As a result, the immersed subject, who is freely suspended in the water mass, remains dry (Navasolava et al., 2011). For a relatively short period, the model can faithfully reproduce most of physiological effects of microgravity, including centralization of body fluids, support unloading,

and hypokinesia. In the last decades, this model has been useful for the space program as a ground simulation tool. Its application to space research contributed to improvement of this model and development of immersion under dry conditions that allows for longer experiments. Dry immersion induces an obvious decline in postural muscle tone and a decrease in the electromyographic activity of extensor muscles with a simultaneous increase in flexor activity within the first few hours. The intensity and rate of neuromuscular changes that occur during dry immersion are much higher than those during head-down bed rest (Navasolava et al., 2011). The unique pathophysiological model of dry immersion lately became relevant for basic research, with potential therapeutic applications. Russian scientists at the Institute of Biomedical Problems (Moscow, Russia) developed this model initially to simulate a microgravity environment. The removal of afferent signals from the support zones of the feet has been shown to trigger a dramatic drop in activity of the postural muscle system (Gevlich et al., 1983; Miller et al., 2004). Today, it also appears to be a model relevant to pathophysiological and aging studies.

### 2.3. Animal ground-based models of muscle disuse

The animals most frequently used for studying intervention strategies against skeletal-muscle aging are rats and mice. Yet even in rodents, life-long interventions still require 18–26 months of follow-up and are therefore costly and time-consuming (Kovanen and Suominen, 1987; Leeuwenburgh et al., 1997; Viidik et al., 1996). Senescence-accelerated mice (SAM) represent rodent models characterized by accelerated senescence and age-related pathologies (Takeda, 1999). Among these murine lineages, SAMP8 mice appear to be a suitable model for studies on skeletal-muscle aging (Derave et al., 2005).

Among rodent models of skeletal muscle disuse and wasting, most of the relevant scientific studies have used experimental models of denervation and hindlimb unloading (Bodine et al., 2001). Denervation has drastic and rapid effects on skeletal muscle, with accelerated protein degradation (Furuno et al., 1990), and denervation's role in aging muscle has always been difficult to identify. In this context, and because advancing age is associated with increased denervation, the experimental procedures of denervation have been largely used to explore and study the molecular consequences for skeletal muscle. As in several models of muscle deconditioning, investigators usually report a disproportional loss of muscle mass and force. The study by Carlson et al. (1996) revealed a 75% decrease of muscle mass and a 99% reduction in maximum isometric contractile force in the rat extensor digitorum longus (EDL) after 4 months of denervation. Moreover, Carlson et al. (2001) showed a decrease of 49% of muscle mass and an 83% reduction of maximum force in EDL of 32-month-old rats. Thus, the losses of muscle mass and force during aging appear much slower. At the histological level, denervated muscle and old muscles share some common signs: reduced microvasculature and increased amounts of interstitial connective tissue and collagen (Borisov et al., 2000). In parallel, the processes of apoptosis and myonuclear death are observed, both in denervated muscles and during aging, but it remains unclear

which pathways lead from nuclear death to cellular death (Borisov et al., 2001). As a general rule, severity and extent of skeletal-muscle damage observed in aging muscles are less than those observed after denervation.

The rodent model of hindlimb unloading (HU) has been developed by the National Aeronautics and Space Administration (NASA) Ames Research Center in the mid-1970s and was validated to simulate weightlessness (Morey, 1979; Morey-Holton and Globus, 2002). Several hundred research articles have been published. The most crucial parameters of this model are the housing unit, the device to which a rat or mouse is attached to allow for mobility, the angle of unloading, and the harness system (Chowdhury et al., 2013; Morey-Holton and Globus, 2002). The skeletal muscle wasting of HU rats is comparable in studies involving tail-HU and pelvic-HU in animals within the same period (usually 2 weeks). Soleus muscle mass, for example, decreases by ~40% after 2 weeks, either in tail-HU or pelvic-HU experiments (Bodine et al., 2001; Chopard et al., 2001; Chowdhury et al., 2013; Cros et al., 1999; Mueller et al., 2005; Picquet and Falempin, 2003). The study by Bodine et al. (2001), which describes a transcript profiling analysis comparing immobilization, denervation, and HU models, emphasized that although most genes perturbed during immobilization are regulated similarly during denervation, most of these genes are unaltered in the HU model, even though similar rates of atrophy are observed in these models. Nonetheless, this study reported for the first time that the two atrogenes, MuRF1 (Muscle RING finger 1) and MAFbx (muscle atrophy F-box), are upregulated in all three models of atrophy (Bodine et al., 2001).

### 2.4. Cell models of muscle wasting

It has been proposed that with various types of muscle atrophy, such as those induced by fasting, unloading (Stevenson et al., 2003), immobilization (Pattison et al., 2003), and diseases, there are subsets of genes whose differential expression is common for these types of atrophy (Lecker et al., 2004b), suggesting that these processes share common mechanisms regardless of the triggering event. Indeed, in muscle-wasting conditions cited above, several subsets of genes show differential expression similar to that in disease models (Lecker et al., 2004b) and thus attribute all kinds of atrophy inducers to a common pathway (Sandri et al., 2004; Sartorelli and Fulco, 2004). To study the molecular details of muscle atrophy, several cell culture models have been developed. Starvation of cultured cells was used as a system for identification of molecular details of whole-muscle atrophy (Sandri et al., 2004). The failure to replenish differentiation media, the simple application of short-term (hours to 2 days) phosphate-buffered saline (PBS) treatment to mature myotube cell lines (C2C12, L6) leads to rapid atrophy (Stevenson et al., 2005). PBS-treated cells showed activation of at least the ubiquitin protein ligase MAFbx (Sandri et al., 2004). These cell culture models have been said to “mimic” atrophy *in vivo* because they have in common at least a few changes in protein and mRNA expression (Sartorelli and Fulco, 2004). On the other hand, starvation of myotubes in culture revealed a distinctive phenotype: not a suitable model to study signaling pathways

**Table 1**

A summary of methods for assessment of muscle mass, muscle strength, and physical performance in humans and rodents.

Measured parameter	Humans	Rodents
Muscle mass	<ul style="list-style-type: none"> <li>• Computed tomography (CT)</li> <li>• Magnetic resonance imaging (MRI)</li> <li>• Dual energy X-ray absorptiometry (DXA)</li> <li>• Bioelectrical impedance (BIA)</li> </ul>	<ul style="list-style-type: none"> <li>• Weighing muscle after euthanasia</li> <li>• Postmortem cross-sectional area</li> <li>• Methods used in humans</li> </ul>
Muscle strength	<ul style="list-style-type: none"> <li>• Handgrip strength</li> <li>• Knee flexion/extension (e.g., Cybex)</li> </ul>	<ul style="list-style-type: none"> <li>• Auto grip strength meter (noninvasive)</li> <li>• Electrostimulation (very invasive)</li> </ul>
Physical performance	<ul style="list-style-type: none"> <li>• Short Physical Performance Battery (SPPB)</li> <li>• Gait speed</li> <li>• “Timed get-up-and-go” test (TUG)</li> <li>• Stair climb power test (SCPT)</li> </ul>	<ul style="list-style-type: none"> <li>• Wire Grip test and Mesh Grip tests</li> <li>• Beam balance test, tightrope test, and rotarod test</li> <li>• Maximal aerobic speed test</li> <li>• Maximal oxygen consumption test</li> </ul>

of whole-muscle atrophy conditions, such as those presented below. Yet the above approach indicated that the overt atrophy phenotype reflects a great many differentially expressed genes and is not simply attributable to a particular set of genes.

Glucocorticoids (GCs) are key mediators of muscle proteolysis and atrophy (Britto et al., 2014; Kuo et al., 2013; Schakman et al., 2013). Metabolic disruptions reported in atrophying skeletal muscle in humans and experimental animal models are also observed in cell experiments involving dexamethasone, and GC-treated cultured myotubes have been used in several studies as an *in vitro* model of muscle wasting (Klaude et al., 2007; Schakman et al., 2013; Thissen, 2005; Thompson et al., 1999; Tiao et al., 1997; Wang et al., 1998). In these experiments, dexamethasone-treated myotubes based on rat (L6) and mouse (C2C12) cell lines have been used to identify the mechanisms of muscle wasting. The concentration of dexamethasone varied among the studies, and the effects of dexamethasone were found to be induced by concentrations ranging from 10–50 nM to 100  $\mu$ M (Latres et al., 2005; Stitt et al., 2004; Sultan et al., 2006; Thompson et al., 1999). One study revealed, for example, that treatment with dexamethasone or corticosterone resulted in dose-dependent increases in protein degradation rates in both L6 and C2C12 myotubes accompanied by 25–30% reduction of myotube diameter (Menconi et al., 2008).

Mechanistically, GCs act via activation of the ubiquitin proteasome (UPS) and the lysosomal systems (Schakman et al., 2013; Thissen, 2005). Stimulation of these proteolytic systems, especially the UPS, is mediated by upregulation of several atrogenes such as FoxO1/3a, MAFbx, and MuRF-1 (Schakman et al., 2013). Aside from the GC-induced atrophy, inflammation-related atrophy has been extensively used for muscle cell culture (Schakman et al., 2013; Thissen, 2005). Tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ), originally called “cachectin” (Mirza et al., 2014a), can induce C2C12 atrophy when added to cell culture. TNF- $\alpha$  is a potent trigger of muscle wasting *in vitro* and *in vivo*, through inhibition of myogenesis and induction of apoptosis and proteolysis, via activation of nuclear factor  $\kappa$  B (NF- $\kappa$ B) and various components of the UPS even at a low concentration (1–3 ng/ml) (Bakkar and Guttridge, 2010; Cai et al., 2004; Li et al., 1998; Magee et al., 2008). In addition to the canonical Akt-dependent pathway, the FoxO4 pathway is stimulated by TNF- $\alpha$  treatment and leads to MAFbx mRNA upregulation (Moylan et al., 2008). A cocktail of proinflammatory cytokines such as TNF- $\alpha$  plus interferon (IFN)- $\gamma$  has also been used to simulate muscle

wasting in the C2C12 cell line (Dehoux et al., 2007; Kimura et al., 2014b). Transforming growth factor  $\beta$ 1 (TGF- $\beta$ 1) belongs to a family of multifunctional cytokines including bone morphogenic proteins (BMPs) and activins (Massagué, 1990). TGF- $\beta$ 1 plays essential roles in various biological processes, including cell growth, differentiation, apoptosis, tissue development, and inflammation (Massagué, 1990; Shull et al., 1992). Besides the fibrogenic effects, studies showed that TGF- $\beta$ 1 is also a potent inhibitor of growth and differentiation of myoblasts (Allen and Boxhorn, 1987) and can suppress division and block fusion of satellite cells both *in vitro* and *in vivo* by suppressing myogenic factors (Allen and Boxhorn, 1987; Zhu et al., 2004). When incubated with C2C12 cells, 1 ng of TGF- $\beta$ 1 reduces myotube diameter and increases protein expression of MuRF-1 and MAFbx (Abrigo et al., 2016; Mendias et al., 2012). More recently, Nozaki et al. (2015) developed the C2C12 cell line from the BubR1 hypomorphic mouse model of accelerated-aging (Baker et al., 2008); these cells are characterized by a subpopulation of muscle stem cells expressing p16Ink4a (Nozaki et al., 2015). This modified cell line shows increased expression of the muscle-specific ubiquitin ligases MAFbx and MuRF-1, reduced expression of MyoD and myogenin, and a decreased fusion index (Nozaki et al., 2015).

### 3. Characteristics of sarcopenia-related muscle deconditioning

The three components of sarcopenia are low muscle mass, low muscle strength, and poor physical performance (Cruz-Jentoft et al., 2010; Fielding et al., 2011; Morley et al., 2011; Muscaritoli et al., 2010). Table 1 presents methodologies used to assess muscle mass, muscle strength, and physical performance in humans and rodents.

Typically, in humans, muscle mass remains stable during early life, but after age ~50 years, muscle mass declines at a rate of ~1% per year in men and ~0.5% in women (Mitchell et al., 2012). Nevertheless, the decline of skeletal-muscle mass may accelerate along with aging, amounting to 6% per decade between 30 and 70 years of age (Fleg and Lakatta, 1988), 1.4% to 2.5% per year after age 60, and can start as early as 35 years of age (Frontera et al., 2000a). In humans, it is accepted that aging is accompanied by a decrease in muscle mass by ~40% from the adulthood to death (Ibebunjo et al., 2013; Kimball et al., 2004; Lexell et al., 1988); this decrease can exceed 50% (Baumgartner et al., 1998). Rodents, especially rats, are experimental animals particularly useful

for studying sarcopenia. Depending on the strain, rats are considered elderly between 18 and 30 months (Hopp, 1993). Fischer 344 Brown Norway F1 hybrid and Wistar rats are the most popular rat strains for studies on sarcopenia. Usually, regardless of the strain, at age ~18 months (middle age for rats), the weight of the soleus, EDL, gastrocnemius, quadriceps, tibialis anterior, and plantaris is reduced as compared to animals aged 6 or 12 months (Ibebunjo et al., 2013; Kimball et al., 2004; Paturi et al., 2010). This decrease is relatively slow and small at 18 months of age (~10%) but accelerates thereafter to reach 30% to 40% at age 24 months (old age in rats) (Ibebunjo et al., 2013; Kimball et al., 2004; Paturi et al., 2010). In very old animals (~30 months old), this decrease can reach 60% in some muscles, notably the gastrocnemius (Ibebunjo et al., 2013; Kimball et al., 2004).

In humans, the age-related decrease in muscle mass is mainly due to a loss of muscle fibers affecting both type I fiber and type II fiber (Aniansson et al., 1986; Lexell et al., 1988; Young et al., 1985). Although a decrease by only 5% in the number of fibers occurs between 24 and 50 years of age, a reduction of 30% to 40% is reported between 50 and 80 years (Aniansson et al., 1992). Nonetheless, atrophy of muscle fibers (reduction of their cross-sectional area) is also implicated in the decrease of muscle mass associated with aging (Aniansson et al., 1986; Lexell et al., 1988). Atrophy does not affect all types of muscle fibers similarly. Indeed, fast type II fibers appear to be the most affected by aging, with a decline from 20% to 60% in their size (Hikida et al., 2000; Larsson et al., 1978; Lexell et al., 1988). Among type II muscle fibers, greater reductions are observed in the fiber IIX type compared to type IIA fibers (Aniansson et al., 1986; Coggan et al., 1992).

The decrease in muscle strength is a key diagnostic criterion of sarcopenia, and the functional consequences are important for autonomy of the affected person (Cruz-Jentoft et al., 2010). Muscle strength of the knee extensors should be considered due to its functional importance (Doherty, 2003). On average, the peak strength decreases by 20–40% between 20–30 and 70–80 years (Larsson et al., 1979; Murray et al., 1985; Young et al., 1985). Similar results were observed with other muscle groups such as shoulder and wrist flexors (Bassey and Harries, 1993; McDonagh et al., 1984). Greater reductions (50%) are still reported in subjects aged over 90 years (Murray et al., 1980, 1985). Decreased muscle strength seems to be accelerated especially between 60 and 70 years. Indeed, longitudinal studies revealed a reduction by 30% to 40% in the peak strength of the knee and shoulder extensors between ages 60 and 70 years (Aniansson et al., 1986; Frontera et al., 2000b; Hughes et al., 2001). Similar results were observed in rodents, but the onset of this phenomenon seems to depend on the strain and age of the rodent. Thus, in Fisher 344 Brown Norway F1 hybrid and aged mice, a decrease in maximal force is generally observed between 32 and 36 months in the soleus, gastrocnemius, and EDL (Brooks and Faulkner, 1988; Ryall et al., 2007; Thomas et al., 2010). In Wistar rats, no difference was observed in the EDL, but maximal force decreased after 24 months in the soleus.

Gait speed is now the recommended parameter for assessment of physical performance for diagnosis of sarcopenia (Cruz-Jentoft et al., 2010; Fielding et al., 2011; Morley et al.,

2011); however, there is still no consensus on the cutoff value for diagnosis of sarcopenia. Gait speed is usually evaluated by the 6-meter test (Fielding et al., 2011; Morley et al., 2011) or the 4-meter test (Cruz-Jentoft et al., 2010). Cutoff levels for sarcopenia are defined as a speed less than 1 m/s in the first case and less than 0.8 m/s in the second (Cesari et al., 2009). Gait speed can be easily used in clinical practice and research. Other tests designed specifically for elderly people are also acceptable. The most popular are the Short Physical Performance Battery (SPPB) (standardized battery of short physical tests), the timed get-up-and-go (TUG), or the stair climb power test (SCPT). The SPPB evaluates balance, gait speed, strength, and endurance by examining an individual's ability to stand with the feet together in side-by-side, semitandem, and tandem positions, time to walk 8 feet, and time to rise from a chair and return to the seated position five times (Guralnik et al., 1994). Each event yields a performance score, and the sum of the scores on all the subtests characterizes overall performance. A score below 8/12 points to probable sarcopenia (Guralnik et al., 2000). SPPB is a standard measure for research and for clinical practice. The TUG is a test of the time required to perform a series of basic motor tasks. The subject must stand up from a chair, walk a short distance, turn around, and come back to sit. This method allows for estimation of the dynamic balance that is assessed on a scale of 1 to 5 (Mathias et al., 1986). A score below 3 means probable sarcopenia (Mathias et al., 1986). Finally, the SCPT when used clinically estimates power of the lower limbs (Bean et al., 2007). The subject must climb 10 steps as soon as possible. The power of the lower limbs is then calculated in relation to the height of the steps and the rate of climbing and is normalized to the weight of the subject (Bean et al., 2007). This test may be useful in some research settings but the cutoff point for sarcopenia needs to be defined. Poor physical performance is also revealed by a decrease in both resting and maximal oxygen consumption with advancing age in humans (Short et al., 2004). In rodents, there is no consensus on how to evaluate the decrease in physical performance in old animals, but numerous tests are available (Table 1). Very few data are available on old rodents. Nevertheless, alterations in the balance and coordination in old rodents (23–24 months) have been reported by Altun et al. (2007) and Emerich et al. (2008), who measured the time that a rodent can stay on a narrow beam (Beam Balance Test) or a tightrope (tightrope test). Decreased physical performance in old rodents is also shown by decreased endurance capacity in old rats compared to young rats (Derbre et al., 2012).

Sarcopenic (and more generally deconditioned) muscle is also characterized by an increase in IMAT infiltrations (Addison et al., 2014; Goodpaster et al., 2000). This point will be discussed in part 5.

#### 4. Cellular and molecular alterations of skeletal muscle

The development of effective treatments or prophylactic measures against sarcopenia requires understanding the cellular and systemic mechanisms as well as the pathways involved in sarcopenia onset and development.

Maintaining muscle mass is mostly a balancing act between protein synthesis and protein degradation systems. It is well established that sarcopenia involves negative protein turnover (Combaré et al., 2009) characterized by the reduction of myofibrillar (especially myosin heavy chain: MyHC) and mitochondrial proteins synthesis (Balagopal et al., 1997; Cuthbertson et al., 2005; Haddad and Adams, 2006) and their increased proteolysis via the UPS and calcium-dependent activation of proteases (i.e., calpains and caspases) (Gumucio and Mendias, 2013; Konopka and Sreekumaran Nair, 2013). The decrease in MyHC synthesis is at least due to a decrease in its transcription because the amounts of mRNA of different isoforms decrease during aging, e.g., MyHC IIa and MyHC IIx isoforms (Balagopal et al., 2001; Short et al., 2005). This may explain in part why MyHC protein content in the muscle of old animals is reduced as compared to young animals (Haddad and Adams, 2006; Thompson et al., 2006) and why MyHC IIa and IIx protein levels decline by 3% and 1% per decade, respectively, in humans (Short et al., 2005). This decrease in the synthesis of myofibrillar proteins appears to be specific because actin synthesis is not affected by aging in humans (Hasten et al., 2000) and its protein expression in muscle is unchanged in aged animals (Haddad and Adams, 2006; Thompson et al., 2006).

Given the vital functions carried out by mitochondria in the context of energy provision, redox homeostasis, and regulation of several catabolic and cell death pathways, it is not surprising that age-related alterations of mitochondrial functions are viewed as the central cause of sarcopenia by numerous authors (Calvani et al., 2013; Konopka and Sreekumaran Nair, 2013; Marzetti et al., 2013). One major consequence of age-associated mitochondrial dysfunction is a decline in bioenergetics, judging by a decrease in both resting and maximal oxygen consumption ( $\dot{V}O_2\text{max}$ ) with advancing age in humans (Short et al., 2004) and mice (Lee et al., 2010) and by decreased endurance capacity in old rats compared to young rats (Derbre et al., 2012). Moreover, perturbations in mitochondrial energetics of skeletal muscle have been shown to correlate with reduced  $\dot{V}O_2\text{max}$  (Short et al., 2005), walking capacity (Coen et al., 2013), and maximal isometric strength (Safdar et al., 2010) in older adults and are associated with an increase in muscle fatigability in old rats (Chabi et al., 2008). The bioenergetic failure of the sarcopenic muscle is related to a reduction in mitochondrial numbers and functions (Calvani et al., 2013; Vina et al., 2009), which is the result of a vicious cycle involving production of reactive oxygen and nitrogen species and damage to (and/or depletion of) mitochondrial DNA (mtDNA), and defective quality control of mitochondria (Marzetti et al., 2013).

The equilibrium between apoptosis and regeneration processes is also involved in the maintenance of muscle mass (Hikida, 2011; Marzetti et al., 2012a; Snijders et al., 2009). Decreased capacity for muscle regeneration (Hikida, 2011; Snijders et al., 2009) and exacerbation of apoptosis (Marzetti et al., 2012a) are believed to be involved in sarcopenia-associated muscle deconditioning. This notion is supported by the observation that apoptotic signaling correlates with slow walking speed and reduced muscle volume in older persons (Marzetti et al., 2012b). Moreover, numerous studies have revealed that the extent of apoptotic DNA fragmentation

increases in skeletal muscle in the course of aging, thus paralleling the development of sarcopenia (Braga et al., 2008; Kovacheva et al., 2010; Marzetti et al., 2012b; Siu et al., 2006; Wohlgenuth et al., 2010); this fragmentation is likely involved in the decrease in transcriptional efficiency observed during sarcopenia (Cuthbertson et al., 2005; Roberts et al., 2010). This phenomenon is probably worsened by alterations in muscle regeneration capacity limiting incorporation of new nuclei into aging muscle fibers by satellite cells (SCs). This point will be addressed in Section 5.2.

Although skeletal muscle atrophy contributes substantially to the loss of muscle strength during aging, it is not considered the only factor involved in this phenomenon. Indeed, data from animal studies have shown that specific strength of isolated muscle fibers (i.e., force normalized to the cross-sectional area of the fiber) also decreases with age (Gonzalez et al., 2000; Renganathan et al., 1998; Thompson, 2009; Thompson and Brown, 1999), but contradictory results have been reported too (Clafin et al., 2011). Several mechanisms are proposed to explain these results, e.g., post-translational modifications of contractile proteins (Lowe et al., 2001), decoupling of complex excitation–contraction (Wang et al., 2000), and decreased myosin ATPase activity (Lowe et al., 2004). Studies focused on permeabilized muscle fibers have shown that the decrease in specific strength is also explained by a reduction in the fraction of myosin heads that bind to actin filaments (Lowe et al., 2001, 2004). The research on isolated intact muscle fibers revealed the involvement of excitation–contraction coupling in age-associated changes of muscle contractile properties. Thus, the maximal release of calcium from the sarcoplasmic reticulum is decreased in aged rodent muscle tissue (Jimenez-Moreno et al., 2008). Neuromuscular impairments such as a loss of motor units and loss of motor neurons are also involved in muscle strength reduction during aging as reviewed well by Piasecki et al. (2015).

These mechanisms contribute to the onset of sarcopenia and are affected by numerous upstream factors including a decrease in the release of anabolic hormones (e.g., growth hormone [GH], insulin like growth factor 1 [IGF-1], or testosterone) (La Colla et al., 2015; Morley and Malmstrom, 2013), increased production of proinflammatory cytokines (e.g., interleukin 6 and TNF- $\alpha$ ) (Lee et al., 2007), insulin resistance (Walrand et al., 2011), neuromuscular aberrations (Edstrom et al., 2007; Piasecki et al., 2015), dysregulation of microRNAs (McGregor et al., 2014; Rivas et al., 2014; Zampieri et al., 2015), and an increase in muscle oxidative stress (Brioché and Lemoine-Morel, 2016; Derbre et al., 2014; Jackson, 2009; Jackson and McArdle, 2011; Ji, 2007).

## 5. IMAT: an important factor of sarcopenia

There is now a growing body of evidence that the loss of strength and power mostly exceeds the loss of muscle mass observed after inactivity (di Prampero and Narici, 2003). Fatty infiltrations or IMAT may be an additional independent variable explaining the loss of muscle strength. IMAT can be first defined as adipocyte deposition located between the muscle fibers and between muscle groups; IMAT should not be confused with intramyocellular triglyceride accumulation (Addison et al., 2014; Karampinos et al.,



2012; Vettor et al., 2009). Although IMAT may be a variable part of healthy human skeletal muscle, its increase and accumulation are linked to muscle dysfunction, deconditioning, and even perturbed regeneration (Addison et al., 2014; Marcus et al., 2010; Sciorati et al., 2015; Uezumi et al., 2014b). These fatty infiltrations are well known to be associated with many conditions: myopathies, diabetes, COPD, cachexia, or even sarcopenia, among many others. To our knowledge, Aherne (1965) was the first to describe fat infiltration in various tissues, including skeletal muscle, in a newborn infant, and this report was followed by other studies (Birkbeck, 1970; Kannan et al., 1976; Kelley et al., 1991; Nordal et al., 1988). Recognition of the role of IMAT in muscle function seems to increase year after year, and scientific knowledge will certainly continue to expand around this subject. This part of the review is focused on IMAT accumulation with aging, its impact on muscle function, and the major role of increasing physical activity as a countermeasure. We will also try to highlight some cellular players possibly responsible for IMAT development with advancing age, in particular, one specific skeletal-muscle mesenchymal-stem-cell population called fibroadipogenic progenitors (FAPs).

### 5.1. The fight against inactivity: the real purpose

The literature highlights a correlation between IMAT levels and muscle function. Indeed, numerous studies have demonstrated that an increase in IMAT accumulation matches a decrease in muscle mass, muscle strength, and insulin-sensitivity (Delmonico et al., 2009; Goodpaster et al., 2000; Miljkovic-Gacic et al., 2008; Visser et al., 2005). First, it has long been well known that IMAT levels increase strongly with age (Addison et al., 2014; Goodpaster et al., 2000; Kirkland et al., 2002) and some relatively old papers have already showed larger fatty infiltrations in old men (Borkan et al., 1983; Rice et al., 1989) and women (Ryan and Nicklas, 1999; Song et al., 2004) using computed tomography, or more recently, magnetic resonance imaging. Two studies, Borkan et al. (1983) and Rice et al. (1989), were the first to demonstrate fatty infiltrations with aging accompanied by a decrease in muscle mass (Gallagher et al., 2000) and in muscle strength (Jubrias et al., 1997). Subsequent studies evaluated the relation between levels of IMAT and muscle function. Two studies, performed on the same elderly cohort, demonstrated a correlation between the degree of IMAT accumulation and the loss of both muscle strength and performance (Goodpaster et al., 2001; Visser et al., 2002). In fact, a study by Visser et al. (2002) showed decreased performance on the 6-meter walk and repeated chair stands tests, with a correlation between IMAT levels and performance on both tests. Later, a study by Delmonico et al. (2009) confirmed that the loss of muscle strength with aging matches IMAT development. Altogether, these studies have helped to identify a new important component of sarcopenia: IMAT accumulation. In the specific context of sarcopenia, other studies have shown that IMAT infiltration into the mid-thigh muscle remains an independent risk factor of mobility limitations (Beavers et al., 2013; Tuttle et al., 2012; Visser et al., 2005). The level of fatty infiltrations was also found to be a good predictor of clinical fracture

in older adults (Schafer et al., 2010). Finally, a study by Marcus et al. (2012) demonstrated again the IMAT contribution to the impaired mobility of older adults and showed once again that IMAT is an essential component of sarcopenia.

Although IMAT is widely known to be strongly linked to sarcopenia, the following studies suggest that IMAT accumulation may also be a result of global muscle inactivity rather than aging *per se* (Leskinen et al., 2009; Manini et al., 2007; Tuttle et al., 2011). Indeed, a study by Manini et al. (2007) demonstrated that only 4 weeks of unilateral limb suspension can increase IMAT accumulation in healthy young adults. Moreover, a longitudinal study based on twin populations showed that physically inactive cotwins have greater IMAT accumulation as compared with the active cotwins (Leskinen et al., 2009). A study by Tuttle et al. (2011) also revealed a correlation between physical activity levels, measured by the average daily step count, and IMAT accumulation in people with diabetes and peripheral neuropathy. One research group also found an increase in IMAT area 6 weeks after incomplete spinal cord injury (Gorgey and Dudley, 2007), and another study showed IMAT accumulation to be associated with mobility limitations in older adults (Murphy et al., 2014). Although these studies were performed on different populations, they contribute to the knowledge on IMAT accumulation and highlight the highly plausible role of physical activity interventions in aging.

A study by Goodpaster et al. (2008) was the first to detail precisely 1 year of increased physical activity among older adults. They demonstrated a clear protective effect of physical activity against the aging-associated IMAT increase and against the decrease in knee extensor strength. Another study, published almost at the same time, by Marcus et al. (2008), revealed similar data on IMAT accumulation during both combined aerobic and eccentric resistance training or only aerobic training in a type 2 diabetes population. Two years later, the same group investigated the effect of 12 weeks of eccentric exercise training in the elderly (Marcus et al., 2010) and found an 11% decrease in thigh IMAT area. Under almost the same conditions, Ryan et al. (2011) also found a decrease in the IMAT area in old stroke survivors. Another study examined the effect of 8 months of endurance exercise on sedentary subjects with dyslipidemia and showed a decrease in IMAT accumulation (Durheim et al., 2008). Two studies also prevented an increase in IMAT with aging by a 1-year diet-and-moderate-exercise intervention in overweight and obese patients with type 2 diabetes (Gallagher et al., 2014) or by a more intensive but shorter exercise intervention in older people prone to falls (Jacobs et al., 2014). A recent study has shown a decrease in the IMAT area after 5 months of moderate resistance training in overweight and obese older adults (Nicklas et al., 2015). Taaffe et al. (2009) also observed an increase in IMAT content among the elderly after a detraining session followed by a decrease in IMAT levels after a retraining resistance exercise session. Lastly, an interesting study by Wroblewski et al. (2011) on old elite athletes, trained for fitness and sports competitions at least four or five times per week, showed that they do not exhibit any loss of lean muscle mass or any increase in IMAT amount with aging. The last two studies strengthen the notion that some muscular alterations

observed in sarcopenia are a direct consequence of a gradual decrease in physical activity among older adults.

Numerous studies also failed to detect any decrease in IMAT content after an exercise program (Christiansen et al., 2009; Jung et al., 2012; Ku et al., 2010; Prior et al., 2007; Santanasto et al., 2011; Walts et al., 2008), even though studies demonstrating no increase in IMAT content after an exercise intervention among the elderly may also be a good result. These discrepancies can be explained from a methodological point of view in relation to the age of participants, their race, and the associated diseases or even the type of exercise used, its intensity, and duration. Altogether, these studies appear to reveal an important role of increasing physical activity in stabilization of (or even a decrease in) IMAT development and accumulation during aging. Further studies are needed to precisely define the most effective way to train the elderly in order to combat the progressive sedentary lifestyle leading to sarcopenia and IMAT accumulation, both affecting muscle function and overall health of older adults.

### 5.2. How does fat infiltration occur in sarcopenia?

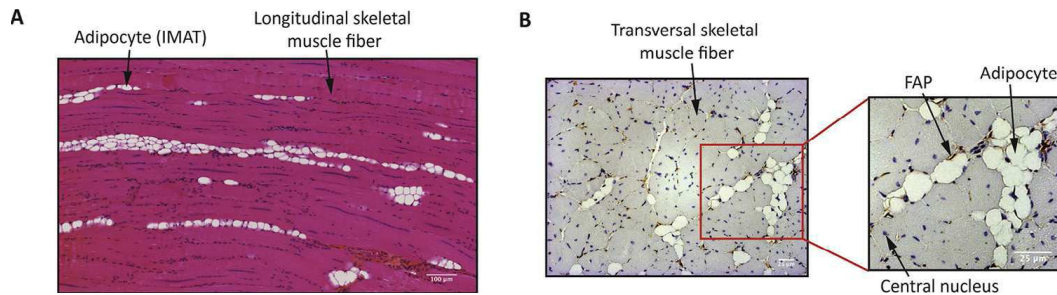
Even if IMAT development and accumulation will be studied more, the mechanisms leading to fatty infiltrations during sarcopenia or a sedentary lifestyle are poorly understood. To our knowledge, a review by Kirkland et al. (2002) was the first to give some clues to IMAT accumulation during aging. They highlighted a possible role of disruption of the leptin pathway in a decreased ability to metabolize fatty acids with aging (Koteish and Diehl, 2001; Wang et al., 2001) and also pointed to potential involvement of SCs and mesenchymal stem cells (MSCs).

Concerning SCs, it appears that the SC pool is maintained until ~70 years of age (Dreyer et al., 2006; Roth et al., 2000) and then declines (Kadi et al., 2004; Renault et al., 2002; Sajko et al., 2004; Verdijk et al., 2014). There is a specific decline in SC content located under the basal lamina of type II muscle fibers (Verdijk et al., 2007, 2012, 2014), which coincides exactly with the preferential development of sarcopenia in the fast-twitch type of muscle. It is also important to note that advancing age is associated with impaired proliferation capacity of SCs (Conboy et al., 2003; Schultz and Lipton, 1982; Suetta et al., 2013) concomitant with the expected decrease in regenerative capacity (Collins-Hooper et al., 2012; Gallegly et al., 2004; Marsh et al., 1997; Renault et al., 2002; Sadeh, 1988). Numerous studies based on parabiosis and whole-muscle grafting experiments have demonstrated that the decline in muscle SC function with aging may be largely attributed to extrinsic/microenvironmental factors (Brack et al., 2007; Carlson and Faulkner, 1989; Conboy et al., 2005; Harrison, 1983; Villeda et al., 2011). However, newer studies, involving more powerful SC isolation/purification tools, have also confirmed SC-specific alterations in sarcopenia (Bernet et al., 2014; Chakkalakal et al., 2012; Cosgrove et al., 2014; Price et al., 2014; Sousa-Victor et al., 2014). A large number of reviews have already discussed the recent research on this subject (Blau et al., 2015; Brack and Muñoz-Canoves, 2015; La Colla et al., 2015; Sousa-Victor et al., 2015) and precisely detail both extrinsic and intrinsic factors that influence SC function during aging. The review by Brack and Muñoz-Canoves

(2015) also highlights the importance of SC heterogeneity as the main source of discrepancies among studies on SC function with advancing age, and those authors argue that distinct SC subsets are differentially sensitive to extrinsic and intrinsic factors probed in these studies. Our review will not elaborate on SC function in sarcopenia or on transdifferentiation as already discussed in other reviews (Sciorati et al., 2015; Vettor et al., 2009) but will instead discuss the potential role of FAPs in IMAT accumulation in conjunction with advancing age.

The muscular environment contains various populations of tissue-resident progenitors, including the MSCs called FAPs. These FAPs, which are positive for the cell surface marker platelet-derived growth factor receptor  $\alpha$  (PDGFR $\alpha$  or CD140a), are a widely studied stem cell population in skeletal muscle. FAPs are able to proliferate quickly after injury, participate in phagocytosis of necrotic muscle fibers, and support SC-mediated efficient muscle regeneration without differentiating into adipocytes (Heredia et al., 2013; Joe et al., 2010; Lemos et al., 2015). In muscle disuse or pathological conditions, such as Duchenne muscular dystrophy, FAPs proliferate and differentiate into adipose and/or fibrous tissue (Uezumi et al., 2011, 2014a). Simultaneously with the decrease in SC content (Blau et al., 1983; Heslop et al., 2000), in this case, FAPs lead to accumulation of IMAT. In order to investigate IMAT development and accumulation in an experimental mouse model, the glycerol model of regeneration is commonly used (Joe et al., 2010; Lukjanenko et al., 2013; Pagano et al., 2015; Pisani et al., 2010; Uezumi et al., 2010) and allows for better characterization of muscle-resident adipocyte precursors (Fig. 2). Even though no study has been conducted to characterize the role of FAPs in sarcopenia, the literature highlights a possible important function of FAPs in age-related development of IMAT (Blau et al., 2015; Farup et al., 2015). Moreover, two *in vitro* studies have demonstrated that skeletal-muscle-FAP-derived adipocytes are insulin resistant (Arrighi et al., 2015; Laurens et al., 2015). These studies support, on the one hand, the correlations between IMAT accumulation and the decrease in insulin sensitivity observed during aging and obesity (Goodpaster et al., 1997, 1999, 2000; Ryan and Nicklas, 1999; Simoneau et al., 1995), and on the other hand, the fact that FAPs can be the main stem cell population implicated in sarcopenia-related IMAT development.

The recent literature underscores the critical role of the immune and inflammatory systems in efficient muscle regeneration, and various reviews excellently summarized recent advances in this field (Aurora and Olson, 2014; Madaro and Bouche, 2014; Maffioletti et al., 2014; Sciorati et al., 2015). A study by Lemos et al. (2015) also showed the crucial effect of the balance between TNF- $\alpha$  and TGF- $\beta$ 1 on FAPs. This study demonstrated that TNF- $\alpha$  that is released by M1 macrophages provokes FAP apoptosis, whereas TGF- $\beta$ 1 (released by M2 macrophages) promotes their survival. Interestingly, regeneration and aging are associated with deregulation of numerous systems including inflammation. Indeed, Butcher et al. (2001) showed alteration of neutrophil function with aging, and these results, in light of those of Heredia et al. (2013), may indicate a defective neutrophil-induced IL-4/IL-13 release and further signaling toward FAPs, promoting their adipocytic differentiation.



**Fig. 2.** Histological analysis of IMAT accumulation induced in the glycerol model of skeletal-muscle regeneration in mice. (A) A histological longitudinal paraffin-embedded muscle section, stained with hematoxylin-eosin-saffron, 21 days after intramuscular injection of glycerol into the murine tibialis anterior muscle. (B) A histological transversal paraffin-embedded muscle section, 21 days after intramuscular injection of glycerol into the tibialis anterior (labeling with an anti-PDGFR $\alpha$  antibody).

A study by Lemos et al. (2015) also elegantly demonstrated the precise and crucial temporal transition of TNF- $\alpha$  (proinflammatory) to TGF- $\beta$ 1 (proregenerative) cytokine production during muscle regeneration. Przybyla et al. (2006) demonstrated defective regulation of muscle macrophage function with advancing age, and some studies also showed an increase in both TNF- $\alpha$  and TGF- $\beta$  levels (Carlson et al., 2008, 2009; Fagiolo et al., 1993; Merritt et al., 2013). Therefore, the inflammatory deregulation and the possible overlap between TNF- $\alpha$  and TGF- $\beta$ 1 signaling may promote survival of FAPs and adipocytic differentiation leading to IMAT development and accumulation during sarcopenia. The upregulation of TGF- $\beta$  observed during aging can also explain the increase in the muscle levels of FAPs discussed in a review by Shadrach and Wagers (2011) although these are unpublished observations. Nevertheless, some other molecular pathways can also modulate the fate of FAPs during aging and will be discussed below.

Numerous studies by the same group evaluated the effect of histone deacetylase inhibitors (HDACi) on dystrophin-deficient mdx mice (characterized by permanent muscle lesion-regeneration cycles). These researchers first showed that HDACi treatment of mdx mice promotes a functional and morphological recovery of skeletal muscle, certainly mediated in part by an increase in follistatin expression (Minetti et al., 2006). They next showed that in young mdx mice, FAPs are sensitive to adipogenesis inhibition mediated by HDACi and thus still support SC differentiation (Mozzetta et al., 2013). However, they also demonstrated in old mdx mice the inability of HDACi treatment to be as effective as in young mdx mice. Another study showed that an epigenetic intervention, again with HDACi, not only suppresses the adipogenic potential of FAPs from young mdx mice but also unexpectedly promotes FAPs' myogenic lineage (Faralli and Dilworth, 2014; Saccone et al., 2014). In contrast, FAPs from old mdx mice fail to adopt a myogenic phenotype, and altogether these studies reveal that FAPs are highly influenced by environmental changes during aging. It is important to note, however, that the population isolated in that study was not only purified FAPs: it may contain other populations such as PW1 interstitial cells (PICs), side population cells (SP), or even type 1 pericytes (Boppart et al., 2013; Judson et al., 2013); this methodological point may explain the unexpected development along the myogenic lineage.

The Notch signaling pathway may also be involved in FAP-driven IMAT development during aging. This pathway is a major one implicated in the maintenance of SC quiescence as well as promotion of their proliferation after injury (Bjornson et al., 2012; Conboy et al., 2003; Mourikis et al., 2012; Pellegrinet et al., 2011; Wen et al., 2012). During aging, an attenuated Notch signaling response results in impaired proliferation of SCs and a regeneration failure (Conboy et al., 2003, 2005). Interestingly, TGF- $\beta$  is a Notch inhibitor upregulated during aging (Buas and Kadesch, 2010; Carlson et al., 2008, 2009) and may explain the spontaneous entry of SCs into the cell cycle during aging. In addition, research has demonstrated that altered Notch signaling in the adult heart leads to abnormal development of fibrosis (Croquelois et al., 2008; Nemir et al., 2014). Notch signaling thereby regulates key mechanisms in mesenchymal cardiac progenitors and controls the balance between fibrosis development and cardiac repair in the adult heart. Nevertheless, there are no studies concerning the function of the Notch signaling pathway in FAPs, and further research is clearly needed to identify a highly possible role of this pathway in aged skeletal muscle.

Plenty of studies have already shown a major role of Wnt signaling in the inhibition of MSC differentiation into adipocytes accompanied by promotion of their myogenic fate (Bennett et al., 2002; Brunt et al., 2012; Chung et al., 2012; Moldes et al., 2003; Ross et al., 2000; Shang et al., 2007; Zaragosi et al., 2008). A study by Vertino et al. (2005) was focused particularly on Wnt10b, and they concluded that during aging, altered Wnt10b signaling increases expression of key adipogenic genes promoting IMAT accumulation. The Wnt signaling pathway appears to be particularly complex, especially when related to aging because expression levels of Wnt proteins will not all vary in the same direction. Further studies are needed to elucidate the mechanisms underlying age-related deregulation of Wnt signaling and IMAT development during sarcopenia.

Aging is also linked to altered synthesis of nitric oxide (NO) in skeletal muscle (Nyberg et al., 2012; Samengo et al., 2012). A study by Cordani et al. (2014) demonstrated *in vitro* that NO regulates FAP fate through inhibition of their differentiation into adipocytes. This mechanism was already reviewed by the same group recently (Sciorati et al., 2015), and these findings reveal another potential

mechanism behind FAPs' driving adipogenesis with advancing age.

Finally, the fibroblast growth factor 2 protein (FGF-2) seems to also play an important role in age-related impairment of skeletal muscle regeneration. FGF-2 expression increases with age, due to decreased expression of its inhibitor Sprouty 1. This mechanism induces a loss of stem cell quiescence, driving SCs to enter the cell cycle, and then most likely contributes to the loss of SCs observed during aging (Chakkalakal et al., 2012). No studies have been conducted on the effect of FGF-2 on FAPs even though this growth factor seems to also perform significant functions in differentiation of MSCs (Bae et al., 2015; Cai et al., 2013; Lee et al., 2013).

In conclusion, this part of the review highlighted the increasing recognition of the role of IMAT in the age-mediated loss of skeletal-muscle function. We first described the now well-known IMAT accumulation during sarcopenia and emphasized that this new important factor is closely linked to inactivity. We next reviewed various studies demonstrating the major role of increasing physical activity in stabilization of (or even a decrease in) IMAT accumulation in the elderly depending on the training characteristics. After that, we tried to emphasize new clues concerning FAPs and aging by linking them to some cellular players. We showed that many signaling pathways including HDAC, Notch, Wnt, NO, and FGF-2 may be implicated in aging-induced IMAT development and accumulation. We are confident that new studies will strengthen the links among FAPs, aging, and the influence of the stem cell niche regulating IMAT development.

## 6. Strategies against sarcopenia

Identification of cost-effective interventions to maintain muscle mass, muscle strength, and physical performance in the elderly is an important public health challenge. The perfect strategy would act on the three components of sarcopenia and would have the fewest possible side effects. Exercise appears to be the perfect strategy against sarcopenia (Montero-Fernandez and Serra-Rexach, 2013; Pillard et al., 2011; Wang and Bai, 2012). However, large-scale implementation of such an intervention is hampered by the lack of motivation among most persons. In addition, many senior citizens are nonambulatory or have comorbidities such as moderate to severe osteoarthritis (Bennell and Hinman, 2011) or certain forms of unstable cardiovascular disease that can preclude participation in resistance training exercises (Williams et al., 2007). To overcome such barriers, development of alternative methods for prevention and treatment of sarcopenia is very important.

Here, we will focus on exercise, hormone replacement therapies, and HMB. In the last part, we will discuss the possibility of targeting the glucose 6-phosphate dehydrogenase (G6PDH) as a new effective strategy against sarcopenia. Other strategies such as pharmacological treatments (angiotensin-converting-enzyme inhibitors, statins, myostatin inhibitors, or anti-inflammatory drugs), nutritional strategies (e.g., whey proteins, branched amino acids, and vitamin D) and antioxidant strategies (e.g., supplementation with antioxidants or pharmacological inhibitors of pro-oxidant enzymes such as allopurinol) have been reviewed well elsewhere (Brioché

and Lemoine-Morel, 2016; Brook et al., 2015; Cruz-Jentoft and Landi, 2014; Gomez-Cabrera et al., 2013; Maggio et al., 2013; Morley and Malmstrom, 2013; Sanchis-Gomar et al., 2011). For each strategy, we will present data on the efficiency and the mechanisms of action on sarcopenia.

### 6.1. Exercise as the most effective strategy against sarcopenia

Exercise appears to be the perfect strategy against sarcopenia because it can increase muscle mass, strength, and physical performance (Brook et al., 2015; Montero-Fernandez and Serra-Rexach, 2013; Pillard et al., 2011; Wang and Bai, 2012). In this work, when not specified, the word "exercise" will refer to repetition of different exercise sessions (i.e., training). Exercise also has positive effects on the metabolic, cardiovascular, and reproductive systems (Montero-Fernandez and Serra-Rexach, 2013; Pillard et al., 2011; Wang and Bai, 2012). In addition, exercise is known to improve quality of life and psychological well-being and is associated with better mental health and social integration: it alleviates anxiety and depression and improves self-efficacy in older adults (Mather et al., 2002). Usually, four types of exercise are recommended for older adults to prevent sarcopenia: aerobic (endurance), resistance (strength), flexibility (stretching), and balance (proprioception) training. Recommendations regarding the prescription of exercise to the elderly are not the objective of this review, but this topic has already been reviewed well elsewhere (Montero-Fernandez and Serra-Rexach, 2013; Phu et al., 2015; Pillard et al., 2011; Wang and Bai, 2012). Here, we will focus on the main mechanisms by which exercise (essentially resistance and endurance exercise) helps to fight sarcopenia.

#### 6.1.1. Exercise during aging improves protein balance

Although whole-body protein synthesis appears to be unchanged by resistance exercise in older people (Hasten et al., 2000; Welle et al., 1995; Yarasheski et al., 1993), numerous studies showed that exercise can specifically increase mixed muscle protein synthesis (Balagopal et al., 2001; Hasten et al., 2000; Short et al., 2004; Welle et al., 1995; Yarasheski et al., 1993, 1999), in particular, the synthesis of myofibrillar proteins such as MyHC (Balagopal et al., 2001; Hasten et al., 2000; Welle et al., 1995). In response to resistance exercise, these increases are always associated with improvement of muscle mass and muscle strength (Balagopal et al., 2001; Hasten et al., 2000; Welle et al., 1995; Yarasheski et al., 1993, 1999). Typically, resistance training programs used in the cited studies lasted 3–4 months, with three sessions per week (separated by a rest day) with two to three sets of multiple exercises alternating between the upper and lower body, at gradually increasing intensity from 50–60% to 75–80% of the maximal repetition (1RM). Moreover, 1 week of resistance exercise is sufficient to obtain these results, and with only 2 weeks, the beneficial effect will persist for as long as 3 months (Hasten et al., 2000). Interestingly, although resistance training is typically associated with the most profound gains in strength, elderly subjects who completed a 3-month moderate-intensity aerobic program (3–5 days per week, with sessions of

20–45 min at 60–80% of the heart rate reserve) also demonstrated a marked increase in whole-muscle size and strength associated with increased mixed muscle protein synthesis and MyHC synthesis (Konopka et al., 2011; Short et al., 2004). Although many studies have shown an increase in muscle protein synthesis after resistance and aerobic training in the elderly, few studies have investigated the signaling pathways involved in this phenomenon. Nevertheless, it seems that activation of the PI3K/Akt/mTOR pathway is involved. Indeed, Mayhew et al. (2009) and Williamson et al. (2010) showed in elderly people that resistance exercise (12–16 weeks, 3 days per week, 80% of 1RM) leads to muscle hypertrophy (cross sectional area increase), an increase in muscle strength, and substantial muscle protein accretion associated with increased Akt, p70S6K, and rpS6 phosphorylation (activation). Data obtained from hypertrophied skeletal muscle of old rats indicated similar mechanisms. Indeed, chronic muscle overload induced by bilateral ablation of the gastrocnemius for 28 days increases plantaris weight in aged animals in conjunction with an increase in mTOR and rpS6 phosphorylation (Chale-Rush et al., 2009). On the other hand, it has been shown that aerobic exercise (lifelong running wheel exercise or treadmill training) in old rats increases IGF-1 and IRS-1 protein content in skeletal muscle and Akt and mTOR activation associated with hypertrophied muscles (Kim et al., 2008; Pagano et al., 2014; Pasini et al., 2012). Finally, there are very few studies on older people or old animals that have explored the protein synthesis signaling pathways in response to training. Further research is needed to test various types and combinations of training and to explore responses in the functions of different muscle types (slow or fast) and evolution of these responses over the decades.

To date, various studies have shown that exercise (resistance and aerobic) in elderly subjects has no effect on proteolysis (Hasten et al., 2000; Welle et al., 1995; Yarasheski et al., 1993). This phenomenon may be explained in several ways: a lack of sensitivity of the techniques used to measure proteolysis; amino acids from proteolysis may be recycled during protein synthesis; exercise increases the activity of several proteolysis systems, while others are downregulated at the same time.

Data on the effects of exercise on the UPS systems in elderly people and old rodents are scarce. Moreover, to the best of our knowledge, proteasome activity has never been measured in older people and rodents in response to physical training. Nevertheless, some available data on the expression of the widely studied ubiquitin ligase E3 (i.e., MuRF1 and MAFbx) and muscle ubiquitinated protein (marker of E3 ligase activity) seem consistent with decreased activity of UPS after training. Indeed, it has been shown that 4 weeks of supervised endurance training in patients with chronic heart failure and muscle atrophy (mean age 72 years) is associated with a decrease in muscle content of ubiquitinated protein along with a marked decrease in muscle MuRF1 mRNA and protein content. In the same way, in old mice subjected to short and low-intensity treadmill training (4 weeks, 5 days per week, 20 min per session at 10 m/min), LeBrasseur et al. (2009) showed a marked decrease in muscle content of MuRF1 protein. Other studies after resistance training (12 weeks, 3 days per week,

70–75% 1RM) or a moderate-intensity aerobic program (12 weeks, 3–5 days per week, 20–45 min per session, 60–80% heart rate reserve) did not detect any change in the expression of MuRF1 or MAFbx (Konopka et al., 2010; Williamson et al., 2010). Finally, more studies are needed to definitively determine the effect of exercise on the UPS in sarcopenic people.

The most pronounced effect of exercise on proteolysis in the elderly and older animals is related to autophagy regulation. In humans and rodents, sarcopenia is associated with decreased autophagy (Carnio et al., 2014; Fry et al., 2013; Kim et al., 2008; McMullen et al., 2009; O'Leary et al., 2013; Wohlgemuth et al., 2010; Zampieri et al., 2015), whereas exercise (endurance as well as resistance training) may reverse this impairment and cause muscle hypertrophy (independently of muscle type), decrease muscle fatigue, and increase strength (Carnio et al., 2014; Kim et al., 2013; Luo et al., 2013; Wohlgemuth et al., 2010; Zampieri et al., 2015). Indeed, in response to 8 weeks of treadmill training (5 days per week, 40 min per session at 16.4 m/min), there was an increase in muscle content of Beclin-1, LC3, and Lamp-2 proteins in old mice along with an increase in EDL and gastrocnemius weight (Kim et al., 2013). Earlier, Wohlgemuth et al. (2010) showed that rats subjected to lifelong exercise show upregulation of Lamp-2 RNA, Atg7, and Atg9 proteins along with increased plantaris weight. On the other hand, similar results were obtained in response to a resistance training protocol (climbing of a 1-m ladder inclined at 85° with weight attached to the tail) in old rats (Luo et al., 2013). Moreover, these authors showed an increase in lysosomal content of protease proteins (e.g., cathepsin L). Recently, similar results were published about old athletes compared to sarcopenic elderly people (Carnio et al., 2014; Zampieri et al., 2015). Indeed, these two studies on the same subjects showed that exercise completely reverses age-associated impairment in muscle content of autophagy markers such as ATG7, LC3II, and Bnip3. Finally, upregulation of these different markers suggests that aerobic exercise as well as resistance exercise during aging should stimulate autophagy induction, autophagosome formation, and fusion with lysosomes. Indeed, because no studies have directly measured the number of autophagy vesicles, the upregulation of various molecules regulating autophagy points only to enhancement of the latter. Because deregulation of the autophagic system is associated with accumulation of dysfunctional mitochondria and unfolded proteins (Carnio et al., 2014; Kim et al., 2013), Kim et al. (2013) speculated that an exercise training-induced autophagic response can be considered one of the mechanisms of cellular “clearance” that may be related to protection against accumulation of dysfunctional mitochondria and unfolded proteins.

#### 6.1.2. Exercise during aging decreases myonuclear apoptosis

Several studies showed that aerobic exercise and resistance training during aging decrease myonuclear apoptosis and increase muscle mass and strength in old animals (Luo et al., 2013; Marzetti et al., 2008; Song et al., 2006; Wohlgemuth et al., 2010). It seems that no data are available on older humans.

Regarding the effects of exercise training on myonuclear apoptotic signaling, [Song et al. \(2006\)](#) showed that 12-week treadmill exercise (5 days per week, 60 min per session) reduces the expression of Bax in gastrocnemius extracts of old rats. Conversely, levels of Bcl-2 are increased in trained rodents, resulting in a dramatic decrease in the Bax-to-Bcl2 ratio, reaching youth-related values. In addition, cleavage of caspase 3 is lowered by 95% in old exercising rats. As a consequence, the extent of apoptotic DNA fragmentation in the gastrocnemius is significantly attenuated by the exercise intervention, such that old trained rats show the levels of apoptosis similar to those observed in young control animals. It is noteworthy that the reduced severity of apoptosis is accompanied by an increased fiber cross-sectional area along with increased muscle weight (soleus and gastrocnemius). Similarly, [Marzetti et al. \(2008\)](#) found that 4-week treadmill exercise training downregulates the death receptor pathway of apoptosis in the EDL of old rats. Indeed, exercise reversed the age-related upregulation of TNF-R1, activated caspase 8, and cleaved caspase 3, resulting in reduced levels of apoptotic DNA fragmentation. These adaptations were accompanied by improvements in exercise tolerance and forelimb grip strength. Furthermore, the same group published similar data on rats subjected to lifelong exercise with free access to a running wheel ([Wohlgemuth et al., 2010](#)). In addition, they showed that exercise reverses the age-related increase in caspase 9 activity. Recently, [Luo et al. \(2013\)](#) found that 9 weeks of resistance training prevent the loss of muscle mass and improve muscle strength, accompanied by reduced cytosolic concentration of cytochrome c in the gastrocnemius and inhibition of cleaved caspase 3 production, thus resulting in a reduced apoptotic index.

The decreased number of apoptotic myonuclei or decreased DNA fragmentation may be explained by a renewal of the latter thanks to SC activation.

#### 6.1.3. Exercise during aging stimulates SCs

This topic was reviewed well by [Snijders et al. \(2009\)](#). Although some studies failed to demonstrate any effect of exercise on SCs in older people ([Leiter et al., 2011](#); [Petrella et al., 2006](#)), most of them showed exercise-related activation of the latter in elderly people and older rodents along with improvement in muscle mass and strength ([Leenders et al., 2013](#); [Mackey et al., 2007](#); [Shefer et al., 2010](#); [Verdijk et al., 2009](#); [Verney et al., 2008](#)). For instance, [Verdijk et al. \(2009\)](#) found that 3 months of resistance training (3 days per week, 80% 1RM) increase muscle mass, reduce fat mass, and increase muscle strength in healthy elderly men. The observed skeletal muscle hypertrophy was specific for type II muscle fibers and was accompanied by a specific increase in SC numbers. These data were recently confirmed by [Leenders et al. \(2013\)](#), who showed that 6 months of resistance-type exercise training (3 days per week, 80% 1RM) lead to increased lean mass of legs and quadriceps CSA, resulting in increased strength of one-repetition maximum leg extension and decreased sit-to-stand time. These results were accompanied by a type II muscle fiber-specific increase in myonuclear and SC content. On the other hand, [Verney et al. \(2008\)](#) and [Shefer et al. \(2010\)](#) reported similar results on the response to endurance training in the elderly

(13 weeks of combined lower-body endurance and upper-body resistance training) and old rats (14 weeks of treadmill training, 6 days per week, 20 min per session): an increased size of type II muscle fiber accompanied by an increase in SC numbers.

#### 6.1.4. Exercise during aging improves mitochondrial function and dynamics

Aerobic exercise of sufficient intensity (at least 60%  $\dot{V}O_2\text{max}$ ) and duration (at least 3 weeks with 3 sessions of 1 h per week) can significantly increase  $\dot{V}O_2\text{max}$  and endurance capacity in older adults and rodents ([Hammeren et al., 1992](#); [Huang et al., 2005](#); [Koltai et al., 2012](#); [Lambertucci et al., 2007](#); [Lanza et al., 2008](#); [Malbut et al., 2002](#); [Radak et al., 2002](#); [Safdar et al., 2010](#); [Short et al., 2003, 2004](#)). An increase in mitochondrial function and number, in the expression of mitochondrial proteins, and/or in the expression of transcription factors involved in mitochondrial biogenesis are mechanisms that explain the above improvements. [Short et al. \(2003\)](#) were among the first to show in humans that endurance training (16 weeks, four sessions per week at 80% of maximal heart rate for 40 min) increases  $\dot{V}O_2\text{max}$  associated with an increase in activities of mitochondrial enzymes (citrate synthase and cytochrome c oxidase), in mRNA levels of mitochondrial genes (e.g., COX4), and in mRNA of genes involved in mitochondrial biogenesis in skeletal muscle (PGC-1 $\alpha$ , NRF-1, and TFAM). These results suggested that aerobic exercise could induce de novo mitochondrial biogenesis and improve mitochondrial function during aging. Indeed, [Lanza et al. \(2008\)](#) demonstrated in older trained people (performing at least 1 h of cycling or running 6 days per week during the past 4 years) an increase in mitochondrial ATP production rate; in citrate synthase activity; in muscle content of PGC1- $\alpha$ , NRF-1 and TFAM proteins; and in mtDNA abundance. Moreover, an increased protein level of Sirt 3 (known to stimulate PGC-1 $\alpha$ ) was also observed. Moreover, [Safdar et al. \(2010\)](#) confirmed such results and showed that they are associated with functional improvements (an increase in maximal isometric strength and a decrease in time to perform the 30-foot walk test and stair climb tests). Furthermore, they found that physical activity in older people increases complex IV activity and protein levels of COX subunits I and II in skeletal muscle. More recently, [Koltai et al. \(2012\)](#) showed in old trained rats (6 weeks of treadmill training at 60%  $\dot{V}O_2\text{max}$ , 1 h/day) that increased mitochondrial biogenesis (according to increased protein levels of PGC-1 $\alpha$ , succinate dehydrogenase, and COX4 in muscle and increased mtDNA abundance) is driven by an increase in Sirt 1 activity and AMPK phosphorylation. Moreover, these authors found that aerobic exercise can restore mitochondrial dynamics (fusion and fission) to levels similar to those observed in young rats (according to quantitative data on protein levels of mitofusin 1, fission protein 1, and Lon protease); this finding may reflect a reduction in the number of impaired mitochondria. [Konopka and Sreekumaran Nair \(2013\)](#) confirmed these results in older people after aerobic training (four sessions of 45 min per week at 80% heart rate reserve). Indeed, trained elderly people showed increased protein levels of mitofusins 1 and 2, fission protein 1, PGC-1 $\alpha$ , and citrate synthase in muscle and increased  $\dot{V}O_2\text{max}$  and

CSA. Because resistance training is usually not associated with improvement of mitochondrial function, very few studies are available on this topic. However, in older people after 12 weeks of whole-body resistance training (three sessions per week, 80% of 1RM), [Parise et al. \(2005\)](#) found an increase in complex IV activity, reflecting improvement of the electron transport chain. Moreover, in older rats, [Luo et al. \(2013\)](#) found increased AMPK phosphorylation associated with an increased mitochondrial concentration of cytochrome C in skeletal muscle after 9 weeks of resistance training. In both studies, however, physical parameters were not measured.

Here, we presented the main molecular mechanisms of prevention of sarcopenia by exercise, but other mechanisms, which are beyond the scope of this review, are also involved, such as better regulation of microRNAs controlling muscle mass ([McGregor et al., 2014](#); [Rivas et al., 2014](#); [Zampieri et al., 2015](#)). Finally, exercise appears to be the best countermeasure against sarcopenia because it can act on all the deleterious effects of aging, and at the same time, improve muscle mass, strength, and physical performance. As discussed here, resistance training leads to the most profound gains in strength and muscle mass, whereas aerobic training enhances  $\dot{V}O_2$ max and endurance capacity. Performing the resistance training cycles and endurance training separately appears to be the best solution to combat sarcopenia.

## 6.2. Hormone replacement therapies as a possible strategy

There is evidence that hormones, in particular testosterone and GH (whose levels decrease with age), play a key role in the age-related onset of sarcopenia ([Giannoulis et al., 2012](#); [Maggio et al., 2013](#); [Sakuma and Yamaguchi, 2012](#)). Consequently, numerous researchers have tried to reverse sarcopenia using hormone replacement therapies.

### 6.2.1. Testosterone replacement therapy

In 2006, the findings from 11 randomized controlled trials were examined using the methods of meta-analysis to determine whether androgen treatment (testosterone or its more potent derivative 5 $\alpha$ -dihydrotestosterone) increases strength in men aged 65 years and older ([Ottenbacher et al., 2006](#)). This meta-analysis was recently completed by [Maggio et al. \(2013\)](#), who reviewed the most recent randomized controlled trials. Those authors concluded that testosterone or 5 $\alpha$ -dihydrotestosterone treatment can effectively increase muscle mass, strength, and physical performance ([Maggio et al., 2013](#); [Ottenbacher et al., 2006](#)). The most convincing and complete data come from the Testosterone in Older Men with Mobility Limitations (TOM) trial conducted by [Travison et al. \(2011\)](#). The aim of this placebo-controlled randomized trial was to determine whether testosterone therapy (10 g of testosterone gel daily for 6 months) in community-dwelling men (age 74 years; with severe limitation in mobility) improves muscle strength and physical function. Muscle strength was assessed by means of leg press and chest press strength. Physical function was evaluated using a 12-step stair climb and 40-meter walk tests. Muscle fatigue was also assessed, by tests involving lifting and lowering a basket of a weight equivalent to 15% of body weight.

Finally, lean body mass was determined by dual energy X-ray absorptiometry (DXA). All these parameters were enhanced by this treatment and were associated with upregulation of serum total and free testosterone. Nevertheless, adverse cardiovascular events occurred in more men receiving testosterone as compared to men receiving placebo, thus causing the organizers to stop the study. This study showed that despite numerous significant beneficial effects of testosterone treatment on elderly men, more studies are needed to find the optimal regimen of administration. Currently, intermittent treatments and/or treatments associated with 5 $\alpha$ -reductase inhibitors (to avoid adverse effects on prostate), injections instead of oral or transdermal treatments, are new approaches being tested to decrease adverse effects of testosterone ([Borst and Yarrow, 2015](#)).

Different mechanisms can explain the beneficial effects of testosterone. Testosterone is known to stimulate muscle protein synthesis, to improve recycling of intracellular amino acids, to decrease the protein breakdown rate, and to enhance neuromuscular function (to increase activity of motoneurons) ([Dubois et al., 2012](#)). Testosterone also promotes SC activation and may inhibit their differentiation into adipocytes via an androgen receptor-mediated pathway ([Grossmann, 2011](#)). Testosterone treatment is also associated with upregulation of hemoglobin; this effect can be considered an additional mechanism by which this hormone improves muscle oxygenation and function ([Fernandez-Balsells et al., 2010](#)). Moreover, testosterone seems to have anti-inflammatory effects because it can reduce the plasma concentration of TNF- $\alpha$  and several interleukins ([Malkin et al., 2004](#)). More recently, it was shown that testosterone is effective at reversing sarcopenia in rodents ([Kovacheva et al., 2010](#)). Those authors showed that testosterone decreases lipid oxidative damage and apoptosis and explained this result by the concomitant increase in muscle content of the glucose 6-phosphate dehydrogenase (G6PDH) protein. Moreover, testosterone treatment led to SC activation though the Notch signaling pathway due to myostatin inhibition and Akt activation ([Kovacheva et al., 2010](#)).

### 6.2.2. GH replacement therapy

GH is a single-chain peptide of 191 amino acid residues; it is produced and secreted mainly by the somatotopic cells of the anterior pituitary gland. GH coordinates postnatal growth of multiple target tissues, including skeletal muscle ([Florini et al., 1996](#)). GH secretion occurs in a pulsatile manner with a major surge at the onset of slow-wave sleep and less conspicuous secretory episodes a few hours after meals ([Ho et al., 1988](#)). GH secretion is controlled by the actions of two hypothalamic factors: GH-releasing hormone (GHRH), which stimulates GH secretion, and somatostatin, which inhibits GH secretion ([Giannoulis et al., 2012](#)). The secretion of GH is maximal at puberty, accompanied by very high circulating IGF-I levels ([Moran et al., 2002](#)), with a gradual decline during adulthood. Indeed, circulating GH levels decline progressively after 30 years of age at a rate of ~1% per year. In aged men, daily GH secretion is 5- to 20-fold lower than that in young adults ([Ryall et al., 2008](#)). Moreover, [Veldhuis et al. \(1995\)](#) found a decrease in GH secretory burst amplitude with age (maximal rate of GH

secretion attained within a release episode). The age-dependent decline in GH secretion is secondary to downregulation of GHRH and to an increase in somatostatin secretion (Kelijman, 1991).

The effects of GH administration in elderly people on muscle mass, strength, and physical performance are still debated (Giannoulis et al., 2012). Some researchers demonstrated an improvement in strength after short- and long-term administration (3–11 months) of GH (Blackman et al., 2002; Brill et al., 2002; Welle et al., 1996). For instance, in healthy subjects over 60 years old, Welle et al. (1996) found that GH treatment for 3 months (0.03 mg per kg of body weight subcutaneously, three times per week) increases lean body mass, muscle mass, and thigh strength. Similar data were published by Blackman et al. (2002) on a 26-week randomized, double-blind, placebo-controlled parallel-group trial in healthy, ambulatory, community-dwelling US men aged 65–88 years, who received GH at 20  $\mu\text{g}/(\text{kg}$  body weight) subcutaneously three times per week. Treated men showed a fat mass decrease along with an increase in lean mass, which was higher as compared to another group (receiving testosterone). Furthermore, men's  $\dot{V}\text{O}_2\text{max}$  increased under the influence of GH and was directly related to changes in lean body mass. Unfortunately, some adverse effects such as arthralgia were more common with GH treatment. Interestingly, in older men, it has been shown that GH therapy leads to substantial upregulation of the MyHC 2X isoform (Lange et al., 2002). In contrast, other researchers did not find any improvement of muscle strength or muscle mass after GH treatment in the elderly (Giannoulis et al., 2012). There may be several reasons for the lack of effectiveness of GH treatment, for example: failure of exogenous GH treatment to mimic the pulsatile pattern of natural GH secretion (Sakuma and Yamaguchi, 2012). In animal models, beneficial effects were also observed when recombinant human GH was used. Indeed, in old rats treated with GH (2.7 mg per kg per day during 12 weeks) (Andersen et al., 2000) observed an increase in maximal tetanic tension of calf musculature (soleus, plantaris, gastrocnemius, tibialis anterior, EDL) associated with muscle hypertrophy (assessed by muscle weight and volume) likely due to the concomitantly increased protein synthesis. Similarly, using a treatment mimicking the pulsatile pattern of natural GH secretion (2 mg/kg per day, diluted in saline, divided into two subcutaneous injections: at 10:00 and 17:00 h), with doses resulting in hepatic and plasma IGF-1 levels of young rats, Castillo et al. (2005) and Brioché et al. (2014) showed that GH treatment during 4 or 8 weeks increases lean mass and gastrocnemius mass and decreases fat mass. Nevertheless, others did not detect such beneficial effects, maybe because of shorter treatment duration or a different dose or source of GH (e.g., recombinant porcine GH) (Marzetti et al., 2008).

Molecular mechanisms by which GH may increase muscle mass, strength, and maximal oxygen consumption in the elderly and in older animals have not been studied well in skeletal muscle. It is clearly established that effects of GH are driven by IGF-1, which can be produced in either the liver or skeletal muscle. Different isoforms of IGF-1 have diverse effects. Liver-derived IGF-1 appears to predominantly increase muscle mass by improving protein synthesis,

whereas muscle-derived IGF-1 has effects on the development of SCs and on maintenance of neuromuscular function (Perrini et al., 2010). Recently, Brioché et al. (2014) showed that GH treatment prevents the muscle mass decrease observed in old rats. These beneficial effects are driven by an increase in protein synthesis concomitant with a decrease in proteolysis, as expected after activation of p70S6K and weaker MuRF1 expression. The downregulation of myostatin observed in the old treated rats could explain these results. Indeed, in the absence of myostatin, mice show increased protein synthesis owing to increased activation of the PI3K/Akt/mTOR pathway (Guo et al., 2009). Moreover, inhibition of myostatin with a specific inhibitor has been shown to decrease MuRF1 expression in the skeletal muscle of mice (LeBrasseur et al., 2009). In the same study, rats treated with GH showed downregulation of the cell cycle inhibitor p21 and upregulation of the myogenic factor Myf-5 implying activation of SCs. These different results may explain how GH treatment prevents a decrease in muscle mass in the elderly. An increase in mitochondrial biogenesis is usually associated with increased maximal oxygen consumption and/or increased endurance capacity in the elderly and older rats (Derbre et al., 2012; Koltai et al., 2012; Short et al., 2003). Interestingly, GH treatment used in the study by Brioché et al. (2014) led to improved mitochondriogenesis in the treated rats. Indeed, GH treatment was associated with increased muscle content of cytochrome c protein and citrate synthase activity, likely because of the concomitant activation of the PGC-1 $\alpha$ /NRF-1 pathway. Earlier, Vescovo et al. (2005) working on the rat soleus muscle, reported that GH activates PGC-1 $\alpha$  via IGF-1 and calcineurin pathways. Similarly, Short et al. (2008) demonstrated in healthy young humans that acute GH action promotes an increase in mitochondrial oxidative capacity and expression of several mitochondrial genes (e.g., COX3, TFAM). However, mitochondrial protein synthesis was not increased likely because of the unique dose of GH. As reviewed well by Derbre et al. (2014), oxidative stress is involved in protein balance impairment and mitochondrial dysfunction in sarcopenia. Interestingly, GH treatment in old rats improves the redox status according to a decrease in oxidative damage to protein and DNA and the upregulation of key intracellular antioxidant enzymes, such as catalase, glutathione peroxidase, and G6PDH. Although the exact mechanisms by which GH activates the expression of antioxidant enzymes are still unknown, the better redox status observed in old rats treated with GH is likely involved in the improvement of protein balance and mitochondriogenesis. These results and those of Kovacheva et al. (2010) suggest that in general, growth factors (at least GH and testosterone) can alleviate oxidative stress. GH treatment may also improve muscle mass and physical performance via a reduction in apoptosis (Forman et al., 2010; Vescovo et al., 2005); however, to our knowledge, this effect has never been demonstrated in aged muscle.

Hormone replacement therapies, notably testosterone and GH, are useful for improving muscle mass in the elderly. Nevertheless, the doses currently used are often associated with side effects. More studies are needed to continue exploration of other parameters of various treatments such as dose (physiological doses), duration, and periodicity



(intermittent versus continuous) to prevent adverse effects. With respect to GH, pilot treatments mimicking its pulsatile secretion in humans could be a new way. Moreover, by elucidating which mechanisms mediate the action of hormones in older animals, it is possible to find new molecules to target in sarcopenia and more generally to combat muscle disuse in various situations.

### 6.3. Nutritional interventions: the case of HMB

Nutritional interventions seem effective at improving muscle strength and physical performance with or without increasing muscle mass (Cruz-Jentoft and Landi, 2014). Nissen et al. (1996) first demonstrated that dietary supplementation with HMB lowers muscle proteolysis after resistance training and augments gains in lean body mass and strength in a dose-dependent manner in young people. A few years later, Vukovich et al. (2001) reported a similar result in the elderly, whereas May et al. (2002) showed that the mixture HMB/Arginine/Glutamine is effective in increasing fat-free mass of aged patients with advanced cancer (May et al., 2002; Vukovich et al., 2001). Since then, HMB has been studied under a variety of anaerobic and aerobic training and wasting conditions (Wilson et al., 2008; Wu et al., 2015). As explained in this section, among nutritional strategies, HMB (a metabolite of the branched-chain amino acid leucine) may be considered an effective strategy against sarcopenia – and other wasting conditions – when taken alone or combined with exercise or other nutritional supplements.

#### 6.3.1. HMB, who are you?

HMB is a metabolite of the essential amino acid leucine, which is generated after a transamination reaction producing  $\alpha$ -ketoisocaproate (KIC; an intermediate of the Krebs cycle) and subsequent conversion to HMB by KIC-dioxygenase in the cytosol (Nissen and Abumrad, 1997; Van Koeveering and Nissen, 1992). The liver is the major site of production of HMB; muscle and other tissues produce HMB but in low amounts (Nissen and Abumrad, 1997). Under normal conditions, approximately 2–10% of the dietary leucine (~6.1 g per day) is converted to HMB corresponding to ~0.3–0.6 g for a subject weighing 70 kg (Van Koeveering and Nissen, 1992). HMB is present in such foods as citrus fruits, some fish, and breast milk (Nissen and Abumrad, 1997). Nonetheless, it may be impractical to provide via diet the quantities of HMB used in human studies that demonstrate efficiency of HMB (3 g/day) (Wilson et al., 2013; Wu et al., 2015). For example, 3 g of HMB would represent 60 g of leucine, which would be supplied by 600 g of high-quality protein and a very large amount of food. Currently, HMB can be used as monohydrated calcium salt [formula:  $\text{Ca}(\text{HMB})_2 \cdot \text{H}_2\text{O}$ ] or a free acid form of HMB (FaHMB) (Fuller et al., 2011). Although currently, the calcic HMB (CaHMB) form is more widely used in humans and in rodent studies, Fuller et al. (2011) found that FaHMB improves HMB availability in human subjects as compared with the CaHMB. Surprisingly, it seems that the opposite occurs in rodents (Shreeram et al., 2014). Whatever the HMB form, HMB half-life is ~2–3 h, with a return to normal values ~9 h postabsorption (Wilson et al., 2013). Nowadays in humans,

3 g of CaHMB taken three times a day (1 g each time) is the optimal posology, which allows for continual bioavailability of HMB in the body (Wilson et al., 2013). In rodents, the optimal dose seems to be 0.250 mg/(kg·day) in mice and 320 mg/(kg·day) in rats (Szczesniak et al., 2015). In rodent studies, HMB is often administered via unique intragastric gavage to better control the treatment; however, this approach may minimize the effect of treatment because plasma will be free of HMB for ~15 h/day. CaHMB is completely safe, and its safety in humans and animals has been well studied and reviewed (Szczesniak et al., 2015; Wilson et al., 2013). However, a possible decrease in insulin sensitivity and higher glucose intolerance have been reported in young healthy rats treated for 4 weeks with CaHMB (320 mg/[kg·day]) in the first case (Yonamine et al., 2014), or in rats treated with glucocorticoids combined with HMB, as compared to rats treated only with glucocorticoids (Nunes et al., 2013). These findings have not been reported in any previous human study but have to be studied in the future along with the safety of FaHMB, which has been studied only in rodents and showed no side effects (Fuller et al., 2014). Moreover, safety of very long treatments can be studied because the longest study lasted for 1 year (Baier et al., 2009), whereas treatment of sarcopenia or other wasting conditions such as cachexia can last longer.

#### 6.3.2. HMB: a very potent strategy against various muscle-wasting conditions

Beneficial effects of HMB in human subjects have been reported in several muscle-deconditioning conditions characterized by a loss of muscle mass and a decrease in muscle strength and physical performance, for example, cancer cachexia (May et al., 2002), AIDS (Clark et al., 2000), chronic obstructive pulmonary disease (Hsieh et al., 2006) and aging (Baier et al., 2009; Deutz et al., 2013; Flakoll et al., 2004; Hsieh et al., 2010; May et al., 2002; Stout et al., 2013; Vukovich et al., 2001). Efficiency of HMB in various muscle-deconditioning conditions in rodents and cell lines has also been reported, e.g., experimental models of cachexia (Aversa et al., 2011; Caperuto et al., 2007; Eley et al., 2007; Mirza et al., 2014b; Nunes et al., 2008; Smith et al., 2005), glucocorticoid treatments (Aversa et al., 2012; Baptista et al., 2013; Giron et al., 2015; Nunes et al., 2013), and experimental models of sepsis and endotoxemia (Eley et al., 2008a; Kovarik et al., 2010; Supinski and Callahan, 2014) in response to muscle-wasting treatments such as TNF- $\alpha$  and angiotensin II (Eley et al., 2008a, 2008b), a mouse model of Duchenne muscular dystrophy (Payne et al., 2006), and hypokinesia and hypodynamia models (HU or immobilization) (Alway et al., 2013; Baptista et al., 2013; Hao et al., 2011; Mirza et al., 2014b). In animals, effects of HMB during aging have been studied only in combination with other muscle-deconditioning circumstances (Alway et al., 2013; Hao et al., 2011), and specific studies are needed.

#### 6.3.3. Efficiency of HMB in various muscle-wasting conditions in the elderly

Now, we will specifically focus on the studies showing HMB efficiency in the elderly and discuss the underlying molecular and cellular mechanisms. Very recently, Wu et al. (2015) published a meta-analysis of the effect of HMB

supplementation on muscle in older adults. Currently, only seven studies have been conducted on the elderly receiving HMB alone (Deutz et al., 2013; Hsieh et al., 2010; Stout et al., 2013) or in combination with exercise (Stout et al., 2013; Vukovich et al., 2001) or with arginine and lysine or glutamine supplementation (Baier et al., 2009; Flakoll et al., 2004; May et al., 2002). Among these studies, four were devoted specifically to the protective effect of HMB on muscle deconditioning related to aging (Flakoll et al., 2004; Stout et al., 2013; Vukovich et al., 2001), whereas two of them dealt with the protective effect of HMB during bed rest (Deutz et al., 2013; Hsieh et al., 2010), and one study involved older cachexic patients (May et al., 2002). According to all these studies, supplementation with HMB alone or in combination with other compounds or exercise has a beneficial effect on body composition (especially, in terms of greater muscle mass) in the groups receiving HMB, without an obvious effect on fat mass in elderly people despite the wasting condition. Effects on muscle strength and physical performance are more controversial. Among these studies, five studies measured muscle strength by different methods (handgrip strength, leg strength, or knee extensor and flexor strength), whereas only four studies evaluated the effect of HMB on physical performance (e.g., SPPB, TUG). Deutz et al. (2013) did not report any effect of HMB on muscle strength after 10 days of bed rest in the elderly (men and women) but showed a clear tendency to limit the decrease observed; the latter result did not reach statistical significance probably due to the small sample size. Baier et al. (2009) observed similar results after 1 year of HMB treatment in aged subjects. After 8 weeks of resistance training with or without HMB supplementation, Vukovich et al. (2001) observed a similar increase in both upper-body strength and lower-body strength in different groups. Stout et al. (2013) reported similar results on elderly people after 24 weeks of resistance training, combined or not combined with HMB supplementation. However, the same authors showed that when used alone for 24 weeks, HMB increases muscle strength as compared to a placebo group. Flakoll et al. (2004) observed similar results in elderly females receiving HMB supplementation for 12 weeks, which significantly improved their muscle strength (measured by leg extensor force and handgrip strength) and physical function.

#### 6.3.4. Molecular and cellular mechanisms by which HMB prevents muscle wasting

Mechanisms by which HMB prevents a loss of muscle mass or muscle strength or deterioration of physical performance have not been studied well in humans, but studies on animals and cell cultures provided a lot of data. As demonstrated above, in Section 4, negative protein turnover and an imbalance between apoptosis and regenerative processes may largely explain the decrease in muscle mass and muscle strength during sarcopenia, while impairment of mitochondrial dynamics and functions may mostly be involved in the deterioration of physical performance.

In elderly subjects, HMB increases muscle mass or prevents a muscle mass decrease by improving protein turnover (Baier et al., 2009; Deutz et al., 2013; Flakoll et al., 2004; Hsieh et al., 2010). Protein balance improvement seems to

be caused by an increase in muscle protein synthesis (Baier et al., 2009; Deutz et al., 2013; Flakoll et al., 2004) and a decrease in protein breakdown (Hsieh et al., 2010). Similar data have been published about animals and cellular muscle-wasting models (Aversa et al., 2012; Eley et al., 2007, 2008a, 2008b; Giron et al., 2015; Kovarik et al., 2010; Smith et al., 2005). The increase in protein synthesis after HMB supplementation may be explained by activation of the PI3K/Akt/mTOR pathway and its targets. Indeed, prevention of gastrocnemius mass loss in cachexic rats by HMB is associated with increased activation of mTOR and p70S6K phosphorylation (Aversa et al., 2011). Similar results were reported in numerous studies on culture of muscle cells exposed to different muscle-wasting treatments such as TNF- $\alpha$ , INF- $\gamma$ , GCs (dexamethasone), and angiotensin II (Aversa et al., 2012; Eley et al., 2007, 2008a, 2008b; Kimura et al., 2014a). The stronger activation of the PI3K/Akt/mTOR pathway and its targets in response to HMB treatment may be explained by an increased activity of the GH/IGF-1 axis, which is known to improve protein synthesis through mTOR activation (Schiaffino et al., 2013). Indeed, healthy young rats treated with GH show higher GH mRNA and protein content in the pituitary gland, along with higher IGF-1 serum and hepatic levels as compared to rats receiving a placebo (Gerlinger-Romero et al., 2011). Similar results were observed in cultured human SCs (Kornasio et al., 2009). The mechanistic link between HMB and the GH-IGF-1 axis is completely unknown.

Decreased protein breakdown observed in the elderly receiving a supplement of HMB may be explained by lesser proteasomal activity and expression of subunits associated with lesser expression of MAFbx and MuRF1 (two important E3 ubiquitin ligases involved in muscle wasting). Indeed, in cachexic mice receiving HMB, Smith et al. (2005) showed a decreased proteasomal activity along with a decrease in protein content of proteasome subunits in hindlimb muscles, as compared to cachexic control mice. In line with these results, Noh et al. (2014) found decreased mRNA and protein content of MuRF1 and MAFbx associated with less ubiquitinated MyHC and higher total MyHC protein content in the soleus of rats treated with GCs and receiving HMB, as compared to a placebo group. Similarly, Russ et al. (2015) showed recently that HMB treatment combined with  $\beta$ -alanine in sedentary aged male rats (20 months) reduces muscle MuRF1 expression as compared to control rats. Similar data have been obtained in muscle cell cultures exposed to wasting treatments such as TNF- $\alpha$ , INF- $\gamma$ , and GCs (dexamethasone) (Aversa et al., 2012; Giron et al., 2015; Kimura et al., 2014a). Inhibition of proteasome and E3 ubiquitin ligases by HMB could be due to its anti-inflammatory and antioxidant effects (Eley et al., 2008b; Hsieh et al., 2006) mediated by decreased expression of TNF- $\alpha$  and activation of the redox-sensible p38 MAPK (Aversa et al., 2012; Townsend et al., 2013), which are known to activate the UPS system (Schiaffino et al., 2013). Nonetheless, these mechanisms need to be confirmed.

HMB's effect on autophagy may be involved in the improvement of the protein balance observed in aged subjects receiving a supplement of HMB. Nonetheless, only data obtained in response to glucocorticoid treatments are available; although autophagy increases in response to GCs

(Giron et al., 2015), it decreases during sarcopenia (Fry et al., 2013; Kim et al., 2013; McMullen et al., 2009; O'Leary et al., 2013; Wohlgemuth et al., 2010). However, HMB treatment of muscle cells normalizes the dexamethasone-induced autophagy pathway through the increased ratio phosphorylated-FoxO3/total-FoxO3 and via Akt activation (Giron et al., 2015). More data are needed to explore the effects of HMB under sarcopenic and more generally under muscle-wasting conditions.

The decrease in apoptosis in response to HMB supplementation may be involved in HMB's protective effect on muscle mass in the elderly. Indeed, HMB can decrease apoptosis, judging by a dramatic decrease in the number of TUNEL-positive nuclei in the soleus and plantaris of hindlimb-unloaded old rats treated with HMB, as compared to a placebo group. This effect of HMB was due to downregulation of cleaved caspases 3 and 9 in the soleus and plantaris (Hao et al., 2011). Similar data were obtained in muscle cell cultures (Kornasio et al., 2009).

Finally, HMB may participate in prevention of muscle mass loss in the elderly by acting on SC proliferation and differentiation. This way, data have been obtained in old rats during muscle recovery after hindlimb unloading. Indeed, those authors showed increased numbers of bromodeoxyuridine-positive, PAX7- and PAX7/Ki67-positive nuclei (markers of SC proliferative state) in the plantaris of HMB-treated rats compared to control rats (Alway et al., 2013). As a result, there was a greater number of differentiated stem cells judging by a greater number of MyoD/myogenin-positive myonuclei relative to all myonuclei, in HMB-reloaded plantaris muscles as compared to reloaded muscles from vehicle-treated animals. Furthermore, HMB increased the nuclear protein levels of proliferation markers – inhibitor of differentiation 2 and cyclin A – as compared to vehicle treatment of reloaded muscles. Enhanced SC proliferation leading to an increased number of differentiated myonuclei should promote the transcriptional potential to support hypertrophic changes in muscle and functional changes in sarcopenic muscles; and this effect may partly explain the reduced apoptosis in HMB-treated muscles (Alway et al., 2013).

Increased physical performance in aged subjects treated with HMB may also involve better mitochondrial functions and dynamics (mitochondrial biogenesis and fusion/fission). Indeed, Pinheiro et al. (2012) found that HMB supplementation increases ATP and glycogen content and citrate synthase activity in the skeletal muscle of young healthy rats and raises specific force generation and resistance to acute fatigue (without changes in contraction) as well as relaxation velocities in electrostimulated hindlimb muscles. It has been demonstrated that leucine stimulates mitochondrial biogenesis genes *Sirt1*, *PGC-1 $\alpha$* , and *NRF-1* through AMPK activation and increases mitochondrial mass (by 30%) and oxygen consumption in C2C12 myotubes (Liang et al., 2014). Moreover, HMB has been reported to activate AMPK synergistically with resveratrol in C2C12 myotubes (Bruckbauer and Zemel, 2013). Consequently, it can be hypothesized that HMB activates mitochondrial biogenesis through AMPK activation, leading to increased citrate synthase activity thus eventually resulting in better physical performance in HMB-treated elderly people. Nevertheless, all of this has to be confirmed.

In conclusion, HMB treatment clearly appears to be a safe potent strategy against sarcopenia, and more generally against muscle wasting, because HMB improves muscle mass, muscle strength, and physical performance. It seems that HMB is able to act on three of the four major mechanisms involved in muscle deconditioning (protein turnover, apoptosis, and the regenerative process), whereas it is hypothesized to strongly affect the fourth (mitochondrial dynamics and functions). Moreover, HMB is cheap (~30–50 US dollars per month at 3 g per day) and may prevent osteopenia (Bruckbauer and Zemel, 2013; Tatara, 2009; Tatara et al., 2007, 2008, 2012) and decrease cardiovascular risks (Nissen et al., 2000). For all these reasons, HMB should be routinely used in muscle-wasting conditions especially in aged people.

#### 6.4. A potential therapeutic target in sarcopenia: glucose 6-phosphate dehydrogenase

G6PDH was first described in 1931 (Kornberg et al., 1955). Most studies were focused on G6PDH deficiency and lipid metabolism until a few years ago. G6PDH deficiency is the most common gene mutation in the world (Nkhoma et al., 2009). During the last decade, studies have started to explore its involvement in diabetes (Park et al., 2005), heart failure (Assad et al., 2011), and cancer (Kuo et al., 2000). It is now clear, however, that G6PDH is a critical metabolic enzyme under complex control and is at the center of an essential metabolic nexus that affects many physiological processes. Surprisingly, its function in skeletal muscle is poorly studied, whereas several clinical cases of rhabdomyolysis due to G6PDH deficiency were reported more than 15 years ago (Kimmick and Owen, 1996). Moreover, numerous studies have shown since the 1980s that deregulation of its activity is associated with myopathies (Elias and Meijer, 1983; Meijer and Elias, 1984). Finally, G6PDH should be studied more because its upregulation in transgenic flies extends their life span (Legan et al., 2008) and mice overexpressing G6PDH have better protection from aging-associated functional decline, for example, an extended median lifespan, higher levels of NADPH, lower levels of reactive oxygen species-associated damage, and better neuromuscular fitness in females compared to their control littermates (Nobrega-Pereira et al., 2016).

G6PDH controls the entry of glucose 6-phosphate (G6P) into the pentose phosphate pathway also known as the hexose monophosphate shunt. The major products of the pentose phosphate pathway are ribose 5-phosphate (R5P) and nicotinamide adenine dinucleotide phosphate (NADPH) generated from NADP by G6PDH and the next enzyme in the pathway, 6-phosphogluconate dehydrogenase (PGD). In the following paragraphs, it will be explained why via NADPH and R5P, G6PDH may be involved in sarcopenia and why enhancing their production by G6PDH should help to combat sarcopenia.

Not long ago, G6PDH was described only as the principal source of NADPH in the cytosol. It was recently shown, however, that G6PDH is present in the mitochondria of skeletal muscle cells and provides NADPH as do isocitrate dehydrogenase (ICDH), malic enzyme (ME), and glutamate dehydrogenase (GDH), which were originally described

as the principal sources of NADPH in mitochondria. Thus, NADPH is mainly produced by five enzymes in mammalian cells: G6PDH, 6-PGD, ICDH, ME, and GDH. All have been studied extensively and play crucial cellular roles. G6PDH, however, appears to be of unique importance to many cellular processes that use NADPH because inhibition of G6PDH lowers NADPH levels, which are not maintained at normal levels by the other enzymes producing NADPH (Hecker et al., 2013; Stanton, 2012).

It has been traditionally thought that G6PDH is regulated by the NADPH/NADP ratio so that as the ratio decreases, activity increases to provide more NADPH. Indeed, G6PDH is activated after exposure of cells to various extracellular oxidants (Kletzien et al., 1994) that lead to a decrease in the level of NADPH. Regulation by the NADPH/NADP ratio has been clearly demonstrated in vitro (Holten et al., 1976) but not in vivo. G6PDH is tightly regulated at the transcriptional, translational, and post-translational levels and by the intracellular location. G6PDH is the downstream target of many molecules (Table 2), in particular growth factors and their downstream effectors. In the skeletal muscle of old rodents, it has been shown that testosterone and GH treatments (known to activate the PI3K/Akt/mTOR pathway) can increase G6PDH activity and protein content associated with muscle hypertrophy (Brioché et al., 2014; Kovacheva et al., 2010; Max, 1984). Aerobic training was also found to increase G6PDH activity in rat skeletal muscle (Barakat et al., 1989). Other factors also regulate G6PDH and are summarized in Table 2. Interestingly, G6PDH is mainly activated by growth factors; this finding is suggestive of a role in cell growth, as discussed below.

Several antioxidant systems depend on the production of NADPH for proper function. The first is the glutathione system dependent on the production of reduced glutathione

by glutathione reductase, which depends on NADPH (Scott et al., 1993). Catalase does not need NADPH to convert hydrogen peroxide to water but has an allosteric binding site for NADPH that can keep catalase in its active conformation (Scott et al., 1993). Superoxide dismutase does not use NADPH to convert superoxide to hydrogen peroxide; however, if this compound is not adequately reduced chemically by catalase or glutathione, the increased hydrogen peroxide levels will quantitatively increase and inhibit the SOD activity (Stanton, 2012). It has been shown in various studies that during sarcopenia and aging, decreased G6PDH activity and/or muscle protein content are associated with depletion of GSH, an increase in the GSSG/GSH ratio associated with decreased activity or protein content of glutathione reductase (GR), glutathione peroxidase (Gpx), catalase, and SOD (Brioché et al., 2014; Kovacheva et al., 2010; Kumaran et al., 2004; Sinha-Hikim et al., 2013). These observations may explain the observed concomitant increase in lipid peroxidation and DNA and protein oxidation (Brioché et al., 2014; Kovacheva et al., 2010; Kumaran et al., 2004; Sinha-Hikim et al., 2013). On the other hand, in response to different antioxidant strategies or testosterone or GH treatment of rats, G6PDH protein content or activity is increased in skeletal muscle; a concomitant increase in GSH, GR, Gpx, Cat, and SOD activities was observed, leading to alleviation of oxidative damage (Brioché et al., 2014; Kovacheva et al., 2010; Kumaran et al., 2004; Sinha-Hikim et al., 2013). These results provided evidence that targeting G6PDH would be a good strategy to combat sarcopenia by restoring youth-associated redox status, which is very important to reestablish protein synthesis and the muscle regenerative potential (Derbre et al., 2014). As mentioned above, Kovacheva et al. (2010) published data on this topic. Indeed, testosterone treatment of old mice is able to increase G6PDH muscle content associated with decreased lipid peroxidation and increased Akt activation and SC activation. Eventually, these mice develop muscle hypertrophy. Similar results were published by Sinha-Hikim et al. (2013) regarding old mice in response to treatment with a GSH precursor. In young animals, it has been shown that aerobic exercise can increase G6PDH activity in the liver and skeletal muscle (Askeq et al., 1975). Moreover, Braga et al. (2008) detected a dramatic decrease in G6PDH protein content in sarcopenic mice. Similarly, G6PDH overexpression in endothelial cells results in decreased reactive oxygen and nitrogen species production as compared to wild-type cells (Leopold et al., 2003).

Various studies in cell culture have shown a direct negative relation between G6PDH activity and/or protein content and apoptosis (Fico et al., 2004; Nutt et al., 2005; Salvemini et al., 1999; Tian et al., 1999). For instance, G6PDH-null embryonic stem cells are more sensitive to hydrogen peroxide-induced apoptosis associated with GSH depletion and high caspase 3 and 9 protein levels (Fico et al., 2004). On the other hand, Nutt et al. (2005) showed that inhibition of G6PDH by dihydroepiandrosterone (DHEA) activates caspase 2 and promotes oocyte apoptosis. In old rodents, decreased activity and/or protein content of G6PDH in skeletal muscle is associated with increased apoptosis and atrophy (Braga et al., 2008; Kovacheva et al., 2010; Sinha-Hikim et al., 2013). Moreover, Braga et al. (2008) confirmed in old mice that

**Table 2**

Positive and negative regulators of G6PDH (adapted from Stanton, 2012).

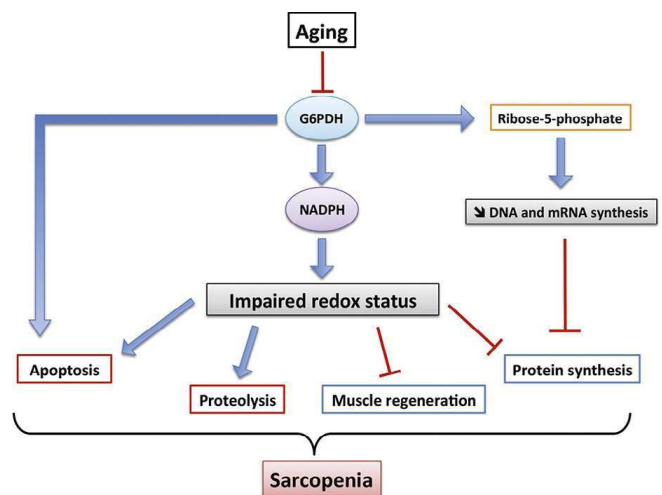
Positive regulators	Negative regulators
PDGF, EGF, VEGF, HGF	TNF- $\alpha$
Insulin	P38 MAPK
Benfotiamine (vitamin B1 analog)	P53
Vitamin D	AMPK
Testosterone, estrogens	Aldosterone
Growth hormone	Angiotensin II
Exercise	Arachidonic acid
PI3K, Akt, mTOR, p70S6K	cAMP
Nrf2	cAMP-dependent PKA
Src	
TIGAR	
Hsp27	
SREBP	
ATM	
Phospholipase C	
cGMP-dependent PKG	
Ras-GTPase	

Abbreviations: PDGF, platelet-derived growth factor; EGF, epidermal growth factor; VEGF, vascular endothelial growth factor; HGF, hepatocyte growth factor; PI-3K, phosphatidylinositol-3-kinase; PKG, protein kinase G; mTOR, mammalian target of rapamycin; TIGAR, TP53-induced glycolysis and apoptosis regulator; Hsp27, heat-shock protein 27; ATM, ataxia telangiectasia mutated; SREBP, sterol-responsive-element-binding protein; PKA, protein kinase A; CREM, cyclic AMP response element modulator; Nrf2, nuclear factor-E2-related factor; TNF- $\alpha$ , tumor necrosis factor  $\alpha$ ; AMPK, adenosine monophosphate-activated protein kinase.

depletion of G6PDH protein is associated with upregulation of caspases 2 and 9 in skeletal muscle. On the other hand, in response to different strategies against sarcopenia, increased G6PDH activity or protein content is associated with decreased apoptosis and with muscle hypertrophy (Brioché et al., 2014; Kovacheva et al., 2010; Sinha-Hikim et al., 2013). This beneficial effect may be mediated by a link between Akt and G6PDH. Indeed, Akt is also known to have antiapoptotic effects (Robey and Hay, 2006). Moreover, in the aforementioned studies, in old muscle, Akt activation and G6PDH protein are both downregulated and associated with muscle atrophy (Brioché et al., 2014; Kovacheva et al., 2010; Sinha-Hikim et al., 2013).

G6PDH activity may play an important role in muscle hypertrophy and regeneration by acting on possible proliferation of SCs, on RNA, and protein synthesis. Indeed, in various old studies on the muscle degeneration-regeneration cycle, it has been shown that during regeneration (known to involve SCs), G6PDH activity is dramatically increased (Wagner et al., 1977, 1978), while protein synthesis and RNA synthesis are also increased (Wagner et al., 1978). Furthermore, inhibition of mRNA and protein synthesis is associated with G6PDH inhibition (Wagner et al., 1978). Thus, it was argued that G6PDH may perform a major function in mRNA and DNA synthesis because it is the rate-limiting enzyme of the pentose phosphate pathway, which is the main pathway synthesizing R5P, an essential component of nucleic acids. Because of this function, G6PDH may indirectly affect protein synthesis. These various hypotheses were confirmed in *in vitro* studies, which have shown that overexpression of G6PDH in various cell lines increases DNA and protein synthesis (Kuo et al., 2000; Tian et al., 1998). On the other hand, G6PDH-deficient cells show a lower growth rate (Ho et al., 2000). Furthermore, inhibition of G6PDH makes cells more susceptible to the growth-inhibitory effects of H<sub>2</sub>O<sub>2</sub> owing to NADPH downregulation leading to decreased GSH content (Tian et al., 1998). Inhibition of G6PDH in cultured cells decreases their proliferation because of decreased protein and DNA synthesis associated with impaired redox status. Therefore, it can be hypothesized that G6PDH downregulation (activity and protein content) – observed in skeletal muscle during aging – may contribute to a decrease in the regenerative capacity of skeletal muscle. On the other hand, increased G6PDH activity should improve this mechanism. Data in this regard have been published by Kovacheva et al. (2010) and Brioché et al. (2014), who showed impaired SC proliferation along with a decrease in skeletal-muscle content of G6PDH protein and an increase in oxidative damage in old sarcopenic mice. Conversely, mice or rats treated with testosterone or GH show increased muscle content of G6PDH protein along with SC proliferation and decreased oxidative damage (Brioché et al., 2014; Kovacheva et al., 2010). G6PDH downregulation during aging should contribute to decreased protein synthesis. Until now, only downregulation of Akt and activation of p70S6K associated with decreased G6PDH activity and atrophy have supported this hypothesis in skeletal muscle (Brioché et al., 2014; Kovacheva et al., 2010; Sinha-Hikim et al., 2013).

Finally, decreased G6PDH activity and/or protein content in skeletal muscle observed during aging may be involved



**Fig. 3.** G6PDH-linked mechanisms possibly involved in sarcopenia. Aging is associated with a decrease in G6PDH activity and/or protein content in skeletal muscle; this change may lead to less active synthesis of NADPH and ribose 5-phosphate. The decrease in NADPH levels should impair the redox status, and this event may exacerbate apoptosis and proteolysis and impede muscle regeneration and protein synthesis. The downregulation of ribose 5-phosphate may impair DNA and mRNA synthesis, thus decreasing protein synthesis. All these mechanisms may lead to sarcopenia.

in sarcopenia by decreasing the antioxidant capacity; this change can impair the PI3K/Akt/mTOR pathway, thereby decreasing protein synthesis. There are no published data on G6PDH and proteolysis; however, by decreasing antioxidant defense, reactive oxygen and nitrogen species should promote their own accumulation and activation of several proteolysis pathways (Derbre et al., 2014). On the other hand, the parallel decrease in Akt phosphorylation and in G6PDH activity lead to apoptosis through activation of caspases. A decrease in G6PDH activity may reduce the regenerative potential of skeletal muscle by limiting SC proliferation. Activated G6PDH should restore the optimal redox status and reverse these adverse effects. All these mechanisms are summarized in Fig. 3.

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Article 5

**Autophagy and Protein Turnover Signaling in Slow-Twitch Muscle  
during Exercise**

Allan F. Pagano, Guillaume Py, Henri Bernardi, Robin B. Candau, Anthony M.J. Sanchez

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# Autophagy and Protein Turnover Signaling in Slow-Twitch Muscle during Exercise

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## ABSTRACT

PAGANO, A. F., G. PY, H. BERNARDI, R. B. CANDAU, and A. M. J. SANCHEZ. Autophagy and Protein Turnover Signaling in Slow-Twitch Muscle during Exercise. *Med. Sci. Sports Exerc.*, Vol. 46, No. 7, pp. 1314–1325, 2014. **Purpose:** The aim of this study was to characterize skeletal muscle protein breakdown and mitochondrial dynamics markers at different points of endurance exercise. **Methods:** Mice run at 10 m·min<sup>-1</sup> during 1 h, and running speed was increased by 0.5 m·min<sup>-1</sup> every minute during 40 min and then by 1 m·min<sup>-1</sup> until exhaustion. Animals were killed by cervical dislocation at 30, 60, 90, and 120 min; at time to exhaustion ( $T_e$ ); and at 3 and 24 h during recovery. The soleus and the deep red regions of the quadriceps muscles were pooled. **Results:** AMPK phosphorylation (Thr172) increased from 30 min to  $T_e$ , and FoxO3a phosphorylation (Thr32 and Ser253) decreased from 120 min to 3 h after exercise. FoxO3a-dependent E3 ligases Mu1 and MuRF1 proteins increased from 30 min to  $T_e$  and at  $T_e$  and 3 h after exercise, respectively, whereas MAFbx/atrogen-1 protein expression did not change significantly. The autophagic markers LC3B-II increased at 120 min and  $T_e$ , and p62 significantly decreased at  $T_e$ . The AMPK-dependent phosphorylation of Ulk1 at Ser317 and Ser555 increased from 60 min to  $T_e$  and at 30 and 60 min, respectively. Akt (Ser473), MTOR (Ser2448), and 4E-BP1 (Thr37/46) phosphorylation decreased from 90 min to  $T_e$ , and the MTOR-dependent phosphorylation of Ulk1 (Ser757) decreased from 120 min to  $T_e$ . Ser616 phosphorylation of the mitochondrial fission marker DRP1 increased from 60 min to  $T_e$ , but protein expression of the fusion markers mitofusin-2, a substrate of Mu1, and OPA1 did not significantly change. **Conclusions:** These results fit with a regulation of protein breakdown triggered by FoxO3a and Ulk1 pathways after AMPK activation and Akt/MTOR inhibition. Furthermore, our data suggest that mitochondrial fission is quickly increased, and mitochondrial fusion is unchanged during exercise. **Key Words:** AMPK, PROTEIN DEGRADATION, MITOPHAGY, MITOCHONDRIA, ENDURANCE EXERCISE

Acute and chronic exercises represent stimuli able to generate profound modulations of cellular signaling mechanisms to promote the accomplishment of specific metabolic and morphological adaptations. According to the intensity considered, acute exercise can induce an alteration in skeletal muscle protein turnover and cause damage to cell constituents. Two major signaling pathways are mainly involved: the PI3K/Akt/MTORC1 pathway associated with protein synthesis and the forkhead box O (FoxO)-related

pathway implicated in the control of protein breakdown (30,35).

The mechanistic target of rapamycin complex 1 (MTORC1) modulates protein synthesis by regulating its downstream effectors, the eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) (1), and the ribosomal protein S6 kinase 1 (S6K1) (10). Phosphorylation of 4E-BP1 at Thr37/46 by MTOR induces its dissociation from the eukaryotic translation initiation factor 4E (eIF4E), providing the assembly of the preinitiation complex (39). In addition, after an initial phosphorylation by MTOR at Thr389, S6K1 phosphorylates substrates involved in the regulation of protein translation, such as the ribosomal protein S6 (rpS6) and eIF4B (13,29).

Conversely to this pathway, FoxO transcription factor-related pathway plays a major role in the control of protein breakdown through the regulation of the ubiquitin-proteasome and autophagy-lysosomal systems (24,34). FoxO1 and FoxO3a are required for the transcription of the E3 ubiquitin ligases MAFbx/atrogen-1 and MuRF1 (16,34), leading to the

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ubiquitination and proteasomal degradation of several proteins involved in skeletal muscle maintenance. MAFbx/atrogen-1 catalyzes the breakdown of the myogenic transcription factors MyoD, myogenin, the eukaryotic initiation factor of translation eIF3f, as well as some sarcomeric proteins (i.e., desmin and vimentin) (22,33). MuRF1 seems essentially implicated in the degradation of myofibrillar proteins like myosin heavy chain protein (6), myosin-binding protein C, and myosin light chain 1 and 2 to date (8). The second system is the autophagy–lysosomal pathway that constitutes a fundamental mechanism for cell maintenance through the degradation of cytoplasmic organelles, soluble proteins and macromolecules, and the recycling of the breakdown products (21). It implicates firstly the sequestration of substrates into a vacuolar system (autophagosome) and secondly their degradation by lysosomal hydrolases. Autophagy requires the Atg (autophagy-related gene) proteins involved in the formation of autophagosomes (7) and two ubiquitin-like conjugation systems. The first one is the Atg12/Atg5/Atg16 complex, which is essential for the formation of autophagosome membrane (38). The second one involves Atg8, also known as microtubule-associated protein 1 light chain 3 (LC3) in mammals. Pro-LC3 is first cleaved by the cysteine protease Atg4 to its mature form, LC3I. This protein is then lipidated by Atg7 and Atg3 to form LC3II, contributing to the membrane fusion and to the substrate selection (27). Two other complexes are also important for the initiation of the autophagic process, the unc-51-like kinase (Ulk1)/Atg1 and the Beclin1/vacuole protein sorting 34 (Vps34)/PI3K complexes (17,25). Importantly, MTOR phosphorylates Ulk1 at Ser757, preventing the initiation of autophagy (15,18). MTORC1 has been proposed as the dominant regulator of autophagy initiation in skeletal muscle (5).

Endurance exercise induces muscular remodeling especially through the AMP-activated protein kinase (AMPK), a serine/threonine protein kinase that acts as a sensor of cellular energy status switch regulating several systems including glucose and lipid metabolism (31). AMPK has been implicated in the control of skeletal muscle protein turnover by decreasing MTORC1 activity and by increasing protein breakdown through regulation of ubiquitin–proteasome and autophagy pathways (32). AMPK can increase FoxO3a-dependent protein expression and also phosphorylate Ulk1 at several sites *in vitro* (18,32). However, little is known about the involvement of muscle protein breakdown systems that occurred in physiological conditions during endurance exercise. Concerning mitochondrial dynamics, a recent study highlights that a 24-h ultra endurance exercise rises the phosphorylation of the dynamin-related protein 1 (DRP1), a key actor of mitochondrial and peroxisomal division, without affecting the protein expression pattern of several mitophagic markers (i.e., PINK1, Parkin, and MFN1) (14). Fission and selective fusion govern mitochondrial segregation as well as elimination by autophagy and an increase in mitochondrial fission has been shown to be permissive for the induction of mitophagy (37). Recently, the involvement of FoxO3a

in mitophagy has been characterized. During muscle-wasting stimuli, enhanced FoxO3a activity results in increased transcription of the mitochondrial E3 ubiquitin protein ligase 1 (Mull), which in turn ubiquitinates and degrades the mitofusin-2 (MFN2), a mitochondrial fusion protein critical for the maintenance and genomic stability of mitochondrial DNA (23). These events promote fragmentation, depolarization, and selective elimination of damaged mitochondria through the autophagy–lysosomal pathway (23).

The present study aimed to characterize the modulation of skeletal muscle protein breakdown and mitochondrial dynamics markers during the progression of endurance exercise and to evaluate whether these regulations were coordinated with potential modulations of protein synthesis markers.

## METHODS

**Ethics statement.** This study was approved by the Committee on the Ethics of Animal Experiments of the Languedoc Roussillon in accordance with the guidelines from the French National Research Council for the Care and Use of Laboratory Animals (Permit Number: CEEA-LR-1069). This study is in adherence to the American College of Sports Medicine animal care standards. All efforts were made to minimize suffering.

**Animals.** Experiments were carried out on 8-wk-old C57BL/6 mice ( $n = 52$ ; body mass =  $26.2 \pm 2.1$  g) from our own stock. Animals were maintained on a 12-h/12-h light–dark cycle and provided with food and water *ad libitum*. Experiments were performed at 22°C.

**Exercise protocol and muscle collection.** The endurance exercise protocol was performed on a motor-driven treadmill (Exer-6M Treadmill; Columbus Instruments). Mice were familiarized to treadmill running during three sessions in which they successively run at  $5 \text{ m}\cdot\text{min}^{-1}$  during 1 min,  $7 \text{ m}\cdot\text{min}^{-1}$  during 4 min, and  $10 \text{ m}\cdot\text{min}^{-1}$  during 2 min. Three days after the familiarization period, mice performed, except a control group ( $n = 8$ ), an endurance exercise until exhaustion. Animals exercised exactly at the same time of the day (i.e., 8:00 a.m.) in the fed state. Mice run at  $10 \text{ m}\cdot\text{min}^{-1}$  during 1 h, and running speed was increased by  $0.5 \text{ m}\cdot\text{min}^{-1}$  every minute during 40 min and then by  $1 \text{ m}\cdot\text{min}^{-1}$  until exhaustion. Mice were killed by cervical dislocation at 30, 60, 90, and 120 min ( $n = 5$  for each time point); time to exhaustion ( $T_e$ ) during exercise; and 3 and 24 h after exercise ( $n = 8$  for each time point). The soleus and the deep red regions of the quadriceps (slow-twitch) muscles of both the left and right hind limbs were rapidly dissected out at the corresponding time and were frozen in liquid nitrogen before storage at  $-80^\circ\text{C}$  for subsequent analyses. Mice that exercised until exhaustion ran  $1957.7 \pm 347.8$  m, with an effective running time of  $151.2 \pm 14.8$  min.

**Proteins isolation and immunoblot analysis.** Muscles samples (i.e., soleus and deep red regions of the quadriceps) were pooled and homogenized in 10 volumes of lysis buffer

(50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EGTA, 100 mM NaF, 5 mM Na<sub>3</sub>VO<sub>4</sub>, 1% Triton X-100, 40 mM β-glycerophosphate, and protease inhibitor mixture [P8340; Sigma-Aldrich]) using FastPrep-24 Instrument (MP Bio-medicals). Cellular debris was removed by centrifugation at 10,000g for 15 min (4°C). Protein concentrations were measured using a Pierce BCA protein assay kit (23225; Thermo Scientific). Proteins (60 μg) were denatured and loaded onto 7% and 15% SDS-polyacrylamide gels before electrophoretic transfer onto a nitrocellulose membrane (Bio-Rad). After transfer, membranes were blocked with 50 mM Tris-HCl pH 7.5, 150 mM NaCl, and 0.1% Tween 20 (TBS-T) containing 5% skimmed milk or BSA and incubated overnight at 4°C with primary antibodies. Membranes were washed three times for 10 min with TBS-T and incubated for 1 h with a peroxidase-conjugated secondary antibody (Sigma-Aldrich). Membranes were washed again three times for 5 min, and immunoblots were revealed by using a Pierce ECL kit (32106; Thermo Scientific) according to the manufacturer's instructions.

**Antibodies.** Anti-phospho-AMPKα (Thr172), AMPKα, phospho-FoxO3a (Thr32), phospho-FoxO3a (Ser253), FoxO3a, phospho-Ulk1 (Ser317), phospho-Ulk1 (Ser555), phospho-Ulk1 (Ser757), Ulk1, phospho-DRP1 (Ser616), DRP1, phospho-Akt (Ser473), Akt, phospho-MTOR (Ser2448), MTOR, phospho-4E-BP1 (Thr37/46), 4E-BP1, phospho-eIF2α (Ser51), eIF2α, phospho-rpS6 (Ser240/244), rpS6, and p62 were purchased from Cell Signaling; anti-Mull1 and VDAC were obtained from Abcam; anti-MFN2 and ubiquitin from Santa Cruz; anti-α-tubulin (DM1A) and LC3 from Sigma-Aldrich; and anti-MAFbx/atrogin-1 and MuRF1 from ECM Biosciences. The OPA1 antibody was a generous gift from Drs. Béatrice Chabi (INRA UMR 866, DMEM, Montpellier, France) and Guy Lenaers (INSERM U-583, Institut des Neurosciences de Montpellier, France).

**mRNAs isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis.** Muscles (i.e., soleus and deep red regions of the quadriceps) were pooled and crushed in liquid nitrogen using mortar and pestle. Total RNA was extracted from powdered muscles by using RNeasy Fibrous Tissue Kit (Qiagen), according to the manufacturer's instructions. RNA concentration was determined by spectrophotometric analysis, and integrity was checked by OD<sub>260nm</sub>/OD<sub>280nm</sub> absorption ratio (>1.95) and by agarose gel analysis. Total RNAs were stored at -80°C until further use. Reverse transcription reaction was performed with 1 μg of total RNA using RevertAid First Strand cDNA synthesis kit (Thermo Scientific). First-strand cDNA synthesis was made by using 200 U of SuperScript II Reverse Transcriptase and Oligo(dT) 12-18 primers (Invitrogen) according to the manufacturer's instructions. cDNAs were diluted (1:10) in water and stored at -20°C until further use. qPCR analysis was performed in a MiniOpticon detection system (Biorad, Hercules, CA) with 10 μL of KAPA SYBR Fast Universal Readymix (Thermo Scientific), 300 nM of both

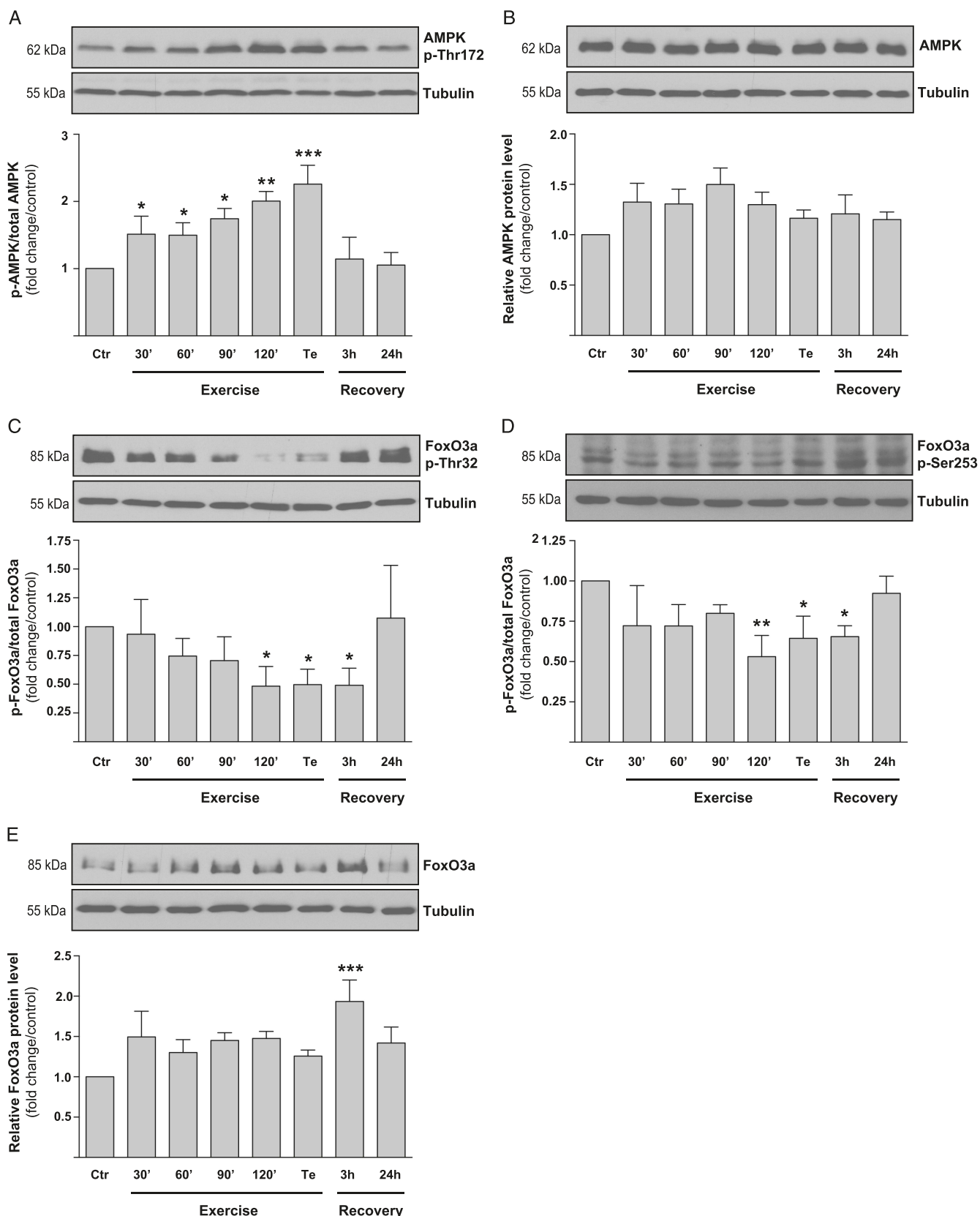
forward and reverse primers, 2 μL of diluted cDNA template and water to a final volume of 20 μL. The primers were designed using Universal ProbeLibrary Assay Design Center (Roche Applied System) and RT Primer Data Base. All PCRs were performed in duplicate using the following cycle parameters: 30 s at 98°C, 40 cycles of 1 s at 95°C and 15 s at 60°C. Relative mRNA expression levels were normalized to ribosomal protein S9 (RPS9) expression, which was unaffected by treatments. Primer sequences: 5'-FoxO3a GGAAATGGGCAAAGCAGA, 3'-FoxO3a AAACGGATC ACTGTCCACTTG, 5'-MAFbx AGTGAGGACCGGCTAC TGTG 3'-MAFbx GATCAAACGCTTGCGAATCT, 5'-MuRF1 TCCTGCAGAGTGACCAAGG, 3'-MuRF1 GGCGTAGAG GGTGTCAAAC, 5'-Mull1 AGGGCATTCTTTCAGAAGCA, 3'-Mull1 GGGGTGGAACCTTCTCGTACA, 5'-RPS9 CGGCC CGGGAGCTGTTGACG, 3'-RPS9 CTGCTTGCGGACCC TAATGT.

**Statistics.** Densitometry analyses of immunoblots were performed using ImageJ software and were normalized to α-tubulin exposure. The statistical analyses were performed using STATISTICA 9.1 software (StatSoft France). All data are expressed as the mean ± SEM. Data were evaluated by one-way ANOVA followed by Fisher's honestly *post hoc* test. Significance was declared when  $P < 0.05$ .

## RESULTS

**Regulation of AMPK and FoxO3a.** We first evaluated AMPK and FoxO3a phosphorylation at the different points of the exercise (Fig. 1). We found a quick increase by 51% ± 27% in AMPK phosphorylation on Thr172 at 30 min, and this level of phosphorylation remained elevated, i.e., +50% ± 18%, +75% ± 15%, +101% ± 14%, and +126% ± 28% at 60 min, 90 min, 120 min, and  $T_e$ , respectively, when compared to non-exercised mice. AMPK phosphorylation returned to baseline at 3 h after exercise (Fig. 1A). Phosphorylation of FoxO3a at Thr32 and Ser253 was significantly decreased at 120 min (-52% ± 17% and -47% ± 13%, respectively),  $T_e$  (-48% ± 11% and -30% ± 12%, respectively), and at 3 h after exercise (-48% ± 13% and -33% ± 6%, respectively; Figs. 1C and D). The total form of FoxO3a was significantly increased at 3 h after exercise (+94% ± 27%; Fig. 1E), whereas the total form of AMPK remained unchanged throughout the protocol (Fig. 1B). We also assessed FoxO3a mRNA level and found a significant increase at  $T_e$  (+173% ± 55%) and at 3 h after exercise (+268% ± 139%; Fig. 2A).

**Ubiquitin-proteasome system markers.** We next characterized the mRNA levels of E3 ubiquitin ligases transcriptionally regulated by FoxO3a (Fig. 2). We found an increase in MuRF1 at  $T_e$  (+300% ± 113%) and at 3 h after exercise (+510% ± 90%; Fig. 2C) and an increase in MAFbx/atrogin-1 at 3 h after exercise (+179% ± 54%; Fig. 2D). Nonetheless, no change in Mull1 mRNA level was found (Fig. 2B). We also determined the protein expression of these three E3 ligases (Fig. 3) and found a significant



**FIGURE 1**—AMPK and FoxO3a phosphorylation state at the different points of the exercise. Total lysates were analyzed by immunoblotting using the indicated antibodies. Values are expressed as percentage of change (means  $\pm$  SEM) compared with the preexercise condition. The most representative Western blot is shown above each histogram. Note that the two bands between 80 and 90 kDa have been quantified for FoxO3a Ser253.  $\alpha$ -Tubulin was used as a loading control, and the phosphorylated proteins were reported in the context of total protein levels. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  compared to control.

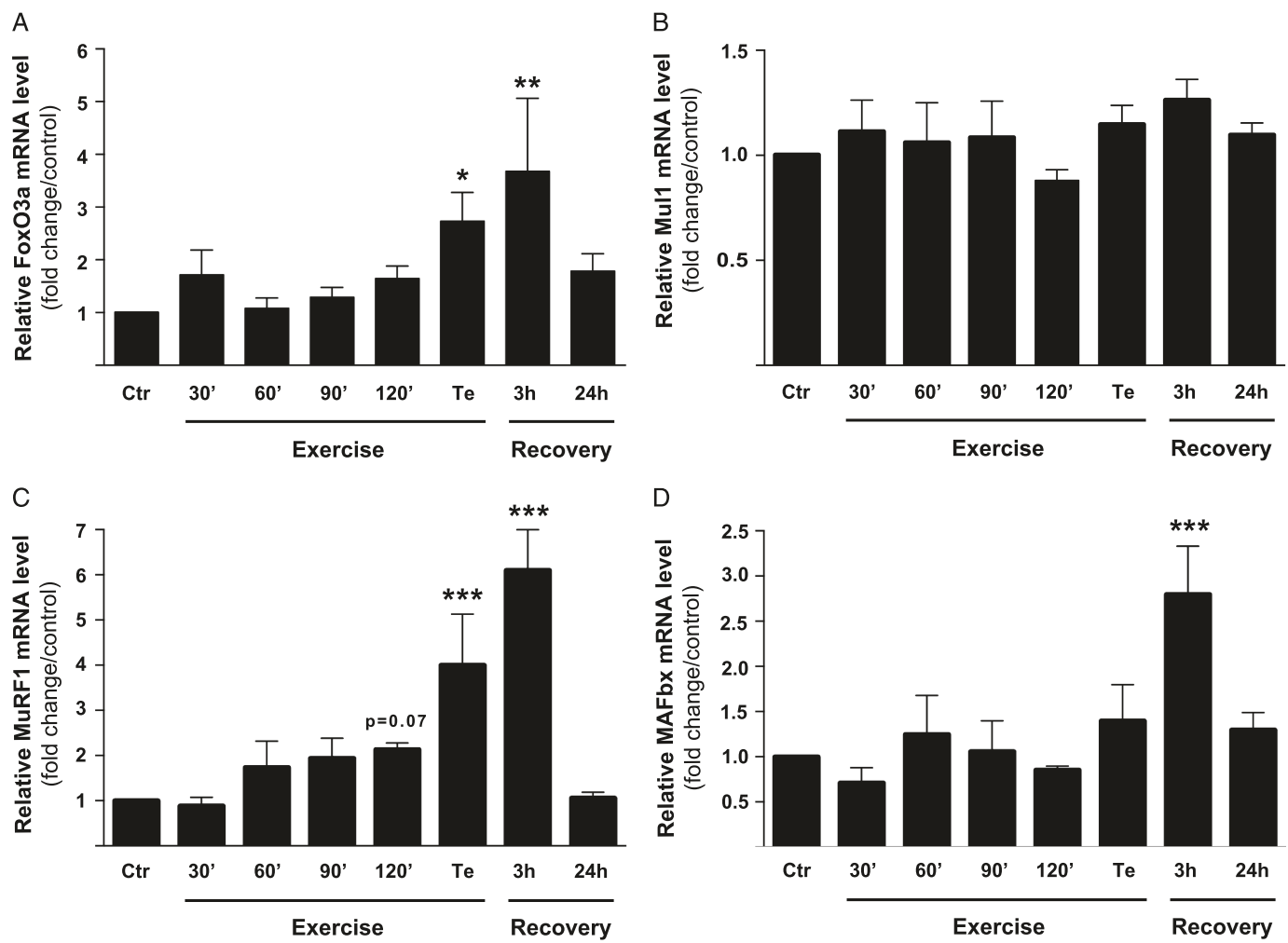
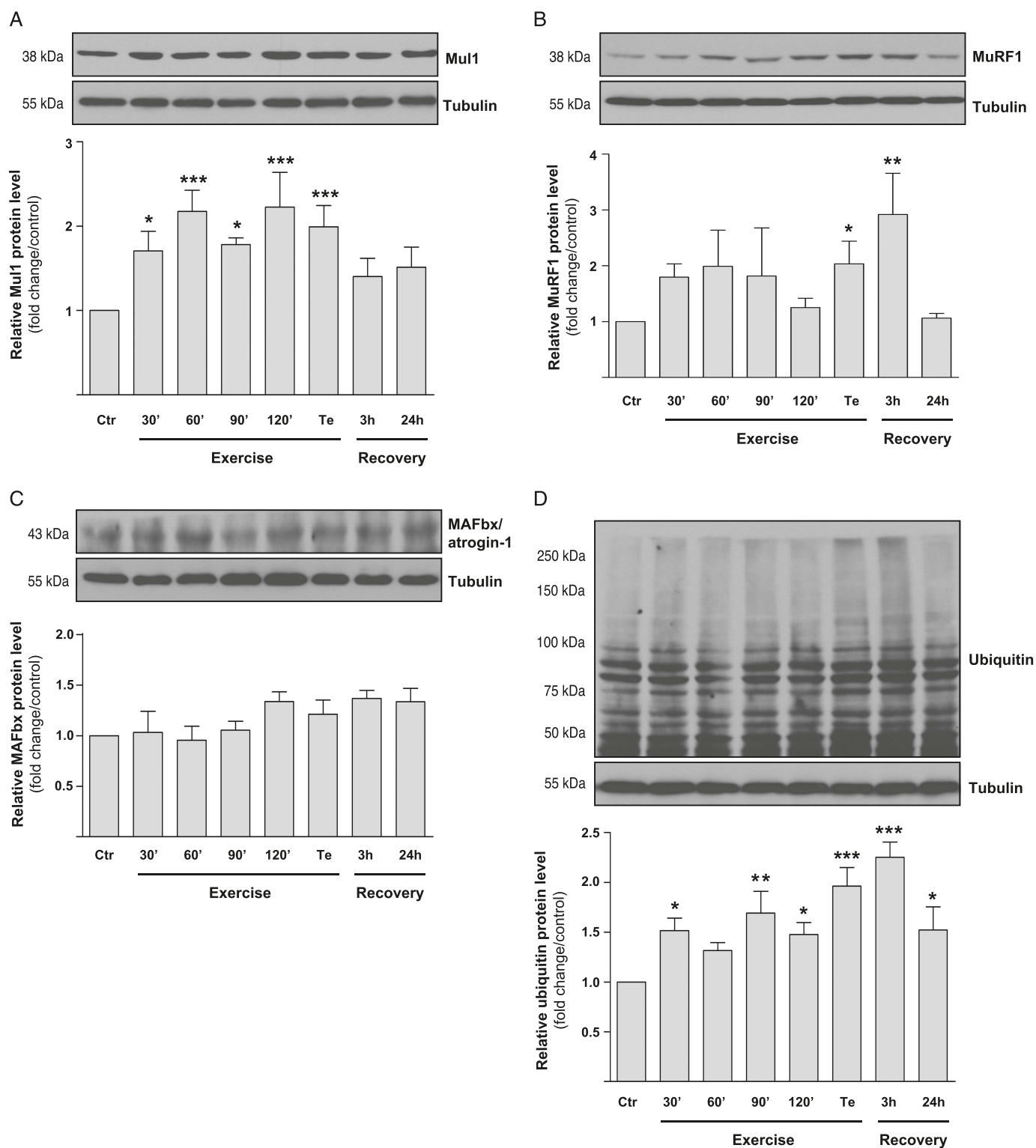


FIGURE 2—Changes in the gene expression of FoxO3a and E3 ubiquitin ligases. Values are expressed as percentage of change (means  $\pm$  SEM) compared with the preexercise condition. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  compared to control.

increase in MuRF1 protein expression at  $T_e$  ( $+104\% \pm 40\%$ ) and 3 h after exercise ( $+192\% \pm 74\%$ ) and an increase in Mu1 protein expression from 30 min to  $T_e$  (Figs. 3B and A, respectively). These rises were  $+71\% \pm 23\%$ ,  $+118\% \pm 25\%$ ,  $+78\% \pm 7.9\%$ ,  $+123\% \pm 41\%$ , and  $+99\% \pm 25\%$  at 30 min, 60 min, 90 min, 120 min, and  $T_e$ , respectively. However, we did not detect any change in MAFbx/atrogin-1 protein level (Fig. 3C). Furthermore, the ubiquitin-conjugated proteins levels were significantly increased by  $52\% \pm 13\%$ ,  $69\% \pm 22\%$ ,  $48\% \pm 12\%$ ,  $96\% \pm 19\%$ ,  $125\% \pm 15\%$ , and  $52\% \pm 23\%$  at 30 min, 90 min, 120 min,  $T_e$ , 3 h, and 24 h after exercise (Fig. 3D).

**Autophagy system markers.** The expression of several markers of autophagy pathway is depicted in Figure 4. We found an increase in LC3B-II/LC3B-I ratio at 120 min ( $+131\% \pm 57\%$ ) and  $T_e$  ( $+100\% \pm 47\%$ ), suggesting an induction of autophagy during exercise (Fig. 4A). In addition, we assessed the protein level of p62, also called sequestosome 1 (SQSTM1). p62 is a ubiquitin-binding scaffold protein that binds directly to LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy-lysosomal

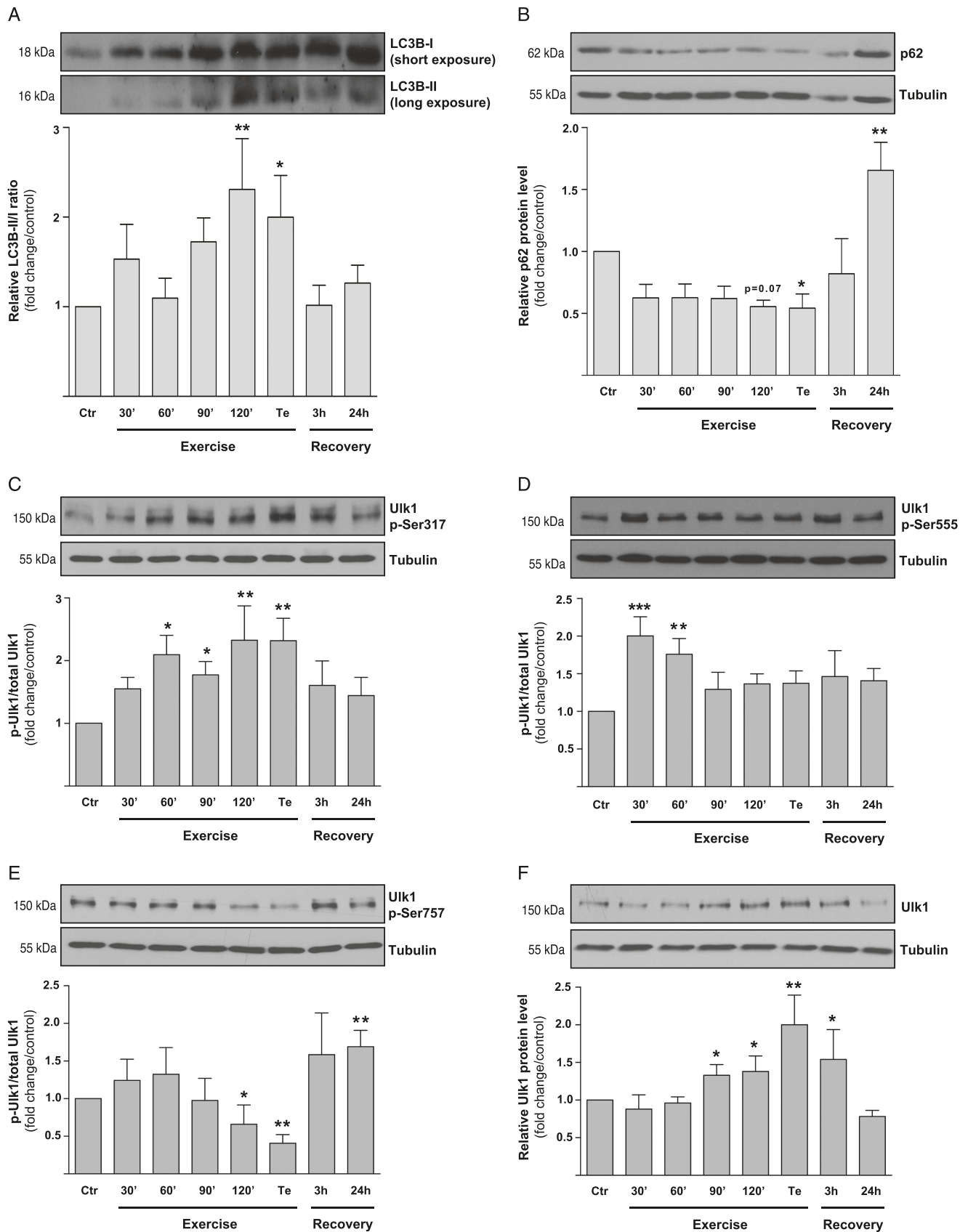
machinery (28). As LC3, p62 is itself degraded by lysosome. Since p62 accumulates when autophagy is inhibited, and decreased when autophagy is induced, p62 is commonly used as a marker to characterize autophagic flux (3). In accordance with LC3 analysis, p62 protein expression was significantly reduced at  $T_e$  ( $-46\% \pm 11\%$ ) and a tendency was observed at 120 min ( $P = 0.07$ ; Fig. 4B). Moreover, a significant increase in p62 protein expression has been found at 24 h after exercise ( $+65\% \pm 23\%$ ). Next, we assessed the phosphorylation level of Ulk1 Ser317, Ser555, and Ser757 that are known to be phosphorylated by AMPK for the first two sites and by MTOR for the last one (9,18). We reported an increase in the phosphorylation of Ser317, i.e.,  $+110\% \pm 31\%$ ,  $+77\% \pm 21\%$ ,  $+133\% \pm 55\%$ , and  $+132\% \pm 36\%$  at 60 min, 90 min, 120 min, and  $T_e$ , respectively, and an increase in Ser555 phosphorylation at 30 min ( $+100\% \pm 26\%$ ) and 60 min ( $+76\% \pm 21\%$ ) of exercise (Figs. 4C and D, respectively). A decrease in Ulk1 Ser757 phosphorylation was found at 120 min of exercise ( $-44\% \pm 26\%$ ) and at  $T_e$  ( $-59\% \pm 12\%$ ), whereas an increase was obtained at 24 h after exercise ( $+69\% \pm 22\%$ ; Fig. 4E). The total form of Ulk1



**FIGURE 3**—Changes in the protein expression of E3 ubiquitin ligases and overall protein ubiquitination. Total lysates were analyzed by immunoblotting using the indicated antibodies. Note that the whole lane has been considered for the ubiquitin blot quantification. Values are expressed as percentage of change (means  $\pm$  SEM) compared with the preexercise condition. The most representative Western blot is shown above each histogram.  $\alpha$ -Tubulin was used as a loading control. \* $P$  < 0.05, \*\* $P$  < 0.01, \*\*\* $P$  < 0.001 compared to control.

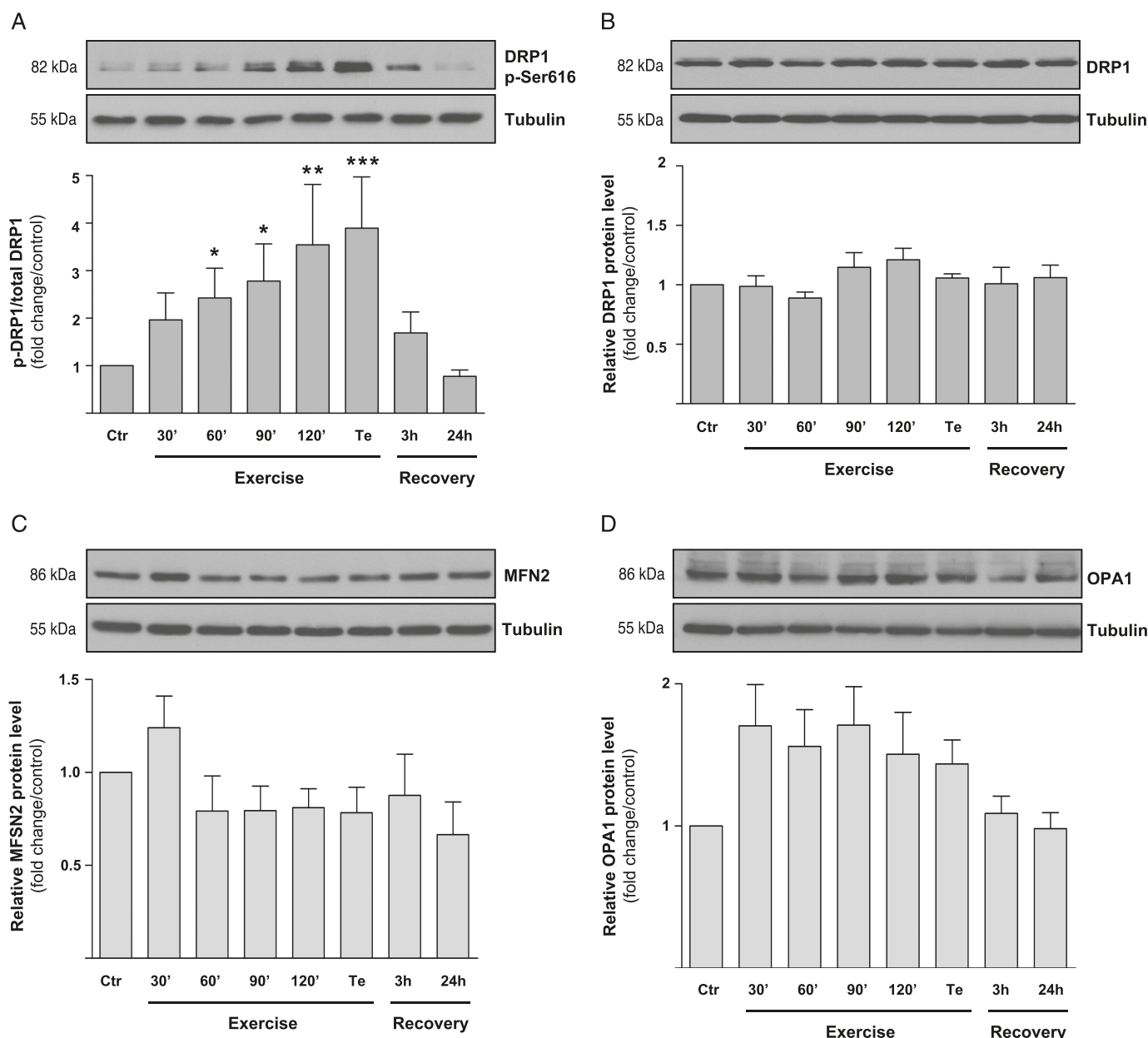
was significantly increased at 90 min ( $+33\% \pm 14\%$ ), 120 min ( $+38\% \pm 21\%$ ),  $T_e$  ( $+100\% \pm 39\%$ ), and at 3 h after exercise ( $+54\% \pm 40\%$ ; Fig. 4F).

**Mitochondrial network remodeling markers.** Next, we evaluated the modulation of major markers of mitochondrial remodeling: the phosphorylation state of DRP1 (Ser616),



**FIGURE 4—Changes in autophagic markers.** Total lysates were analyzed by immunoblotting using the indicated antibodies. Values are expressed as percentage of change (means  $\pm$  SEM) compared with the preexercise condition. The most representative Western blot is shown above each histogram.  $\alpha$ -Tubulin was used as a loading control, and the phosphorylated proteins were reported in the context of total protein levels. \* $P$  < 0.05, \*\* $P$  < 0.01, \*\*\* $P$  < 0.001 compared to control.

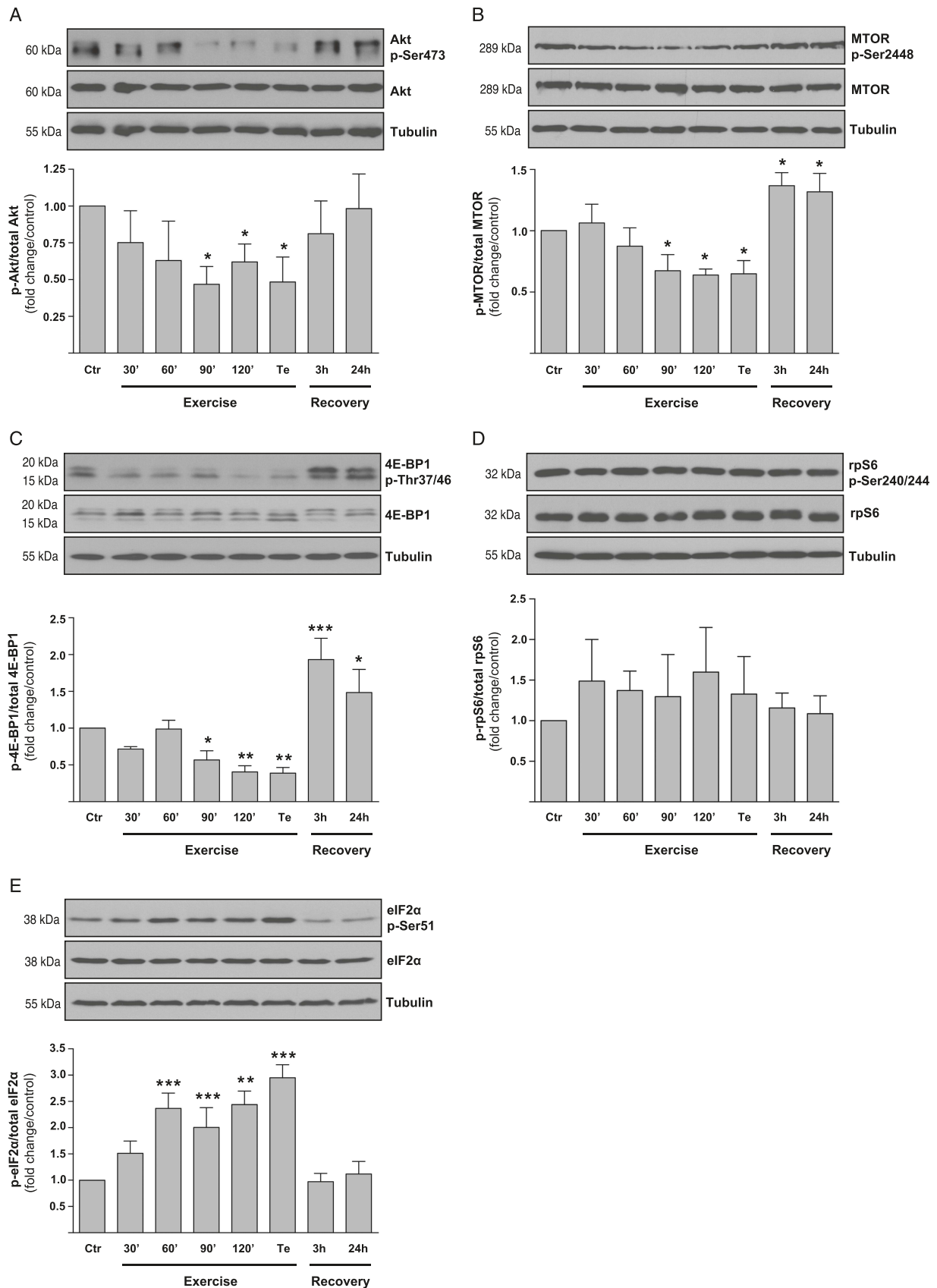




**FIGURE 5**—Changes in mitochondrial remodeling markers. Total lysates were analyzed by immunoblotting using the indicated antibodies. Values are expressed as percentage of change (means  $\pm$  SEM) compared with the preexercise condition. The most representative Western blot is shown above each histogram.  $\alpha$ -Tubulin was used as a loading control, and the phosphorylated proteins were reported in the context of total protein levels. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  compared to control.

which is known to affect mitochondrial morphology through stimulation of mitochondrial fission, the protein levels of MFN2, a substrate of Muf1, and OPA1. The last two proteins are critical regulators of mitochondrial homeostasis because they are involved in mitochondrial fusion. Figs. 5A and B show a rise in the phosphorylation of DRP1 on Ser616 by  $143\% \pm 63\%$ ,  $178\% \pm 78\%$ ,  $255\% \pm 127\%$ , and  $290\% \pm 108\%$  at 60 min, 90 min, 120 min, and  $T_e$ , respectively, without any change in the expression of its total form. Concerning MFN2 and OPA1, we did not find any significant variation of their protein content throughout the exercise and during recovery (Figs. 5C and D, respectively).

**Protein synthesis markers.** Last, we examined several major components of the Akt/MTORC1 pathway to establish whether endurance exercise may induce a decrease in protein synthesis flux (Fig. 6). We found that exercise induces hypophosphorylation of Akt Ser473 at 90 min, 120 min, and  $T_e$  ( $-53\% \pm 12\%$ ,  $-38\% \pm 12\%$ , and  $-52\% \pm 17\%$ , respectively); MTOR Ser2448 at 90 min, 120 min, and  $T_e$  ( $-33\% \pm 13\%$ ,  $-36\% \pm 5\%$ , and  $-35\% \pm 11\%$ , respectively); and 4E-BP1 also at 90 min, 120 min, and  $T_e$  ( $-43\% \pm 13\%$ ,  $-59\% \pm 8\%$ , and  $-61\% \pm 8\%$ , respectively; Figs. 6A, B, and C, respectively). In addition, we assessed the Ser240/244 phosphorylation of the direct target of S6K1, rpS6, but we did not find any significant modulation,



**FIGURE 6**—Time course of markers involved in canonical protein synthesis pathway. Total lysates were analyzed by immunoblotting using the indicated antibodies. Values are expressed as percentage of change (means  $\pm$  SEM) compared with the preexercise condition. The most representative Western blot is shown above each histogram.  $\alpha$ -Tubulin was used as a loading control, and the phosphorylated proteins were reported in the context of total protein levels. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  compared to control.

compared to preexercise condition (Fig. 6D). In sum, these results suggest that the translational machinery could be less functional, especially for the latest points of the exercise. We also assessed the phosphorylation state of the regulatory subunit of the eukaryotic initiation factor 2 (eIF2), a heterotrimeric complex that mediates the binding of tRNA<sup>met</sup> to the ribosome in a GTP-dependent manner (Fig. 6E). The  $\alpha$ -subunit of this factor contains the main target for phosphorylation (Ser51) and is considered as the regulatory subunit of eIF2, with its phosphorylation leading to a stabilization of the eIF2–GDP–eIF2B complex that inhibits the turnover of eIF2B (36). Thus, phosphorylation of eIF2 $\alpha$  at Ser51 is associated with the inhibition of protein synthesis. As expected, a significant increase in the phosphorylation of this residue occurred at 60 min, 90 min, 120 min, and  $T_e$  (+137%  $\pm$  29%, +101%  $\pm$  38%, +144%  $\pm$  26%, and +195%  $\pm$  25%, respectively).

Furthermore, phosphorylation of MTOR and 4E-BP1 was significantly increased at 3 h (+37%  $\pm$  11% and +93%  $\pm$  29%, respectively) and at 24 h (+32%  $\pm$  15% and +48%  $\pm$  32%, respectively) after exercise when compared to non-exercised mice.

## DISCUSSION

In this study, we showed that autophagy and ubiquitin–proteasome markers, notably Mull1 protein expression, are upregulated during endurance exercise, coordinately with a downregulation of the protein synthesis pathway. While autophagy markers and phosphorylation of the mitochondrial fission marker DRP1 returned to basal level after exercise, ubiquitin–proteasome machinery markers, especially MuRF1 mRNA and protein levels, reached a peak at 3 h after exercise.

AMPK plays a major role in skeletal muscle homeostasis in response to energy stress conditions, including exercise (31). It was therefore not surprising to observe an increase in the phosphorylation state of AMPK on Thr172, which witnesses the kinase activation in response to the exercise. In our study, during the first 60 min corresponding to a low-intensity exercise, we were able to detect a fast increased AMPK phosphorylation. The low level of aerobic fitness of the sedentary mice used in the present study may contribute to explain the activation of the kinase from such a low intensity, since it is known that AMPK activation depends on the training status (26). Concerning FoxO3a, we found a decrease of Ser253 and Thr32 phosphorylation (i.e., two Akt-linked inhibiting phosphorylation) from 120 min of exercise and during the recovery period (i.e., at 3 h after exercise). This result suggests that FoxO3a is activated during exercise, especially when intensity is near to exhaustion, and also during the first hours of the recovery period. Accordingly, FoxO3a mRNA and protein levels were markedly increased at 3 h after exercise. Furthermore, among FoxO posttranslational modifications, phosphorylation of FoxO3a Ser413/588 by AMPK has been found to increase FoxO3a transcriptional activity *in vitro* (11,32).

Thus, AMPK-mediated phosphorylation of FoxO3a could also be involved in FoxO3a activation during exercise, although we did not test this possibility because mouse-specific antibodies are not currently available.

Autophagic activity characterization represents a challenge because of the dynamics of the system. Among Atgs proteins, LC3B-II has been positively correlated with an increased number of autophagosomes and is therefore commonly used as a marker of autophagy activation (2). Nonetheless, an increase in LC3B-II expression strengthens the hypothesis of an enhanced number of autophagosomes but does not allow making clear whether this raise is the consequence of an increased autophagosome formation or a defect in their degradation through the lysosome. p62, a ubiquitin-binding scaffold protein that binds with LC3, may serve to link ubiquitinated substrates to the autophagic machinery and is itself degraded by the lysosome. Thus, modulation of p62 levels may be used as a marker to study autophagic flux (3,20). In our study, we used several markers involved in different steps of the autophagic process, LC3B-II and p62 levels, and the phosphorylation level of Ulk1 at several sites. We found that exercise increases LC3B-II and induces a drop in p62 expression near to exhaustion, suggesting an increase in autophagic flux at high intensities. These elements are in agreement with others emergent studies (12,14,19) and strengthen the idea that autophagy–lysosomal pathway is induced by exercise. Ulk1 is a key initiator of autophagy, and Ser317/555 has been recently found to be phosphorylated by AMPK *in vitro* during glucose starvation and autophagy activation (18). MTOR phosphorylates Ulk1 at Ser757, resulting in loss of Ulk1 kinase activity, thus preventing the initiation of autophagy. Here we showed that endurance exercise leads to an increase in the phosphorylation state of Ser317 and Ser555, suggesting that AMPK plays a role in the early step of autophagosome formation during exercise. However, these two phosphorylation sites are differentially regulated. Indeed, Ser555 is quickly and strongly phosphorylated at 30 and 60 min and thereafter go back to its basal phosphorylation level, whereas Ser317 stays phosphorylated from 60 min to  $T_e$ . Although the dynamics of Ulk1 complex is not clearly established in skeletal muscle, some allosteric modifications could explain such differences. These results imply that autophagy machinery would be quickly initiated during exercise, and not only for high intensities, suggesting that autophagy may constitute a generic response to any kind of endurance activity. Moreover, Ulk1 Ser757 phosphorylation was decreased from 120 min to  $T_e$  concurrently to MTOR hypophosphorylation, supporting a potential role of the inhibition of the MTOR signaling pathway in autophagy induction by exercise.

Furthermore, the present study shows for the first time a rise in the protein expression level of the E3 ligase Mull1 in response to exercise. Nonetheless, we did not detect any variation in Mull1 mRNA level, suggesting that exercise induces a stabilization of Mull1 protein. The molecular

mechanisms underlying this stabilization remain to be clarified. Mitochondrial remodeling through fission and fusion is essential for the removal of damaged mitochondria. Mulf has been shown to ubiquitinate the mitochondrial protein MFN2, causing its degradation through the proteasome. Nevertheless, we did not observe a decrease in MFN2, suggesting that its degradation rates were unaltered. Thus, the rise in Mulf expression that we observed during exercise was probably not sufficient to induce MFN2 degradation. In the same way, OPA1 protein level, another GTPase required for mitochondrial fusion, was not significantly altered during exercise. Mulf has also been reported to stabilize the mitochondrial fission protein DRP1 *in vitro*, resulting in mitochondrial fragmentation (4), but these events remain to be tested in skeletal muscle. Phosphorylation of DRP1 at Ser616 stimulates mitochondrial fission and such an increase has been reported to be permissive for mitophagy (37). Here, we found that DRP1 phosphorylation state was progressively increased until exhaustion. All together, these results indicate that endurance exercise quickly promotes DRP1 activation, potentially stimulating mitochondrial fission, but not Mulf-dependent degradation of mitochondrial fusion markers contrary to what it has been reported during muscle wasting (23). Although we did not establish whether Mulf regulates mitophagy in this study, this certainly warrants further investigations especially with a view to characterize other Mulf partners or targets than MFN2 and to investigate the possible differential effect of acute exercise between slow and fast skeletal muscles.

Concerning protein synthesis pathway, we found that Akt, MTOR, and 4E-BP1 phosphorylation was inhibited from 90 min to  $T_e$ . While protein flux synthesis has not been performed, these results suggest that protein synthesis is inhibited during endurance exercise. The significant increase in eIF2 $\alpha$  phosphorylation observed from 60 min of exercise highlights these hypotheses. Furthermore, the slow but significant increase in MTOR phosphorylation and the subsequent rise in 4E-BP1 phosphorylation found at 3 and 24 h after exercise suggest an increase in protein translation and possibly protein synthesis during the recovery period. Endurance training is well known to promote a fast-to-slow muscle phenotype shift and mitochondrial biogenesis but not to induce muscle growth. Thus, a rise in protein synthesis in response to endurance exercise would be necessary for tissue repair and remodeling, especially for the synthesis of specific subcellular protein not involved in muscle hypertrophy as mitochondrial proteins. In contrast, force and

resistance training are two major stimuli of muscle protein synthesis resulting in hypertrophy.

The physiological relevance of these events can be speculated. In our model, AMPK and/or Akt inhibition would modulate FoxO3a activity and Ulk1 axis to mobilize protein degradation as source of alternative nutrient production and energy substrates when exercise intensity/duration becomes high. In addition, autophagy system is known to be required for normal cellular function and for response to multiple types of stress to maintain skeletal muscle function. Thus, during prolonged exercise, THE autophagy–lysosomal pathway may represent a compensatory mechanism to prevent cellular loss function caused by constraints linked to metabolic stress.

In conclusion, the present study aimed to identify specific signaling events related to cellular component turnover that are modulated during endurance exercise. To the best of our knowledge, this study is the first to give a picture of the regulation of pathways implicated in protein balance and which consider mitochondrial dynamics markers at different points of endurance exercise. Our data support the idea that Akt/MTOR pathway inhibition and AMPK activation modulate protein turnover through FoxO3a and Ulk1 axes. Noticeably, the mitochondrial Mulf ubiquitin ligase is quickly induced by endurance exercise, but this induction is not sufficient to trigger mitochondrial fusion markers alteration. Further investigations are needed to better understand the overall implications of AMPK or other metabolic sensors in the regulation of mitophagy and to also identify the precise role of Mulf in these events. On the basis of these data, it is clearly conceivable that the involvement of the autophagic system in response to exercise must be considered not only in muscle homeostasis but also in disease. Because exercise is associated with improved quality of life and constitutes one of the best approaches to limit atrophy and metabolic disorders, these research directions are crucial in the fight against a wide spectrum of metabolic and muscle diseases.

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The results of the present study do not constitute endorsement by American College of Sports Medicine.

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Article 5

**The role of AMP-activated protein kinase in the coordination of skeletal muscle turnover and energy homeostasis**

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## The role of AMP-activated protein kinase in the coordination of skeletal muscle turnover and energy homeostasis

Anthony M. J. Sanchez,<sup>1,2</sup> Robin B. Candau,<sup>1,2</sup> Alfredo Csibi,<sup>3</sup> Allan F. Pagano,<sup>1,2</sup> Audrey Raibon,<sup>1</sup> and Henri Bernardi<sup>1</sup>

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**Sanchez AM, Candau RB, Csibi A, Pagano AF, Raibon A, Bernardi H.** The role of AMP-activated protein kinase in the coordination of skeletal muscle turnover and energy homeostasis. *Am J Physiol Cell Physiol* 303: C475–C485, 2012. First published June 13, 2012; doi:10.1152/ajpcell.00125.2012.—The AMP-activated protein kinase (AMPK) is a serine/threonine protein kinase that acts as a sensor of cellular energy status switch regulating several systems including glucose and lipid metabolism. Recently, AMPK has been implicated in the control of skeletal muscle mass by decreasing mTORC1 activity and increasing protein degradation through regulation of ubiquitin-proteasome and autophagy pathways. In this review, we give an overview of the central role of AMPK in the control of skeletal muscle plasticity. We detail particularly its implication in the control of the hypertrophic and atrophic signaling pathways. In the light of these cumulative and attractive results, AMPK appears as a key player in regulating muscle homeostasis and the modulation of its activity may constitute a therapeutic potential in treating muscle wasting syndromes in humans.

AMPK; autophagy; Ulk1; metabolism; ubiquitin-proteasome; FoxO

### Structure and Regulation of AMPK

The AMP-activated protein kinase (AMPK) is a serine-threonine kinase highly conserved through evolution. AMPK is a heterotrimeric complex composed of a catalytic subunit (AMPK- $\alpha$ ) and two regulatory subunits (AMPK- $\beta$  and AMPK- $\gamma$ ) (Fig. 1). In humans, AMPK subunits are encoded by seven genes ( $\alpha 1$ ,  $\alpha 2$ ;  $\beta 1$ ,  $\beta 2$ ;  $\gamma 1$ ,  $\gamma 2$ ,  $\gamma 3$ ) that can form at least 12  $\alpha\beta\gamma$  heterotrimers, thus increasing the diversification of its functions (17, 109, 117). The most studied of these subunits is the catalytic subunit, which contains the threonine residue (Thr172) located in the activation loop of the kinase domain. Phosphorylation of Thr172 by upstream kinases leads to AMPK activation (47, 49). AMPK is considered as a key enzyme in conditions of cellular energy deficit and is able to inhibit metabolic pathways that consume energy and reciprocally to increase mechanisms that produce energy.

AMPK is activated by a large variety of cellular stresses that increase cellular AMP and decrease ATP levels such as electrical-stimulated muscle contraction (24, 120) and vigorous exercises (15, 20, 92, 125) and by hypoxia (79), ischemia (71), oxidative stress (18), metabolic poisoning (124), or nutrient deprivation (103). In response to energy depletion, AMPK activation promotes metabolic changes to maintain both bioenergetic state and cell survival. Moreover, by modulating

multiple metabolic pathways and by regulating several transport proteins, AMPK potentially couples transport activity to cellular stress and energy levels. Pharmacological molecules such as 5-aminoimidazole-4-carboxamide-1- $\beta$ -D-ribofuranoside (AICAR), metformin, clozapine, or 2-deoxy-D-glucose (2DG) also lead to AMPK activation (21, 35, 48, 98, 102, 129, 130).

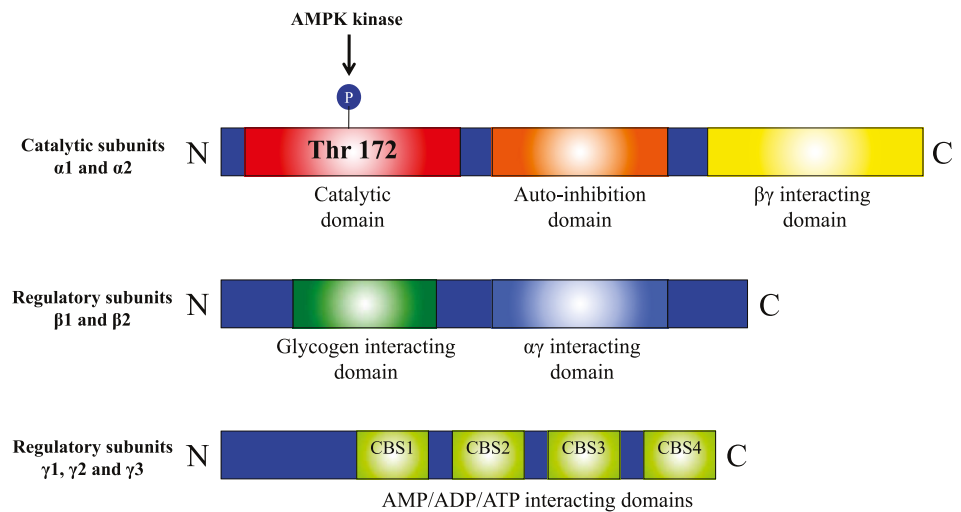
AMPK activity is modulated in an allosteric way by AMP that promotes the phosphorylation on Thr172 by the AMPK kinases (AMPKK), and by ATP that inhibits phosphorylation on this site (110). AMP and ATP competitively bind to AMPK  $\gamma$  subunits on four sites formed by cystathionine  $\beta$  synthase (CBS) domains (17). Moreover, binding of AMP to AMPK inhibits dephosphorylation of Thr172 by phosphatases, an effect that is antagonized by high concentrations of ATP (22). Interestingly, Xiao and colleagues (127) have recently determined the crystal structure of an active AMPK complex and have explored how the kinase region interacts with the regulatory nucleotide-binding site that mediates protection against dephosphorylation. They found that the binding of AMP or ADP to the regulatory domain protects AMPK dephosphorylation, although ADP does not lead to allosteric activation (127). Finally, it was shown that AMPK is inhibited by glycogen in an allosteric manner, leading to inhibition of Thr172 phosphorylation by upstream kinases (80). Thus, these data reveal that AMPK constitutes a sensor of the status of cellular glycogen reserves.

The phosphorylation of AMPK at Thr172 residue is regulated by three AMPKK identified to date. The serine threonine kinase LKB1, a tumor suppressor, and the Ca<sup>2+</sup>/calmodulin-dependent protein kinase  $\beta$  (CaMKK $\beta$ ) were the primary characterized upstream kinases of AMPK (51, 60, 113, 131,

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Fig. 1. Structure of AMP-activated protein kinase (AMPK). AMPK is composed of a catalytic subunit (AMPK- $\alpha$ ) and two regulatory subunits (AMPK- $\beta$  and AMPK- $\gamma$ ). AMPK- $\alpha$  contains a catalytic domain in the NH<sub>2</sub>-terminal that can be phosphorylated by the AMPKK enzymes on the residue Thr172, an autoinhibition domain and a domain interacting with the subunits  $\beta$  and  $\gamma$ . The regulatory  $\beta$  subunits have interacting domains with glycogen and subunits  $\alpha$  and  $\gamma$ . The regulatory  $\gamma$  subunits present four cystathionine  $\beta$ -synthase (CBS) domains interacting with AMP, ADP, and ATP.



132). More recently, the transforming growth factor  $\beta$ -activated kinase 1 (TAK-1) was also found to phosphorylate AMPK (52).

Analysis of AMPK substrates suggests a consensus recognition sequence in which the phosphorylated serine residue is close to a hydrophobic residue on the NH<sub>2</sub>-terminal side (i.e., at -1) and at least one arginine residue at -2, -3, or -4. Substrates for cyclic AMPK which lack the hydrophobic residue at -1 are not substrates for AMPK (13).

#### Glossary

2DG	2-deoxy-D-glucose
ACC	acetyl-CoA carboxylase
AICAR	5-aminoimidazole-4-carboxamide-1- $\beta$ -4-ribofuranoside
AMPK	adenosine monophosphate-activated protein kinase
AMPKK	AMPK kinases
Atg	autophagy-specific gene
atrogin-1/MAFbx	muscle atrophy F-box
CaMKK	Ca <sup>2+</sup> /calmodulin-dependent protein kinase kinase
CBS	cystathionine $\beta$ -synthase
ChREBP	carbohydrate response element-binding protein
CPT-1	carnitine palmitoyltransferase 1
CREB	cAMP response element-binding
CRT	creatine transporter
eEF2	eukaryotic elongation factor-2
eIF4E	eukaryotic translation initiation factor 4E
4E-BP1	eukaryotic translation initiation factor 4E-binding protein 1
FAT/CD36	fatty acid translocase/cluster of differentiation 36
FIP200	focal adhesion kinase family interacting protein of 200 kDa
FoxO	Forkhead box O
GLUT-4	glucose transporter-4
GS	glycogen synthase
HK-2	hexokinase-2

HMGR-CoA	hydroxymethyl-glutaryl-coenzyme A reductase
HSL	hormone-sensitive lipase
LC3	microtubule-associated protein light chain 3
LCFA CoA	long-chain fatty acyl-CoA
LKB1	liver kinase B1
mLST8	mammalian target of rapamycin-associated protein LST8 homolog
mTOR	mammalian target of rapamycin
mtTFA	mitochondrial transcription factor A
mTORC1	mTOR complex 1
MuRF1	muscle RING finger 1
NRFs	nuclear respiratory factors
PDK1	phosphoinositide-dependent kinase 1
PFK-2	6-phosphofructo-2-kinase
PGC-1	peroxisome proliferator-activated receptor- $\gamma$ coactivator 1
PPAR	peroxisome proliferator-activated receptor
PRAS40	proline-rich Akt substrate of 40 kDa
raptor	regulatory-associated protein of mTOR
Rheb	Ras homolog enriched in brain
rpS6	ribosomal protein S6
S6K1	ribosomal protein S6 kinase 1
SIRT1	sirtuin 1
SREBP1c	sterol regulatory element-binding protein 1c
TAK-1	transforming growth factor $\beta$ -activated kinase 1
TSC1/2	tuberous sclerosis complex 1/2
Ulk1	unc-51-like kinase 1

#### Role of AMPK in Metabolic Regulations: AMPK Regulates Anaerobic Metabolism, Fatty Acid Oxidation, and Cholesterol Synthesis

AMPK is a regulator of anaerobic metabolism allowing the insulin-independent transport of glucose and its subsequent metabolism in skeletal muscle and in the heart (4, 50, 99) (Fig. 2). Activation of AMPK induces the expression and the translocation of the glucose transporter-4 (GLUT-4) to the plasma membrane, resulting in an increase in glucose uptake and blood glucose oxidation (53). AMPK regulates glycolysis through

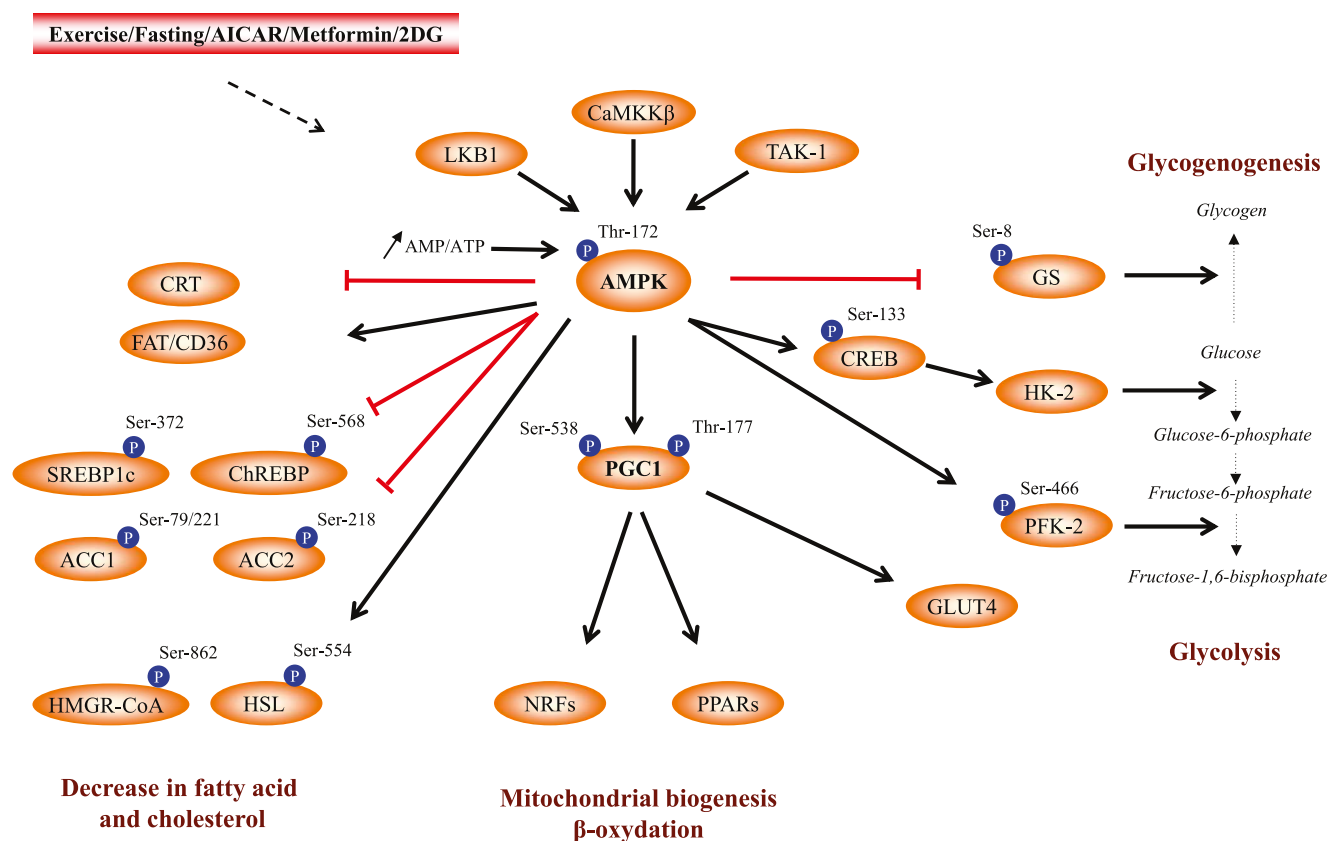


Fig. 2. AMPK regulates metabolic pathways in skeletal muscle. AMPK is directly activated by the enzymes LKB1, CaMKK $\beta$ , and TAK-1 upon high AMP levels, and indirectly by exercise, fasting, AICAR, metformin, or 2-deoxy-D-glucose (2DG) treatments. AMPK positively regulates glycolysis by phosphorylating 6-phosphofructo-2-kinase (PFK-2), by increasing hexokinase-2 (HK-2) transcription through cAMP response element-binding (CREB) phosphorylation, and by increasing the expression and translocation of the glucose transporters (GLUT4). AMPK inhibits the activities of the creatine transporter (CTR), glycogen synthase (GS), acetyl-CoA carboxylase (ACC), sterol regulatory element-binding protein 1c (SREBP1c), and carbohydrate response element-binding protein (ChREBP), and activates hormone-sensitive lipase (HSL) and hydroxymethyl-glutaryl-coenzyme A reductase (HMGR-CoA). AMPK increases fatty acid uptake through the translocation of fatty acid translocase (FAT)/CD36 to the plasma membrane. Activation of AMPK also leads to an increase of the transcriptional activity of the peroxisome proliferator-activated receptors and the nuclear respiratory factors (NRFs) via phosphorylation of PGC-1 $\alpha$ .

phosphorylation of 6-phosphofructo-2-kinase (PFK-2), a key enzyme responsible for fructose 2,6-bisphosphate synthesis, a rate-limiting step in glycolysis (79). A dominant-negative form of AMPK could prevent both the activation and the phosphorylation of PFK-2 by oligomycin (79), which confirms the major role of AMPK in the regulation of PFK-2 activity. Moreover, AMPK increase hexokinase-2 (HK-2) transcription, an enzyme responsible for glucose-6-phosphate synthesis, by phosphorylating cAMP response element-binding (CREB) at Ser133 (111, 116). Furthermore, AMPK phosphorylates and inactivates glycogen synthase (GS), inhibiting glycogenogenesis (1, 13). This action seems to preferentially occur through the regulation of AMPK  $\alpha$ 2 isoform (62). In addition to the regulation of glycolysis and glycogenolysis, Li and colleagues (75) have recently shown that AMPK inhibits apical membrane creatine transporter (CRT) expression in kidney proximal tubule cells. CTR inhibition by AMPK is important because unnecessary creatine reabsorption and cellular energy expenditure are decreased under conditions of metabolic stress.

AMPK also modulates fatty acid and cholesterol metabolism in specialized tissues, such as adipose tissue, liver, and muscle (Fig. 2). AMPK increases fatty acid uptake due to translocation of the fatty acid translocase (FAT)/CD36 transporter to the

cellular membrane (77). AMPK inhibits fatty acid and cholesterol synthesis through direct phosphorylation of the metabolic enzymes acetyl-CoA carboxylase 1 (ACC1), the hydroxymethyl-glutaryl-coenzyme A reductase (HMGR-CoA), and the hormone-sensitive lipase (HSL) (12, 14, 37). Furthermore, AMPK inhibits the expression and the activity of the transcription factors SREBP1c (sterol regulatory element-binding protein 1c) (33, 129) and ChREBP (carbohydrate response element-binding protein) (33, 68). The latter action represses the transcription of lipogenic genes and fatty acid synthesis (33). Furthermore, via phosphorylation and inhibition of ACC2, AMPK induces a drop in the production of malonyl-CoA, an allosteric inhibitor of carnitine palmitoyltransferase 1 (CPT-1) (83, 122, 123). CPT-1 controls the transfer of cytosolic long chain fatty acyl CoA (LCFA CoA) into mitochondria (81) and represents the rate-limiting step of fatty acid oxidation. Thus, AMPK activation involves a reduction in the cytosolic concentration of malonyl-CoA and facilitates the penetration and the oxidation of fatty acids in mitochondria.

The role of PPAR- $\gamma$  coactivator 1 $\alpha$  (PGC-1 $\alpha$ ) in adaptive responses after AMPK activation is now quite detailed (Fig. 2). PGC-1 is a master protein involved in the regulation of oxidative metabolism of brown fat and muscle that upregulates

mitochondrial respiration and biogenesis through an increase in the expression of enzymes implicated in the electron transport system and uncoupling proteins, and through regulation of the nuclear respiratory factors (NRFs) (94, 126). PGC-1 binds to and activates NRF-1 on the promoter of the mitochondrial transcription factor A (mtTFA), a direct regulator of mitochondrial DNA replication and transcription (126). In skeletal muscle, Canto and colleagues (11) have recently shown that inhibition of AMPK activity compromises the histone deacetylase sirtuin 1 (SIRT1)-dependent responses to exercise or fasting by decreasing cellular  $\text{NAD}^+$  levels. This results in impaired PGC-1 $\alpha$  deacetylation and blunted induction of mitochondrial gene expression (11, 60). The effects of AMPK on gene expression of GLUT-4, mitochondrial genes, and PGC-1 $\alpha$  itself are almost entirely dependent on the function of PGC-1 $\alpha$  protein (60). PGC-1 $\alpha$  gene expression is increased with exercise, AICAR, and metformin treatments (54, 112, 113). AMPK directly phosphorylates PGC-1 $\alpha$  at Ser538 and Thr177 and these phosphorylation events are required for induction of the PGC-1 $\alpha$  promoter (60).

#### *Role of AMPK in the Control of Skeletal Muscle Mass*

The maintenance of muscle mass is controlled by a fine balance between catabolic and anabolic processes, which determine the level of muscle proteins and the diameter of muscle fibers. Skeletal muscle hypertrophy can be defined as an overall augmentation in muscle mass, as a result of an increase in the size of preexisting skeletal muscle fibers accompanied by enhanced protein synthesis without an apparent increase in the number of myofibers (39). Muscle hypertrophy is associated with a strong rate of protein synthesis more than with a change in protein degradation. This physiological process stimulates muscle growth in response to mechanical loading or nutritional stimuli (39). On the contrary, skeletal muscle atrophy can be defined as a decrease in muscle fiber diameter, protein content, force production, and fatigue resistance (34, 59). This process results from a plethora of causes including immobilization, denervation, aging, and neuromuscular diseases. Moreover, muscle atrophy can be secondary to some devastating pathologies or health problems, such as spinal cord injury (16), cancer cachexia, sepsis, diabetes, or AIDS, and exacerbated by microgravity, glucocorticoid treatment, and starvation (31, 73, 84).

When the cellular energy level is low, AMPK promotes ATP production by switching on catabolic pathways and conserves ATP levels by switching off ATP-consuming processes, including most biosynthetic pathways. In muscle, this action occurs when AMPK is activated because of energy deprivation, and it results in the arrest of protein synthesis and cell growth, with the stimulation of muscle proteolysis. Thus, AMPK, as the main energy sensor in muscle cells, modulates muscle turnover and skeletal muscle mass.

*AMPK and inhibition of protein synthesis.* Several data suggest that AMPK acts as a negative regulator of protein synthesis by reducing both the initiation and the elongation of ribosomal peptide synthesis. Thus, it was shown that AMPK activation leads to the Thr56 phosphorylation of the eukaryotic elongation factor-2 (eEF2) (55). Upstream of this phosphorylation, activation of AMPK inhibits the mammalian target of rapamycin complex 1 (mTORC1), a multiprotein complex composed of mTOR (also known as FRAP, RAFT1, or RAPT),

the regulatory associated protein of mTOR (raptor), the proline-rich Akt substrate of 40 kDa (PRAS40), and the mTOR-associated protein LST8 homolog (mLST8) (69, 70, 119). This protein complex controls skeletal muscle hypertrophy (6, 97) through modulation of protein synthesis by phosphorylation toward its downstream effectors, the ribosomal protein S6 kinase 1 (S6K1) (2) and the eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) (38). Its inhibition by AMPK results in decreased protein synthesis in both in vivo and in vitro models (7, 93). Phosphorylation of 4E-BP1 at Thr37/46 by mTOR promotes its dissociation from eIF4E bound to the mRNA 7-methylguanosine cap structure, allowing the assembly of the preinitiation complex (128). S6K1 activation needs initial phosphorylation by mTORC1 at Thr389 (101) and additional inputs on Thr229 for full activation by the phosphoinositide-dependent kinase 1 (PDK1) (95). S6K1-mediated regulation of translation is thought to occur in part through phosphorylation of the 40S ribosomal protein S6 (rpS6) at Ser235/236 (30). Several studies highlighted that AMPK activation by resistance exercise (27) or high-frequency electrical stimulation (114) leads to the inhibition of S6K1 and 4E-BP1. In vitro, pretreatment with AICAR completely inhibited the insulin-induced activation of mTORC1 and its downstream effectors (25). Two models have been proposed for the AMPK-mediated inhibition of mTORC1 activity (Fig. 3). The first involves the phosphorylation of the tuberous sclerosis complex 2 (TSC2) on residues Thr1227 and Ser1345, thus enhancing its GTPase activity towards the Ras homolog enriched in brain (Rheb), the direct activator of mTOR (58). The second model implicates the direct phosphorylation of raptor at Ser722/792, leading to the cytoplasmic retention of raptor by 14-3-3 binding (45).

Lantier and colleagues (72) reported that *AMPK*<sup>-/-</sup> mice exhibited a shift of muscular fiber size distribution toward higher values correlated with increased phosphorylation of S6K1 Thr389 and rpS6 Ser235/236 associated with a stimulation of protein synthesis. In this study, the size of AMPK-deficient myotubes was 1.5-fold higher than for controls and soleus mass was significantly higher by 42% in muscle AMPK-deficient mice compared with control mice. Another recent study performed by the same group demonstrates that AMPK-deficient mice exhibit enhanced hypertrophy after mechanical overloading, a phenomenon associated with increased activity of the mTORC1 signaling (87). Moreover, the overloading-induced hypertrophy was associated with a significant increase in AMPK  $\alpha$ 1 expression and activity after 7 and 21 days in non-transgenic muscle, probably to limit muscle mass growth (82). In the absence of AMPK  $\alpha$ 1, unphosphorylated eEF2 levels are higher in response to chronic overload with a parallel increase in mTORC1 signaling leading to greater muscle hypertrophy (87). Thus, these data show that AMPK  $\alpha$ 1 behaves as a negative effector required to limit mTORC1 activity and to inhibit overgrowth of skeletal muscle in response to hypertrophic stimuli.

In an aged rat model, Thomson and Gordon (115) showed that activation of AMPK is linked to the diminished overload-induced hypertrophy in fast-twitch skeletal muscle. An age-related increase in AMPK phosphorylation may partly contribute to the attenuated hypertrophic response observed in overloaded fast-twitch plantaris muscle. Furthermore, AMPK is upregulated with age in resting and overloaded fast-twitch skeletal

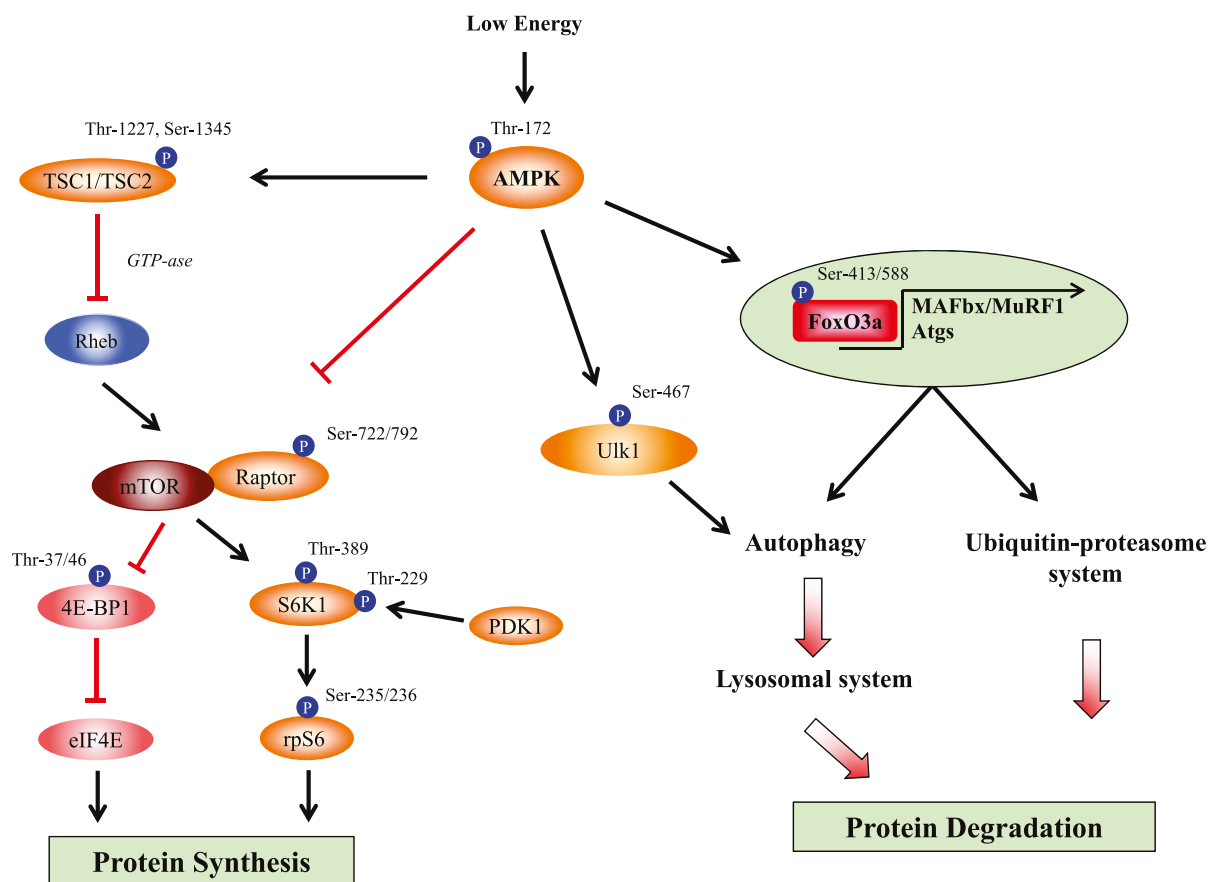


Fig. 3. AMPK regulates both anabolic and catabolic pathways in skeletal muscle. AMPK decreases protein synthesis by phosphorylating the tuberous sclerosis complex 1/2 (TSC1/2), an inhibitor of the mammalian target of rapamycin (mTOR) activator Ras homolog enriched in brain (Rheb), and by phosphorylating the regulatory associated protein of mTOR (raptor). mTOR modulates protein synthesis by phosphorylation toward its downstream effectors, the ribosomal protein S6 kinase 1 (S6K1) and the eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1). S6K1 activation needs initial phosphorylation by mTORC1 and additional inputs for full activation by the phosphoinositide-dependent kinase 1 (PDK1). S6K1-mediated regulation of translation occurs in part through phosphorylation of the 40S ribosomal protein S6 (rpS6). Phosphorylation of 4E-BP1 by mTOR promotes its dissociation from eukaryotic translation initiation factor 4E (eIF4E) and allows the assembly of the preinitiation complex. Activation of AMPK leads to an increase in Forkhead box O3a (FoxO3a) activity that induces the expression of the muscle atrophy F-box (MAFbx), the muscle RING finger 1 (MuRF1), and autophagy genes (Atgs). AMPK also interacts with, and phosphorylates, the unc-51-like kinase 1 (Ulk1) leading to autophagy induction.

muscle but not in slow-twitch muscle. Thus, the modulation of AMPK activity by pharmacological agents could constitute a relevant therapeutic tool to prevent sarcopenia.

Taken together, AMPK appears to be an essential contributor to the control of muscle cell size and adaptation to muscle hypertrophy. AMPK is involved in the cell size maintenance through the regulation of mTORC1 pathway and appears to play a major role in the metabolic program that controls muscle plasticity. The severe activation of AMPK in the overload model could contribute to restoring metabolic homeostasis that has been disturbed by the increased protein synthesis. Thus, AMPK seems to act as a homeostatic factor and to determine the extent to which protein translation is allowed under various energetic circumstances. Additionally, by limiting muscular hypertrophy, AMPK would act as negative feedback in the control of skeletal muscle mass.

**AMPK and stimulation of protein degradation.** In addition to the inhibitory effect of AMPK on protein synthesis, this enzyme has recently been associated with increased myofibrillar degradation in muscle cells. Nystrom and coworkers showed that AICAR and metformin treatments decreased protein syn-

thesis and increased protein degradation in an AMPK-dependent manner in C2C12 myotubes (91).

**AMPK regulates FoxO3a-dependent protein degradation.** Nakashima and Yakabe (88) reported that AMPK activation stimulates myofibrillar protein degradation through increased expression of FoxO transcription factors. Protein degradation is mediated by two conserved pathways: the ATP-dependent ubiquitin-proteasome system and the autophagy-lysosomal pathway. The first one implicates a cascade of enzymatic reactions that labels substrate proteins with ubiquitin chains for degradation by the 26S proteasome. This system involves the activity of an E1 (ubiquitin-activating enzyme), an E2 (ubiquitin-conjugating enzyme), and an E3 ubiquitin ligase, which confers substrate specificity for ubiquitination. Two major E3 ligases have been described to be essential for muscle atrophy, atrogin-1/MAFbx (muscle atrophy F-box) and MuRF1 (muscle RING finger 1) (5, 32, 40). The second pathway implicates lysosomes and represents an important mechanism in the maintenance of protein turnover and cellular metabolism. This process requires Atg (autophagy-specific gene) proteins, which are necessary for the formation of autophagosomes (19). The

formation of these vesicles is required to drive substrates to lysosomes to achieve substrate degradation. mRNAs encoding Atg proteins are very abundant in skeletal muscle (86). This process is constitutively active in skeletal muscle and is enhanced in human myopathies caused by a genetic deficiency of lysosomal proteins, as evidenced by the accumulation of autophagosomes in Pompe's and Danon's diseases (89, 100, 121). Importantly, autophagy sequestration under starvation conditions requires the conjugation of LC3 (microtubule-associated protein light chain 3) with the phospholipids of the vacuolar membrane (65, 66).

Expression of a constitutively activated FoxO3a increases the transcription of many autophagy-related genes, including *LC3B*, *Gabrap11*, *Beclin1*, *PI3KIII*, *Ulk2*, *Atg4b*, and *Atg12l* (78). FoxO proteins are an evolutionarily conserved subfamily of transcription factors involved in tumor suppression, regulation of protein degradation, and development in several tissues, and they are regulated by phosphorylation-dependent nuclear/cytoplasmic shuttling (9, 10). Besides its role in regulating the Atg, FoxO3, as FoxO1, positively controls the transcription of the E3 ligases MAFbx and MuRF1 (67, 107) and upregulation of MAFbx and MuRF1 expression leads to muscle atrophy. Akt phosphorylates FoxO1, FoxO3, and FoxO4 (Thr32, Ser253, and Ser315 in the FoxO3a sequence), leading to its inhibition by cytosolic retention via 14-3-3 (8).

The activation of FoxO3a by AMPK in skeletal muscle and its implication in regulating both autophagy and the ubiquitin-proteasome pathways are illustrated in Fig. 3. It was shown that AMPK activation by AICAR increases the mRNA and the protein content of MAFbx and MuRF1 in C2C12 and primary myotubes (88, 104). However, to the best of our knowledge, no supplementary data exist regarding the regulation of E1, E2, and proteasome subunits by AMPK in skeletal muscle.

Recently, we have described that activation of AMPK induces autophagy pathway in C2C12 cells and primary myotubes (104). The activation of FoxO3a by AMPK leads to an increase in the expression of Beclin, LC3-II and Gabrap11, which are necessary for the promotion of autophagosome formation. AMPK phosphorylates FoxO3a at Ser413/588, residues known to lead to FoxO3a activation and protein degradation (41, 47). Nevertheless, no variations in nuclear content of FoxO3a were detected following AMPK activation after long treatments (i.e., 24 h), but an increase in the total protein level from 30 min was found. After a short time course (30 min–6 h) the activation of AMPK by AICAR induces accumulation of FoxO3a in the nucleus, consistent with the results of Tong and colleagues (118), who reported that AICAR treatment caused FoxO3a nuclear relocation correlated to a decrease in FoxO3a phosphorylation at Thr318/321. However, Greer and colleagues (41) have reported an increase of FoxO3a transcriptional activity without any change in the nuclear content of the factor after AMPK activation by 2-deoxyglucose in HEK293T cells. Nevertheless, these apparent divergent data strongly suggest that FoxO3a relocation into the nucleus after AMPK activation is not necessarily required to increase its transcriptional activity. As demonstrated by Davila et al. in neurons, it can be suggested that AMPK activates FoxO3a directly into the nucleus (23) and may be implicated in the stability of the protein.

*AMPK initiates autophagy by regulating Ulk1 complex.* Another major signaling pathway has been identified in

AMPK-induced skeletal muscle autophagy. It concerns the regulation of Ulk1 complex by AMPK (Fig. 4). This complex is composed of AMPK, mTORC1, Ulk1, FIP200, and Atg13 and has been identified in muscle cells. Ulk1, the homologue of yeast Atg1, is a serine/threonine-protein kinase that plays a key role in the initial stages of autophagy induction, particularly the nucleation and formation of the pre-autophagosome structures (36, 85, 90). Data obtained in skeletal muscle cells are consistent with the model described in HeLa, HEK293T, and mouse embryonic fibroblast cells and show that, under basal conditions, mTORC1 interacts with Ulk1 and prevents autophagy in opposition to energy stress conditions (56, 63, 64). Under nutrient-rich conditions, phosphorylation of Ulk1 by mTORC1 decreases Ulk1 kinase activity and its ability to interact with cofactors Atg13 and FIP200, a necessary interaction in coordinating the autophagic response (36, 46). It was reported in muscle cells that activation of AMPK by AICAR or inhibition of mTORC1 with either Torin1 or amino acid starvation leads to the dissociation of AMPK and mTOR/raptor from the Ulk1 complex (104). This process is thought to result in initiation of the Ulk1-dependent phosphorylation of Atg13 and FIP200 leading to the activation of autophagy (63).

A proteomic analysis of the autophagy system and a coimmunoprecipitation study performed in HEK293T cells have shown that AMPK interacts with Ulk1 and Ulk2 (3, 74). In muscle cells, Ulk1 was identified as a new interacting partner of AMPK that induces Ser467 phosphorylation (104). The phosphorylation of Ulk1 could lead to a conformational change and thereby disrupt the Ulk1-mTORC1 interaction, in line with the suppression of mTORC1 activity in the Ulk1 complex (56, 57, 64). Moreover, Ulk1 phosphorylation by AMPK may directly upregulate Ulk1 kinase activity. Indeed, it was shown in vitro that purified Ulk1 can phosphorylate itself and requires autophosphorylation for stability (26). In mammals, reconstitution of Ulk1-deficient cells with a mutant Ulk1 that cannot be phosphorylated by AMPK revealed that such phosphorylation is required for mitochondrial homeostasis and cell survival following starvation (28, 29). A lack of association between AMPK and Ulk1 resulted in an accumulation of abnormal mitochondria and cell death. Finally, AMPK controls Ulk1 activity by suppressing mTOR activity and by interacting and phosphorylating Ulk1.

Coimmunoprecipitation time course studies demonstrate that, following autophagy induction, AMPK dissociates from Ulk1 after 3 h of AICAR treatment (47). Shang and colleagues (108) reported that AMPK is associated with Ulk1 only under nutrient-rich conditions and dissociated from Ulk1 5 min after starvation in HeLa cells. Thus, altogether these data suggest that Ulk1 is associated with AMPK in normal conditions. However, upon AICAR-induced autophagy, the complex remains stable for 3 h and starts to dissociate later. It is conceivable, as suggested by Shang and colleagues (108), that Ulk1 dissociates from AMPK and thus becomes more active. Conversely, it is also possible that AMPK-Ulk1 dissociation is responsible for a negative regulatory feedback loop as described by Loffler and colleagues (76), who showed that Ulk1 could mediate phosphorylation of AMPK on its regulatory subunits. Additional experiments are necessary to define the molecular mechanisms for these events in skeletal muscle. We only conclude that AMPK takes part in the initiation of

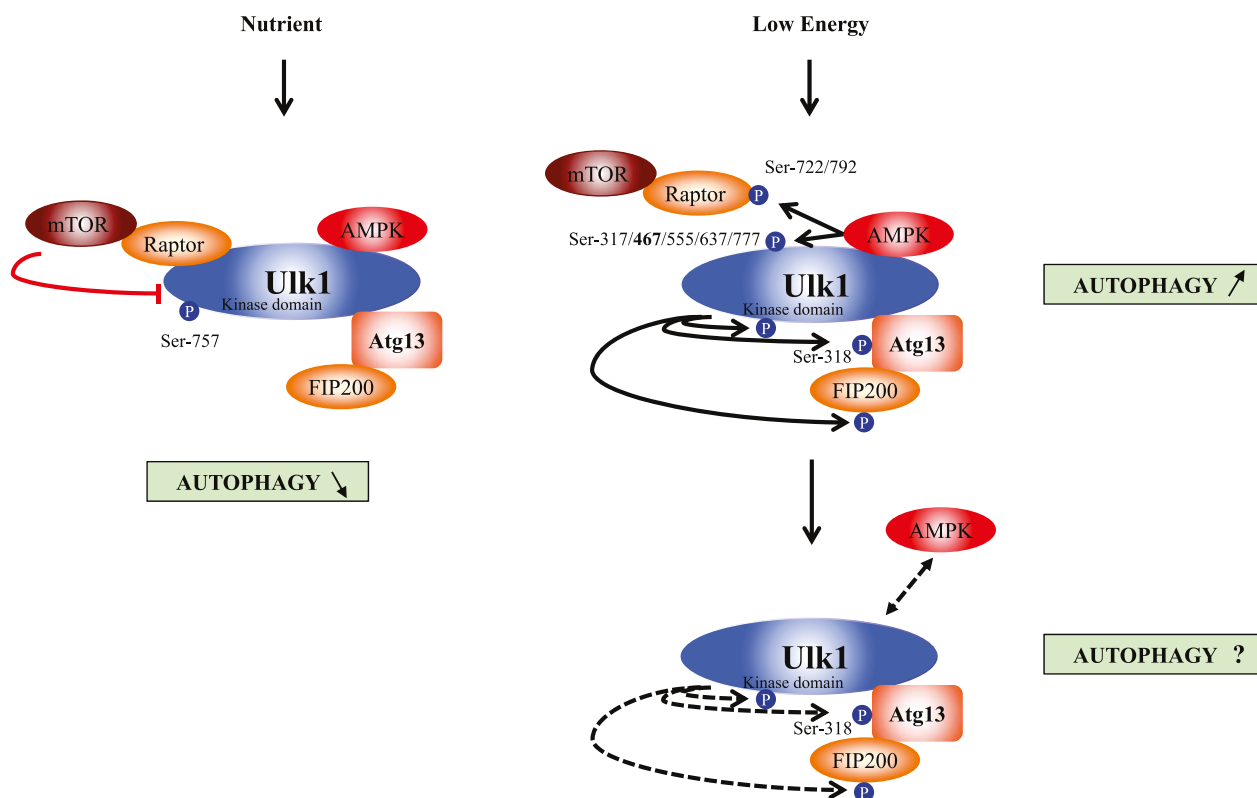


Fig. 4. Regulation of the unc-51-like kinase 1 (Ulk1) complex by AMPK in skeletal muscle. In basal condition, mTOR/raptor (mammalian target of rapamycin/regulatory associated protein of mTOR) interacts with, and phosphorylates, Ulk1 to inhibit its kinase activity and its ability to phosphorylate the autophagy protein 13 (Atg13) and the focal adhesion kinase family interacting protein of 200 kDa (FIP200). In this complex, AMPK also interacts with Ulk1. During cellular stress, AMPK phosphorylates raptor, leading to its dissociation from Ulk1, and phosphorylates Ulk1. Consequently, Ulk1 is activated and phosphorylates itself and its interacting partners Atg13 and FIP200. During autophagy process, AMPK dissociates from Ulk1 and two hypotheses can be formulated: Ulk1 dissociates from AMPK and thus may become more active, or, conversely, AMPK-Ulk1 dissociation may be responsible for a negative regulatory feedback loop. Further exploration will be necessary to better characterize these events. Note that Ulk1 phosphorylation at Ser467 has been identified in myoblasts while the other sites of Ulk1 and Atg13 site have been identified in other cell lines.

autophagosome formation by interacting with, and phosphorylating, Ulk1.

**Physiological relevance.** The physiological relevance of these findings is emerging. Sandri's group has provided evidence of the existence of an amplifying loop of mitochondrial fission in atrophying muscles. Romanello and coworkers (96) have shown that mitochondrial-dependent muscle atrophy requires AMPK activation and inhibition of AMPK restores muscle size in myofibers with altered mitochondria in a FoxO3a-dependent manner. Interestingly, data related to the role of autophagy during exercise are accumulating; thus Grumati and colleagues (44) have shown that autophagy is stimulated during endurance exercise. These authors found that exercise induced mitochondria and myofibrillar degeneration in type VI collagen knockout mice. This deleterious effect was explained by autophagy failure in this model (42–44). He and coworkers have reported that autophagy is required for beneficial metabolic effects of exercise in skeletal and cardiac muscle of fed mice (51). Indeed, BCL2 AAA mice, which have mutations in BCL2 phosphorylation sites preventing stimulus-induced disruption of the BCL2-beclin-1 complex and autophagy activation, presented a decreasing endurance and altered glucose metabolism during acute exercise. The authors also showed chronic exercise-mediated protection against high-fat-diet-induced glucose intolerance. These studies sup-

port a new and essential role of muscular autophagy in the adaptation to exercise. However, excessive autophagy can also lead to atrophy and muscular diseases (105, 106). Lastly, in humans, Jamart and coworkers (61) have found that AMPK and FoxO3a regulate autophagy and ubiquitin proteasome-mediated proteolysis in a coordinated way during ultra-endurance exercise. Thus, AMPK promotes availability of internal energy sources and enhances cellular survival under conditions of energy stress.

#### Summary and Conclusion

In skeletal muscle, AMPK appears to be a master regulator of metabolism; it contributes to decreasing protein synthesis through inhibition of mTORC1 activity, and it plays a role in protein turnover through increased activity of the ubiquitin-proteasome and autophagy-lysosomal pathways. Accumulating data suggest that modulation of activity of AMPK substrates will constitute good candidates for muscle wasting therapy. In Pompe's disease, like in many myopathies, autophagy levels are modified and contractile proteins can be degraded. In this context, it appears important to understand the precise mechanisms that regulate autophagy to develop and improve therapeutic strategies against muscular dystrophies. Among the signaling pathways that control proteolysis and especially

autophagy, AMPK may play a key role that should be further addressed. In addition, attempts to evaluate the effects of physical exercise should be considered. AMPK has become a target for the development of new drugs for the treatment of type II diabetes, obesity, and even cancer, and we can assume that in the near future it will become a target for fighting myopathies.

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#### DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

#### AUTHOR CONTRIBUTIONS

A.M.J.S. and H.B. conception and design of the research; A.M.J.S. prepared the figures; A.M.J.S. and H.B. drafted the manuscript; A.M.J.S., R.B.C., A.C., A.F.P., A.R., and H.B. approved the final version of the manuscript; R.B.C., A.C., and A.R. edited and revised the manuscript.

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## RESUME / ABSTRACT

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### **Déconditionnement et régénération du muscle strié squelettique : rôle du niveau d'activité contractile sur le développement d'infiltrations graisseuses**

Le muscle strié squelettique est un tissu fascinant qui permet d'assurer les fonctions essentielles à notre existence : se mouvoir, maintenir sa posture, se nourrir, communiquer ou tout simplement respirer. De nombreuses situations, engendrant principalement une hypoactivité, peuvent provoquer un déconditionnement musculaire caractérisé par une perte de masse et de force ainsi qu'un développement d'infiltrations graisseuses (IMAT), altérant ainsi la fonction musculaire. Le développement d'IMAT est également observé lorsque les processus de régénération musculaire sont altérés. Les fibro-adipogéniques (FAPs) représentent la population de cellules souches principalement impliquée dans le développement d'IMAT. L'interaction entre FAPs et cellules satellites/immunitaires semble être un trio indispensable pour une régénération optimale, sans développement d'IMAT. Au regard de la littérature scientifique, une modulation du niveau d'activité contractile permet de faire varier le niveau d'expression de nombreuses cytokines impliquées dans la modulation des FAPs et donc dans l'apparition d'IMAT. Nos travaux ont contribué à l'accroissement des connaissances scientifiques relatives à la thématique des infiltrations graisseuses et à leurs exacerbations dans des situations d'hypoactivité ou de régénération musculaire. Nous avons montré que 3 jours d'hypoactivité chez l'homme, induite par le modèle novateur de dry immersion, suffisent à augmenter le contenu musculaire en IMAT. Dans un contexte de régénération musculaire, induite par le modèle glycérol chez la souris, nous avons démontré une inhibition de l'apparition des IMAT en diminuant les contraintes mécaniques appliquées au muscle lésé. Nous avons également précisé le rôle de l'axe  $TNF\alpha/TGF-\beta 1$ , et donc celui des processus inflammatoires nécessaires dans l'apoptose des FAPs afin de limiter le développement des IMAT dans ce modèle. Ces trois études ouvrent de nombreuses perspectives, afin i) de préciser le rôle des IMAT dans la dysfonction musculaire, ii) de définir les mécanismes de régulation qui contrôlent le développement et l'accumulation d'IMAT.

Mots-clés : Plasticité musculaire, hypoactivité, régénération, infiltrations graisseuses, activité musculaire, fibro-adipogéniques (FAPs)

### **Skeletal muscle deconditioning and regeneration: effects of the contractile activity degree on fat infiltration development**

Skeletal muscle is a fascinating tissue that ensures core functions: moving, maintaining postures, feeding, communicating or just breathing. Many situations, associated with hypoactivity, are able to involve muscle deconditioning defined by a loss of mass and strength, as well as fat infiltration development (IMAT), altogether impairing muscle function. IMAT development occurs also with disrupted regeneration processes. Fibro-adipogenic progenitors (FAPs) appear as the main stem cell population involved in IMAT development. The interaction between FAPs and satellite/immune cells seems to be a crucial trio for an efficient regeneration, without IMAT development. According to the literature, the degree of contractile activity is able to affect the expression levels of different cytokines involved in FAPs fate, and therefore in IMAT accumulation. Our work contributed to increase scientific knowledge on muscle fatty infiltrations and their exacerbations in hypoactivity or regeneration situations. We showed that 3 days of hypoactivity in human, induced by the innovative model of dry immersion, are sufficient to promote an increase in IMAT content. In a context of muscle regeneration, induced by the mouse glycerol model, we highlighted an almost complete inhibition of IMAT accumulation by decreasing mechanical constraints applied to the injured muscle. We also investigated the role of the  $TNF\alpha/TGF-\beta 1$  axis, and therefore the potential role of the inflammatory stage in FAPs apoptosis and inhibition of IMAT development. Our work open up new prospects 1°) clarifying the role of IMAT in muscle dysfunction, and 2°) defining the underlying mechanisms controlling IMAT development and accumulation.

Key words: hypoactivity, muscle regeneration, fat infiltrations, muscle activity, stem cells, fibro-adipogenic progenitors (FAPs)