The skeletal muscle stem cell niche : defining hierarchies based upon the stem cell marker PW1 to identify therapeutic target cells

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THÈSE DE DOCTORAT DE L'UNIVERSITÉ PIERRE ET MARIE CURIE (PARIS VI)
Spécialité : Complexité du Vivant

Présentée par
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Pour obtenir le grade de Docteur de l'Université Pierre et Marie Curie

THE SKELETAL MUSCLE STEM CELL NICHE: DEFINING HIERARCHIES BASED UPON THE STEM CELL MARKER PW1 TO IDENTIFY THERAPEUTIC TARGET CELLS

Soutenue le 28 Septembre 2012

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ACKNOWLEDGMENTS

First I want to thank David SASSOON, my PhD supervisor, for guiding me and supporting me during all these years. Thank you for helping me to interpret my results, teaching me to write as a scientist, pushing me to interact with other people and allowing me to participate to high level international meetings; in other words thank you for showing me what research is. I particularly appreciate that you always managed to spare some time for me when I requested your help and that you supported me in good and hard times (concerning the hard times, congratulations for not killing me!!). I am also very grateful to you for giving me many times the opportunity to present my results and to interact with the best researchers of our field.
David, you trusted me and treated me as a colleague from the beginning and this really touched me.

I also want to thank Giovanna MARAZZI for helping me, supporting me, and teaching me all about immunofluorescence. Thank you for cheering me up when I was depressed, and thank you for being my "personal agent". I appreciate that you were always keen on correcting and improving my documents.
Giovanna, in other words, grazie di rompermi sempre le scatole!!

I particularly want to thank Pura MUNOZ and Emilio CLEMENTI for accepting to take the time to read and judge this manuscript. I am honored and very pleased to have you in my jury.

Thank you to Margaret BUCKINGHAM, Pascal MAIRE and Thierry JAFFREDO for being part of my jury but also for having followed my work during several years as my PhD Committee. Thank you for your time, support, and sharp advice.

J’aimerais maintenant remercier toutes les personnes qui m’ont aidée dans mon travail, que ce soit au niveau technique, relationnel ou moral.

Kathryn, merci de m’avoir gentiment et patiemment appris toutes les techniques de base durant mon Master 2. Grâce à toi j’ai appris à être organisée et à ne pas foncer tête baissée dans une manip !

Luigi, sans toi rien n’aurait été possible… Je ne saurai jamais te remercier assez pour les innombrables heures de FACS, culture et microscope qui ont abouti à ces jolis résultats. Merci pour ta patience et ton soutien à toute épreuve. Je suis fière de te compter parmi mes amis et n’oublie jamais : WE LOVE TAPAS !!

Nath, merci pour tes conseils avisés à n’importe quelle heure du jour et de la nuit, tu m’as appris à avoir l’œil critique sur mes résultats. Merci aussi de m’avoir tout appris du western blot, et surtout un grand merci pour ta re-lecture de mon introduction.

Vanessa et Keren, merci pour vos conseils au quotidien, votre esprit critique, vos encouragements, et votre soutien à la fois scientifique et psychologique. C’est vous qui m’avez remonté le moral quand j’avais l’impression de ne pas y arriver, et je dois dire que je suis fière d’avoir votre estime.

Piera, ta bonne humeur et ta convivialité ont illuminé notre U ! Toujours prête à rendre service, tu as le cœur sur la main et c’est une qualité rare. Mais j’ai aussi appris beaucoup de toi grâce à tes vastes connaissances scientifiques, alors merci pour toutes ces petites discussions enrichissantes entre deux manips.

Ludo, un grand merci pour tes leçons d’anatomie murine et ton aide pour les chirurgies. Je te dois tout mon savoir-faire d’expérimentation animale.

Sandrine, je crois que sans toi j'aurais brûlé tous mes western blot !! Un grand merci à toi qui a régulièrement spontanément pris du temps pour m'aider.

Bernie, à nos pétages de plombs de 20h30 accompagnés de rigolades à refaire le monde… et à ce mémorable « corde à sauter » de la retraite de l'unité !

Edgar, se eu fosse de novo uma pequena estudante de mestrado, viria para a tua equipa sem hesita ! Thank you for having always been so keen to me, especially when I was still a poor little Master student without a computer. Your advice and judgment are so valuable to me.

Jess, Val et Claire, vous resterez toujours mes copines de thèse. Etre embarquées dans la même galère, ça crée des liens ! Jess, merci pour avoir refait ma discographie des années 80, 90, 2000 et 2010, pour les craquinettes, les petits potins, nos gros craquages, bref tu es la meilleure face à face de bureau. Tu m'as également remonté le moral dans un moment difficile, pour tout cela merci. Val, entre les épopées à travers Paris à chaque nouvelle inscription, les bavardages pendant les temps d'incubation, quelques soirées mémorables et beaucoup de potins, je me suis bien amusée avec toi ! Claire, pour les soirées au bar ou au confocal… bref, les filles, courage vous y êtes presque !

Et bien sûr tous ceux que je n'ai pas encore cités, Bruno, Anne, Sonia, Revital, Ara, Vincent, Daniel, Max, Erwan, Charline, Frédéric, Shin, Sestina, Laurianne, Thibaud, Maria-Grazia, Aurore, P-O, Muriel, Alessia, Cédric, et pardonnez-moi si j'oublie quelqu'un. Merci d'avoir été là, pour nos rires, nos délires, nos goûters-apéros interminables dans la cuisine.

J'aimerais également remercier tout le personnel du laboratoire, ceux qui rendent notre vie plus facile sans même que l'on ne s'en rende compte.

Bénédicte HOAREAU et Catherine BLANC pour la cytométrie de flux. Merci pour votre aide et vos conseils techniques. Bénédicte, tu es tout simplement adorable et grâce à toi mes figures de profils FACS sont magnifiques.

Lidia, Isolina et Abdou pour la laverie. Merci pour votre bonne humeur et votre disponibilité. Vous êtes tous les 3 de sacrés phénomènes. Entre les chansons et les petites boutades, on ne s'ennuie jamais avec vous ! Surtout continuez comme ça, ces petites bulles de bonne humeur ont rendu mes journées tellement plus agréables.

Serban MOROSAN, Oliver BRÉGERIE, Bocar KANE, Benoit, Tiffany et Kim pour l'animalerie. Merci pour votre gentillesse et votre dévouement, qui m'ont permis de réaliser mes expérimentations animales dans les meilleures conditions. J'ai conscience de vous avoir souvent sollicités, et j'ai beaucoup apprécié votre professionnalisme. Un merci spécial à toi Serban pour ce dimanche après-midi où tu es venu jusqu'au labo pour m'ouvrir l'animalerie. Quand à toi Benoit, j'ai rarement vu quelqu'un d'aussi dévoué à son travail. Je te souhaite de réussir tes études et d'enfin obtenir le job que tu désires et mérites.

Enfin je voudrais remercier ceux qui ont toujours été là pour moi, famille et amis, sans qui je ne serais jamais arrivée jusqu'ici et à qui je dois tellement.

Les kikis ! Mes chers kikis ! Joana, Maëva, Anne-So, Anne-Lyse et Luigi, je vous adore. Tellement de bons souvenirs ont marqués ces quatre ans que je ne sais par où commencer… On a voyagé ensemble à la campaaaagne, en Bretagne, en Italie, à Brétigny et même à l'institut « coquin ». On a rencontré des aigles et des cachalots, on s'est déguisés n'importe comment, on a fait des quiches, de la tyrolienne, du vélib', on a vidé toutes les sauces soja de Paris, on a dansé, on s'est mis de la biafine (ou pas), on a mangé des pastas et des pizzas de la Mamma mais aussi des desserts offerts par « la famille », on a refait la culture française, on a chanté les Disney, etc… Merci pour tout, vous êtes géniaux !!!

Ma choupette, pour tous ces bons moments à la Belette et au Frog mais aussi au labo, et nos soirées pyjama à papoter de tout et de rien. Si ma thèse est belle c'est en partie grâce à toi, j'ai eu un très bon modèle.

Lucie, je n'ai qu'une chose à dire : La Bretagne, ça vous gagne !

Charles et Rodolphe, vous avez été l'oreille attentive des moments difficiles. Merci de m'avoir supporté, écouté, et remonté le moral.

Juju, ça fait 10 ans qu'on se connait et je vais enfin terminer mes études ! Merci pour ta bonne humeur, tes petites blagues, les (très) bons restos, merci d'être là tout simplement.

Bibou, je ne sais pas dans quel état psychologique j'aurais fini si tu n'avais pas été là. Merci pour ton soutien, ta patience, ton écoute, mais surtout tes attentions, nos délires, ta tendresse et notre complicité qui illuminent ma vie.

Et enfin, ma chère famille. Merci d'être toujours là pour moi, pour votre compréhension, vos attentions, votre soutien inconditionnel. Merci aussi pour votre intérêt pour mon travail. On pourra toujours compter les uns sur les autres, et c'est ça le plus important.

SUMMARY

ABBREVIATIONS

AAV: adeno-associated virus

ActR2a: activin receptor type IIa

AP: alkaline phosphatase

BMPs: bone morphogenic proteins

Cdk: cyclin-dependent kinases

Dapi: 4',6-diamidino-2-phénylindole

DKK1: dickkopf-related protein 1

E11: 11^{th} day of embryonic development

EDL: extensor digitorum longus

eIF3-f: eukaryotic initiation factor 3 subunit 5

FACS: fluorescence activated cell sorting

FAPs: fibro/adipogenic progenitors

FGF: fibroblast growth factor

FLRG: follistatin-related gene

FSH: follicle-stimulating hormone

GASP-1: growth and differentiation factor-associated serum protein-1

GDF-8: growth and differentiation factor 8

HGF: hepatocyte growth factor

HMGB1: high-mobility group protein B1

IGF: insulin-like growth factor

IGFR: IGF-1 receptor

Mabs: mesoangioblasts

MAFbx: Muscle Atrophy F-box

MAPK: mitogen-activated protein kinase

MGF: mechano-growth factor

MHC: myosin heavy chain

MMP9: matrix metallopeptidase 9

MRF: myogenic regulatory factors

Mst$^{-/-}$: myostatin-null

mTOR: mammalian target of rapamycin

MuRF1: muscle ring finger 1

Myf5: myogenic factor-5

PCR: polymerase chain reaction

PDGFRα: platelet-derived growth factor receptor alpha

PI3K: phosphatidylinositol-3 kinase

PICs: PW1+ interstitial cells

PW1/Peg3: paternally expressed gene 3

Rb: retinoblastoma protein

Sca1: stem cell antigen 1

Shh: sonic hedgehog

Sma: smooth muscle actin

SP: side population

TA: tibialis anterior

TAK1: TGFβ-activated kinase 1

Tcf4 : transcription factor 4

TGFβ: transforming growth factor beta

TNFα: tumor necrosis factor alpha

VCAM-1: vascular cell adhesion molecule 1

VEGF: vascular endothelial growth factor

WT: wildtype

YFP: yellow fluorescent protein

RÉSUMÉ

Les cellules satellites ont longtemps été considérées comme seules cellules souches musculaires. Cependant, ces dix dernières années, d'autres populations cellulaires au potentiel myogénique ont été découvertes. Parmi celles-ci nous avons mis en évidence une population localisée dans l'espace interstitiel du muscle et caractérisée par l'expression du gène PW1/Peg3, que nous avons dénommée PICs (PW1+ Interstitial Cells). Bien que les PICs participent à la régénération musculaire lorsqu'elles sont greffées dans un muscle blessé, elles n'expriment aucun marqueur musculaire ni ne dérivent du même lignage embryonnaire que les cellules satellites. Notre analyse transcriptomique comparant les gènes exprimés par les PICs et les cellules satellites a montré que les PICs ont un profil d'expression typique de cellules souches mésenchymateuses alors que les cellules satellites ont un profil d'expression très engagé dans le lignage myogénique. *In vitro* les cellules satellites ne peuvent se différencier qu'en muscle squelettique tandis que les PICs ont la capacité de se différencier en muscle lisse, muscle squelettique et adipocytes. Ces propriétés ont été observées pour des cellules isolées de muscle jeune ou adulte. De plus nous avons démontré que le potentiel adipogénique des PICs est issu de la population cellulaire co-exprimant PW1 et PDGFRα, ce qui correspond à la population de progéniteurs fibro/adipogéniques récemment caractérisés comme jouant un rôle dans la régénération musculaire. Par ailleurs, nous avons mis en évidence que les cellules satellites expriment la myostatine qui est un puissant inhibiteur de croissance musculaire alors que les PICs expriment son antagoniste la follistatine. Ceci suggère des interactions paracrines entre les deux populations de cellules. *In vitro*, nous avons démontré que la myostatine inhibe la prolifération des cellules satellites mais pas celle des PICs. De plus les PICs sont capables de restaurer la prolifération des cellules satellites en présence de myostatine, suggérant qu'elles sécrètent des facteurs inactivant la myostatine tels que la follistatine. *In vivo*, l'injection d'un produit pharmaceutique inactivant la myostatine induit une très forte croissance musculaire accompagnée d'une augmentation du nombre de PICs. Il a récemment été montré que la régénération musculaire est impossible sans les cellules satellites, malgré la présence *in situ* des autres populations au potentiel myogénique. Afin de tester si l'inhibition de la myostatine peut favoriser la régénération musculaire en l'absence de cellules satellites, nous avons utilisé le modèle murin $Pax7^{DTR/+}$ qui permet de détruire spécifiquement les cellules satellites. Dans ce modèle, la régénération musculaire n'a pas lieu lorsque les cellules satellites ont été détruites avant la blessure. Cependant si nous traitons au préalable les animaux avec l'inhibiteur de myostatine nous observons une régénération musculaire presque complète, suggérant que les PICs ont favorisé la différenciation des quelques cellules satellites restantes ou permis le recrutement d'autres progéniteurs myogéniques. Dans l'ensemble, nos résultats démontrent que les PICs sont multipotentes et jouent un rôle important dans la régulation de l'homéostasie musculaire.

Mots-clés : cellule souche musculaire, PICs, PDGFRα, myostatine.

ABSTRACT

The satellite cell is considered to be the unique skeletal muscle stem cell underlying muscle regeneration. However, the past ten years have seen the identification of a variety of non-satellite cell skeletal muscle progenitors, suggesting that the muscle stem cell niche is more complex than previously thought. We identified recently a new muscle-resident stem cell population termed PICs (PW1+ Interstitial Cells) that can participate in myofiber formation following engraftment into damaged muscle. However PICs do not express satellite cell markers nor do they share the same embryonic lineage. Micro-array profiling revealed that PICs express genes typical of mesenchymal stem cells while satellite cells display a profile committed to skeletal muscle. *In vitro*, satellite cells differentiate into skeletal muscle whereas PICs can differentiate into smooth muscle, skeletal muscle and adipocytes upon isolation from both young and adult mice. We further demonstrated that the adipogenic potential of PICs correlates to the PW1+ population that also expresses PDGFRα, corresponding to the recently described fibro/adipogenic populations capable of mediating muscle regeneration. In addition, we found that satellite cells express the strong inhibitor of muscle mass myostatin while PICs express its antagonist follistatin, suggesting a paracrine relationship between PICs and satellite cells. *In vitro*, we demonstrated that satellite cell but not PIC proliferation is abrogated by myostatin, however, PICs are capable of restoring satellite cell proliferation in transwell co-cultures. *In vivo*, pharmacological blocking of myostatin leads to an increase in muscle mass due to stem cell recruitment coupled with a dramatic increase in PICs number. Since it was recently demonstrated that skeletal muscle regeneration cannot occur in the absence of satellite cells despite the presence of other progenitors, we tested whether previous activation of PICs by myostatin blockade could rescue regeneration in a conditional Pax7-depleted mouse model. We found that muscle regeneration is rescued when myostatin is inhibited prior to muscle damage, suggesting that PICs can either directly participate in fiber formation or improve the stem cell environment allowing other progenitors and/or any remaining satellite cells to more efficiently function. Taken together, our results reveal that PICs constitute a multipotent muscle-resident population and an important constituent of the stem cell niche. As such, these results have potential impact for muscle degenerative diseases in which regeneration does not occur despite the presence of reduced numbers of satellite cells.

Key words: skeletal muscle stem cell niche, PICs, PDGFRα, myostatin.

RÉSUMÉ DE LA THÈSE

Les muscles striés squelettiques sont composés de fibres musculaires, encore appelées myocytes, qui sont de très longues cellules contenant plusieurs noyaux et constituant les unités contractiles du muscle (Harris, Duxson et al. 2005; Frontera, Reid et al. 2008). Les muscles squelettiques sont mis en place durant le développement embryonnaire grâce à des cellules progénitrices exprimant les facteurs de transcription Pax3 and Pax7, et migrant du dermomyotome vers les bourgeons des membres (Franz, Kothary et al. 1993; Goulding, Lumsden et al. 1994; Williams and Ordahl 1994; Relaix, Rocancourt et al. 2005). Sous l'influence des signaux Wnt et Shh produits par les tissus environnants, les progéniteurs musculaires induisent l'expression des facteurs de régulation myogénique (MRFs) Myf5, MyoD, myogénine et MRF4 (Tajbakhsh 2003). Ainsi engagés dans le lignage myogénique, les progéniteurs prolifèrent puis fusionnent entre eux pour former les fibres musculaires embryonnaires (Stockdale and Holtzer 1961; Ontell, Ontell et al. 1993). Cependant certains d'entre eux n'induisent pas l'expression des MRFs mais s'insèrent entre la lame basale et la membrane plasmique des fibres en formation, donnant ainsi naissance aux cellules satellites.

Les cellules satellites, découvertes par Alexander Mauro en 1961 (Mauro 1961), représentent la source majeure de cellules souches musculaires après la naissance et chez l'adulte (Wang and Rudnicki 2012). Dans le muscle adulte, elles sont normalement quiescentes et expriment des marqueurs tels que Pax7, CD34, α7-intégrine ou M-cadhérine (Beauchamp, Heslop et al. 2000; Seale, Sabourin et al. 2000). Suite à une blessure la majorité des cellules satellites sont réactivées, prolifèrent, différencient et fusionnent entre elles pour former de nouvelles fibres musculaires (Cornelison and Wold 1997; Halevy, Piestun et al. 2004). Une petite proportion de cellules satellites ne se différencie pas mais au contraire retourne à l'état quiescent, permettant ainsi de garder le pool de progéniteurs

musculaires pour de futures réparations (Olguin and Olwin 2004). Ces dix dernières années, d'autres populations cellulaires au potentiel myogénique ont été découvertes. Celles-ci se trouvent dans la circulation sanguine, telles que les cellules hématopoïétiques (Gussoni, Soneoka et al. 1999; Benchaouir, Meregalli et al. 2007), associées aux vaisseaux sanguins tels que les mésoangioblastes et péricytes (De Angelis, Berghella et al. 1999; Dellavalle, Sampaolesi et al. 2007), ou dans l'espace interstitiel du muscle telles que les PICs et les cellules β4-intégrine+ (Mitchell, Pannerec et al. 2010; Liadaki, Casar et al. 2012). Toutes ces populations possèdent la capacité de former des cellules musculaires *in vitro*, et de participer à la régénération musculaire *in vivo* lorsqu'elles sont injectées dans un muscle blessé. Cependant, deux récentes études utilisant des modèles murins pour lesquels les cellules satellite peuvent être spécifiquement détruites ont démontré que ces populations myogéniques ne permettent pas la régénération musculaire en l'absence de cellules satellites (Lepper, Partridge et al. 2011; Sambasivan, Yao et al. 2011). Malgré tout, les interactions entre les cellules satellites et des types cellulaires voisins sont extrêmement importantes pour une réparation musculaire efficace. D'une part les cellules immunitaires arrivant au niveau de la blessure permettent d'éliminer les débris cellulaires, favorisent le recrutement des populations myogéniques et promeuvent la croissance musculaire (Arnold, Henry et al. 2007; Lolmede, Campana et al. 2009). D'autre part, des cellules présentes dans le tissu interstitiel produisent des facteurs favorisant la régénération. Une sous-population de fibroblastes exprimant Tcf4 permet de réguler la prolifération des cellules satellites (Murphy, Lawson et al. 2011), tandis que des progéniteurs adipogéniques (FAPs) favorisent leur différenciation (Joe, Yi et al. 2010). A l'inverse les cellules satellites activées empêchent ces populations de prendre trop d'ampleur, ce qui gênerait la formation de nouvelles fibres musculaires. Ainsi, les interactions réciproques entre les cellules myogéniques et d'autres populations non-myogéniques par l'intermédiaire de signaux moléculaires permettent une régénération musculaire efficace.

Dans notre laboratoire, nous avons mis en évidence une population de cellules myogéniques située dans l'espace interstitiel et caractérisée par l'expression du gène imprimé PW1/Peg3, que nous avons nommée PICs (PW1+ Interstitial Cells) (Mitchell,

Pannerec et al. 2010). Bien que ne dérivant pas du même lignage embryonnaire que les cellules satellites, i.e les progéniteurs Pax3+, les PICs sont capables de reconstituer des fibres musculaires ainsi que des cellules satellites et de nouvelles PICs lorsqu'elles sont injectées dans un muscle blessé (Mitchell, Pannerec et al. 2010). Une autre étude de notre laboratoire, utilisant la souris rapportrice $PW1^{nLacZ/+}$ pour qui la β-galactosidase est exprimée sous le contrôle du gène PW1, a démontré que PW1/Peg3 est non-seulement exprimé par les PICs et les cellules satellites dans le muscle mais aussi par les cellules souches de beaucoup d'autres tissus, suggérant PW1 comme marqueur universel de cellules souches (Besson, Smeriglio et al. 2011).

Dans la première partie de notre étude, nous nous sommes attachés à clarifier la hiérarchisation cellulaire au sein du muscle squelettique par l'intermédiaire de PW1.

Afin de pouvoir étudier les PICs dans le muscle adulte, nous avons mis au point une technique de séparation cellulaire à partir de la souris rapportrice $PW1^{nLacZ/+}$ et des marqueurs de surface CD34 et Sca1. Dans notre précédente étude chez le muscle jeune (1-2 semaines), nous avions observé que les PICs sont présentes dans 2 populations cellulaires : les cellules $CD34+Sca1_{MED}$ étant >90% pures pour PW1 et les cellules $CD34+Sca1_{HIGH}$ étant pures à seulement 50% pour PW1 (Mitchell, Pannerec et al. 2010). Chez l'adulte, nous observons que la population $CD34+Sca1_{MED}$ n'est plus présente et que les PICs se trouvent donc seulement dans la population $CD34+Sca1_{HIGH}$. En regardant les âges intermédiaires nous trouvons que la population $CD34+Sca1_{MED}$ est présente seulement entre 1 et 3 semaines puis disparaît. Grâce à un substrat fluorescent pour la β-galactosidase, nous avons pu purifier les PICs de la fraction $CD34+Sca1_{HIGH}$ et comparer leur potentiel *in vitro* avec les PICs de la fraction $CD34+Sca1_{MED}$. Alors que les PICs isolées de muscle jeune ou adulte sont capable de se différencier en muscle lisse, muscle squelettique et adipocytes, les cellules satellites ne se différencient qu'en muscle squelettique suggérant que les PICs constituent une population multipotente plus « souche » que les cellules satellites. Cette hypothèse a été confirmée par une analyse

transcriptômique comparant le profil d'expression des PICs et des cellules satellites : alors que les PICs présentent un profil de cellules souches mésenchymateuses avec notamment l'expression de gènes relatifs à l'angiogenèse, les cellules satellites sont déjà très engagées dans le lignage myogénique. Nous remarquons néanmoins que les PICs de la fraction CD34+Sca1$_{MED}$ ont un potentiel myogénique plus élevé que les PICs de la fraction CD34+Sca1$_{HIGH}$ qui sont préférentiellement adipogéniques. Ces résultats suggèrent que les PICs de la fraction CD34+Sca1$_{MED}$ jouent un rôle dans la croissance musculaire postnatale, soit par fusion directe avec les fibres en croissance soit par la production de signaux favorisant la différenciation des cellules satellites. A l'âge de 3 semaines, alors que la phase de croissance par fusion nucléaire s'achève (White, Bierinx et al. 2010), nous postulons que les PICs de la fraction CD34+Sca1$_{MED}$ ont soit fusionné avec les fibres soit induit l'expression de Sca1 conduisant à la disparition de la fraction CD34+Sca1$_{MED}$. Ainsi, le niveau d'expression de Sca1 pourrait représenter l'état d'activation des PICs.

Dans le muscle adulte, nous observons que les PICs sont préférentiellement adipogéniques. Afin de déterminer si les PICs représentent la même population que les progéniteurs adipogéniques récemment mis en évidence (Joe, Yi et al. 2010; Uezumi, Fukada et al. 2010), nous avons regardé l'expression du marqueur adipocytaire PDGFRα dans les cellules CD34+Sca1$_{HIGH}$. Nos résultats démontrent que seules les cellules exprimant PDGFRα sont capables de former des adipocytes *in vitro*, et que les toutes les cellules exprimant PDGFRα expriment également PW1, démontrant ainsi que les FAPs constituent une sous-population des PICs. La petite fraction de PICs qui n'expriment pas PDGFRα est quant à elle fortement myogénique. Nous n'avons pas encore déterminé de marqueur spécifique pour cette seconde sous-population, mais nous testons présentement la β4-intégrine qui a récemment permis d'identifier une population de cellules myogéniques résidant dans l'espace interstitiel du muscle et constitue donc un bon gène-candidat (Liadaki, Casar et al. 2012).

En conclusion, nos résultats suggèrent que les cellules souches présentes dans le muscle expriment toutes PW1 et peuvent être différenciées par des marqueurs plus spécifiques. Ainsi les cellules satellites expriment PW1 et Pax7 alors que les FAPs expriment

PW1 et PDGFRα. En accord avec cette hypothèse, notre collaboration avec le laboratoire du Dr Cossu a permis de déterminer que les mésoangioblastes, cellules myogéniques associées aux vaisseaux sanguins et caractérisées par l'expression de l'alkaline phosphatase, expriment aussi PW1. Ce modèle permet de clarifier la hiérarchisation des cellules souches au sein du muscle. Par ailleurs nos résultats suggèrent que les PICs, initialement décrites comme résidant dans l'espace interstitiel et exprimant PW1, sont en fait constituées de 2 populations cellulaires correspondant à ces critères mais au potentiel bien différent puisque l'une est adipogénique alors que l'autre est myogénique.

Nous avons ensuite cherché à comprendre le rôle des PICs dans le muscle adulte. L'un des principaux régulateurs de la masse musculaire est la myostatine, appartenant à la famille des TGFβs (McPherron, Lawler et al. 1997). L'effet inhibiteur de la myostatine sur la croissance musculaire a été démontré par l'observation de mutations naturelles sur ce gène conduisant à des phénotypes hyper-musclés chez beaucoup d'espèces, dont l'homme (Grobet, Martin et al. 1997; Schuelke, Wagner et al. 2004; Clop, Marcq et al. 2006). La myostatine, préférentiellement exprimée dans les muscles cardiaques et squelettiques, est sécrétée sous une forme latente qui devient active après plusieurs clivages au niveau N-terminal (McPherron, Lawler et al. 1997). *In vitro*, la myostatine a pour effet de bloquer le cycle cellulaire en phase G_1 par l'intermédiaire des protéines Smad qui induisent l'expression de p21 tout en bloquant l'activité de Cdk2 (Thomas, Langley et al. 2000; Joulia, Bernardi et al. 2003). En parallèle, l'expression des facteurs de régulation myogénique Myf5 et MyoD est inhibée (Langley, Thomas et al. 2002). Les souris pour lesquelles la myostatine a été génétiquement inactivée ($Mst^{-/-}$) présentent des muscles deux fois plus gros que les souris de type sauvage (WT), résultant de la combinaison de phénomènes d'hyperplasie (i.e augmentation du nombre de fibres musculaires) et d'hypertrophie (i.e augmentation du volume des fibres musculaires) (McPherron, Lawler et al. 1997; Matsakas, Otto et al. 2010). De plus les muscles $Mst^{-/-}$ régénèrent plus rapidement grâce à une réponse immunitaire prononcée et une activation intense des précurseurs myogéniques (McCroskery, Thomas et al. 2005; Wagner, Liu et al. 2005). L'autre régulateur important de la masse musculaire est l'IGF-1, qui lui favorise la croissance musculaire (Adams 1998; Musaro, McCullagh et al.

2001). IGF-1 agit par la voie de signalisation Akt/mTOR pour d'une part activer la synthèse protéique et d'autre part inhiber les voies de dégradation protéiques (Bodine, Stitt et al. 2001; Rommel, Bodine et al. 2001; Stitt, Drujan et al. 2004). La myostatine bloque cette voie de signalisation en inactivant Akt et ainsi renforce son effet inhibiteur sur la croissance musculaire (Sartori, Milan et al. 2009; Lokireddy, McFarlane et al. 2011), cependant l'effet de IGF-1 est dominant sur celui de la myostatine lorsque les 2 facteurs sont en compétition (Trendelenburg, Meyer et al. 2009).

Préserver ou augmenter la masse musculaire aurait de nombreux avantages dans le traitement des maladies musculaires, de certains cancers, ou pour endiguer la perte de masse musculaire survenant avec l'âge. La myostatine constitue une cible attractive pour de tels traitements, ainsi de nombreuses techniques pour inactiver la myostatine ont été mises au point et testées sur modèles murins. L'injection d'un anticorps neutralisant la myostatine a permis d'augmenter significativement la masse et la force musculaire de souris WT ainsi que de souris *mdx* (modèle pour la dystrophie de Duchenne) (Bogdanovich, Krag et al. 2002; Whittemore, Song et al. 2003), mais seulement pour des animaux relativement jeunes (Parsons, Millay et al. 2006). La forme soluble du récepteur à la myostatine, ActR2b, permet également d'augmenter la masse musculaire de près de 50% et indépendamment du type de fibre (Lee, Reed et al. 2005; Cadena, Tomkinson et al. 2010). Enfin, des inhibiteurs de la myostatine tels que GASP-1 ou la follistatine peuvent être utilisés sous forme d'AAV et conduisent à une forte augmentation de la masse musculaire ainsi que l'amélioration de la force chez des souris *mdx* (Haidet, Rizo et al. 2008). Cependant, il n'est toujours pas clairement établi si l'hypertrophie musculaire induite par ces méthodes requière la participation de progéniteurs musculaires. Les cellules satellites permettent la croissance postnatale ainsi que la régénération musculaire et sont inhibées *in vitro* par la myostatine, ce qui suggère qu'en l'absence de myostatine elles pourraient différencier et fusionner avec les fibres. Dans ce sens, McCroskery *et al*. ont observé 2 fois plus de cellules satellites activées dans les muscles $Mst^{-/-}$ par rapport aux muscles contrôles (McCroskery, Thomas et al. 2003), et Zhou *et al*. ont observé 5 fois plus de cellules satellites après injection de la forme soluble de l'ActR2b (Zhou, Wang et al. 2010). A l'inverse,

d'autres groupes n'observent pas de différence en terme de nombre de cellules satellites ou de nombre de noyaux par fibre suite à l'inhibition de la myostatine, suggérant que l'hypertrophie résulterait simplement d'une augmentation du volume cytoplasmique (Amthor, Otto et al. 2009; Blaauw, Canato et al. 2009). Une étude récente suggère que l'inhibition de la myostatine induit d'abord une augmentation de la taille des fibres puis l'activation des cellules satellites et l'augmentation du nombre de noyaux par fibre (Wang and McPherron 2012). Ainsi, l'augmentation de la taille des fibres se déroulerait en 2 étapes et nécessiterait l'addition de nouveaux myonuclei pour maintenir l'hypertrophie.

Dans la seconde partie de notre étude nous avons cherché à comprendre le rôle des PICs dans la croissance musculaire, par inhibition de la myostatine.

Lorsque nous traitons des souris WT avec la forme soluble de l'ActR2b, RAP-031, les animaux présentent une forte augmentation de leur poids et de leur masse musculaire ainsi qu'une hypertrophie des fibres, comme l'ont observé d'autres équipes (Cadena, Tomkinson et al. 2010). Le nombre de PICs est multiplié par 4 alors que le nombre de cellules satellites est seulement doublé, et la proportion de PICs en prolifération est bien supérieure à celle des cellules satellites, suggérant que les PICs sont préférentiellement recrutées suite à l'inhibition de la myostatine. Nous observons de plus une augmentation significative du nombre de myonuclei, indiquant que l'hypertrophie a résulté au moins en partie de la fusion de progéniteurs musculaires. Le même traitement chez des souris $Pax7^{DTR/+}$, pour lesquelles l'injection de toxine diphtérique a détruit les cellules satellites, conduit également à une hypertrophie musculaire mais sans augmentation du nombre de myonuclei. Ainsi, en accord avec une précédente étude (McCarthy, Mula et al. 2011), nous démontrons que les cellules satellites constituent la seule source de noyaux additionnels dans le cas d'hypertrophie induite à l'état basal mais que celle-ci résulte en partie d'une augmentation cytoplasmique.

Ces résultats démontrent que les PICs ne participent pas directement à l'hypertrophie musculaire à l'état basal. Notre analyse transcriptômique révèle que les cellules satellites expriment la myostatine alors que les PICs expriment son antagoniste la

follistatine, suggérant de possibles interactions entre ces 2 populations par l'intermédiaire des voies de signalisation TGFβ. *In vitro* nous observons que la myostatine inhibe la prolifération des cellules satellites d'une manière dose-dépendante comme précédemment décrit (Thomas, Langley et al. 2000; Taylor, Bhasin et al. 2001), alors qu'elle n'a aucun effet sur les PICs. De plus, les PICs permettent de rétablir la prolifération des cellules satellites lorsque les 2 types cellulaires sont co-cultivés en présence de myostatine. En utilisant un anticorps bloquant la follistatine nous démontrons que l'effet bénéfique des PICs sur la croissance des cellules satellites passe en partie par la follistatine, qui se lie à la myostatine et l'inactive. D'après l'analyse transcriptômique, IGF-1 est fortement exprimé par les PICs et pourrait donc également participer à l'activation de la prolifération des cellules satellites. Nous sommes actuellement en train de tester cette hypothèse par l'utilisation d'anticorps bloquant IGF-1. Finalement ces résultats démontrent que les PICs ont un effet pro-myogénique sur les cellules environnantes par la production de signaux favorisant la prolifération cellulaire, tout comme précédemment démontré pour les FAPs (Joe, Yi et al. 2010) qui constituent la majeure partie des PICs.

De récentes études ont démontré que les cellules satellites sont absolument indispensables pour la régénération musculaire (Lepper, Partridge et al. 2011; Murphy, Lawson et al. 2011; Sambasivan, Yao et al. 2011). Comme les PICs sont pro-myogéniques et fortement activées suite à l'inhibition de la myostatine, nous avons testé si le traitement RAP-031 permet la régénération musculaire en l'absence de cellules satellites. Nous avons donc pré-traité des souris $Pax7^{DTR/+}$ avec RAP-031 et induit une blessure par l'injection de cardiotoxine. Alors que les muscles sans cellules satellites n'ayant pas été traités avec RAP-031 présentent beaucoup d'infiltration de cellules fibreuses et d'adipocytes sans régénération musculaire (Sambasivan, Yao et al. 2011), les muscles sans cellules satellites pré-traités avec RAP-031 présentent une régénération quasi-équivalente à celle des muscles contrôles. De plus nous observons beaucoup de cellules Pax7+ dans les muscles pré-traités, indiquant que la régénération a eu lieu par l'activation de myoblastes. Cependant nos expériences n'ont pas permis de déterminer si ces myoblastes Pax7+ proviennent des quelques cellules satellites ayant survécu à la toxine, ou ont été recrutés à partir d'autres progéniteurs myogéniques. Cette hypothèse sera bientôt testée grâce à de

nouveaux modèles murins générés en collaboration avec le Dr Tajbakhsh. D'autres équipes ont démontré l'importance des cellules endothéliales et inflammatoires dans le recrutement des cellules satellites et des mésoangioblastes (Arnold, Henry et al. 2007; Lolmede, Campana et al. 2009), et l'effet pro-myogénique des FAPs (Joe, Yi et al. 2010). Alors que ces signaux sont peut-être insuffisants pour activer la régénération musculaire en l'absence de cellules satellites, l'inhibition de la myostatine favoriserait la production de facteurs pro-myogéniques par les PICs et/ou d'autres populations cellulaires conduisant au recrutement de précurseurs myogéniques.

Finalement nous avons démontré que le tissu musculaire possède une grande plasticité dans ces types cellulaires, et peut être activé par des molécules pharmaceutiques pour induire une régénération musculaire en présence de nombres très réduits de cellules satellites. Ces résultats peuvent avoir d'importantes applications thérapeutiques dans le cas des myopathies avancées où les cellules satellites ne fonctionnent plus.

CHAPTER 1. THE SKELETAL MUSCLE NEIGHBORHOOD

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1. **Generalities about skeletal muscle**
    - Skeletal muscle structure and function
    - Embryonic development of vertebrate limb skeletal muscles

2. **Postnatal skeletal muscle stem cells**
    - The satellite cell
    - Non-satellite muscle progenitors

3. **Influence of the niche on stem cell behavior**
    - “External” signals: the action of inflammatory cells
    - “Internal” signals: the myogenic/adipogenic balance

1. Generalities about skeletal muscle

1.1. Skeletal muscle structure and function

Muscles are contractile tissues comprising ~40% of the human body mass. They are classified in 3 categories: cardiac, smooth and skeletal muscles. Cardiac and smooth muscles are "involuntary" muscles, meaning that their action is not under conscious control, that allow movements of internal organs such as heart, stomach, intestine, bronchi, blood vessels or uterus. Their actions permit vital functions such as blood circulation, breathing and digestion. In contrast skeletal muscle activity is determined by conscious decisions and allows movements of the organism itself for locomotion and interactions with the environment, with the singular exception of diaphragm which controls breathing and is under both autonomic and direct motor control. Cardiac and skeletal muscles have a "striated" structure upon histological examination reflecting the cytoskeleton of the contractile apparatus whereas smooth muscle cells do not have a striated appearance despite their contractile cytoskeleton (**Fig.1**).

Skeletal muscles are anchored to at least one bone by tendon and are composed of several groups of fibers termed fascicles (**Fig.2**). Each fascicle is surrounded by a layer of conjunctive tissue, the perimysium, and all fascicles of the muscle are ensheathed by a layer of connective tissue called epimyisum. Myofibers are multinucleated cells constituting the force producing units that can measure up to 100µm in diameter and 30cm in length in human thigh muscles (Harris, Duxson et al. 2005; Frontera, Reid et al. 2008). Skeletal muscle contraction is driven by motor neurons that attach through the neuromuscular junction. The motorneuron activity consists of an action potential that is propagated down the axon to the neuromuscular junction, leading to the release of the neurotransmitter acetylcholine into the extracellular milieu. Acetylcholine binding to the myofiber transmembrane acetylcholine receptors induces a depolarization of the myofiber membrane which is propagated through the fiber membrane and internal membrane structures leading to Ca^{2+} release and subsequent contraction (Hirsch 2007). Contraction is the result of the simultaneous myofibrils shortening. Myofibrils consist of highly ordered longitudinal

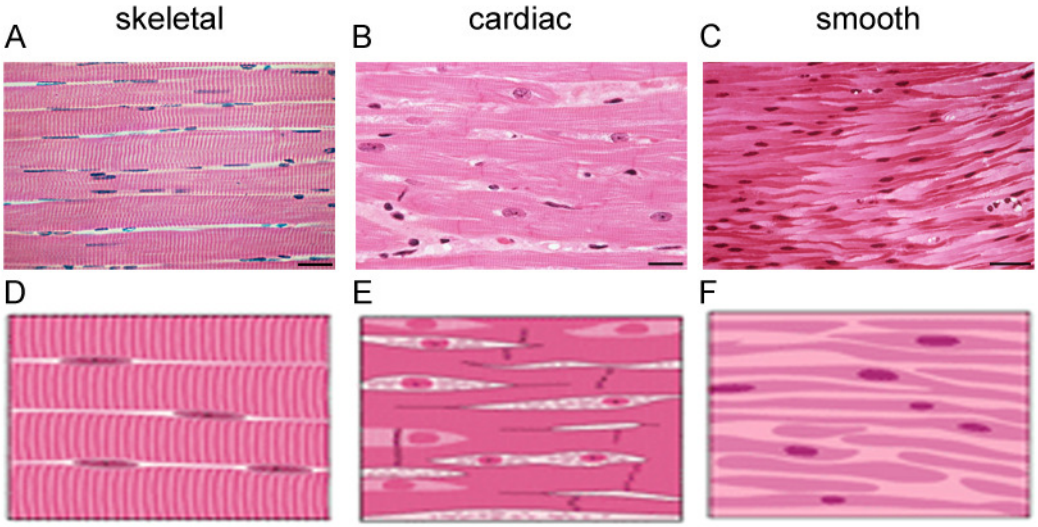

Figure 1. The muscle types. (**a-c**) Light micrographs of longitudinal sections through skeletal (a), cardiac (b) and smooth (c) muscles stained with hematoxylin and eosin. (**d-f**) Schematic representations of light micrograph images presented in (a-c). Skeletal muscle (a, d) is composed of individual muscle fibers separated by connective tissue. Myofibrils, cylindrical structures lying parallel to each other and which contain the contractile proteins, can be observed as regular cross-striations. Cardiac muscle (b, e) is composed of individual cells containing one or two elongated nuclei and extensive, branching cytoplasm giving the appearance of a continuous network of tissue. Smooth muscle (c, f) is composed of mononuclear spindle-shaped cells grouped in irregular bundles. Scale, µm. *Source: Science Photo Library (http://www.sciencephoto.com/)*.

filaments called sarcomeres (**Fig.3**). A sarcomere unit is composed of thick myosin filaments that slide along thin actin filaments, held together by titin, nebulin and other supporting proteins (Clark, McElhinny et al. 2002; Luther 2009). During contraction, the actin filaments are pulled along myosin towards the central M-band of the sarcomere until the actin and myosin filaments completely overlap (Huxley 1969). As the Z-lines move closer together, the muscle shortens. There are two types of muscle fibers: slow twitch (type I) and fast twitch (type II). Slow twitch or "red" muscle is dense with capillaries and rich in mitochondria and myoglobin, giving the muscle tissue its characteristic red color. These fibers are highly oxidative such that they can contract for long periods of time but do not exert much force. Fast twitch or "white" muscle can contract more quickly and with a greater amount of force but can sustain only short, anaerobic bursts of activity due to a lower mitochondrial content (Schiaffino and Reggiani 2011).

1.2. Embryonic development of vertebrate limb skeletal muscles

Vertebrate skeletal muscle arises from the embryonic mesoderm. Paraxial mesoderm separates into two regions, head and trunk, and segments along the neural tube to form metameric structures termed somites (Perry and Rudnick 2000). Molecular signals produced by the neural tube and notochord induce the somites to become subdivided into distinct domains. Sonic hedgehog (Shh) produced by the notochord induces the formation of the ventral sclerotome that gives rise to vertebrae, ribs, and tendons (Johnson, Laufer et al. 1994). Wnt molecules secreted by the neural tube induce the formation of the dorsal dermomyotome which gives rise to dermis and trunk skeletal muscles (Fan, Lee et al. 1997; Marcelle, Stark et al. 1997). Cells present in the lateral region of the dermomyotome initiate the expression of the transcription factors Pax3 and Pax7, and then migrate to the limb bud or ventrally in the body where they subsequently differentiate to form the primary myotubes of the limb and trunk skeletal muscles, respectively (Franz, Kothary et al. 1993; Goulding, Lumsden et al. 1994; Williams and Ordahl 1994; Relaix, Rocancourt et al. 2005). Cell migration is dependent upon Pax3, which induces the expression of the tyrosine kinase receptor c-met that allows epithelium dissociation

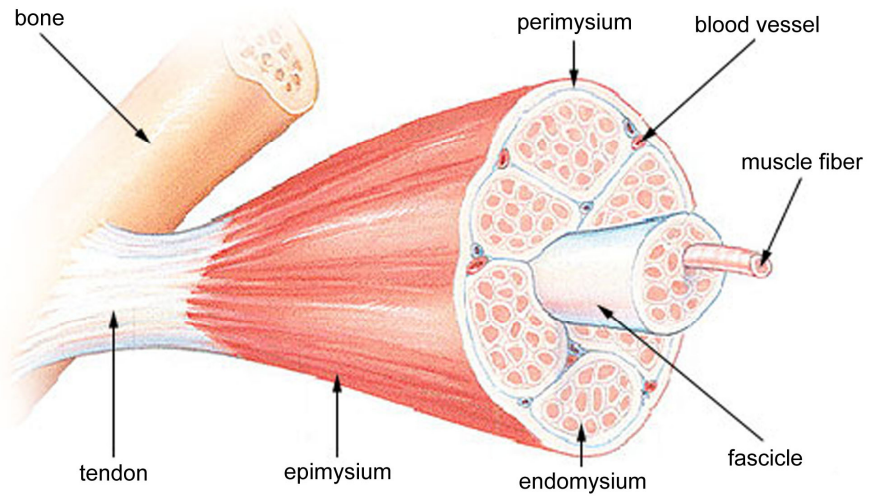

Figure 2. The skeletal muscle structure. Each skeletal muscle is composed of several groups of fibers (fascicles) surrounded by perimysium and irrigated by blood vessels. Fascicles are grouped by the epimysium and anchored to bone by tendon.

through interaction with its ligand HGF (Bladt, Riethmacher et al. 1995; Epstein, Shapiro et al. 1996). Pax3 also induces the expression of Lbx1, which is essential for cells to migrate in the proper direction (Brohmann, Jagla et al. 2000) and has also been suggested to have a role in maintaining them in a proliferative state (Martin and Harland 2006).

Cells that migrate to the limb bud activate the expression of the myogenic regulatory factors (MRFs) under the influence of Wnt and Shh signals received from surrounding tissues, that is mediated by Pax3 (Maroto, Reshef et al. 1997; Tajbakhsh 2003; Bajard, Relaix et al. 2006). The MRFs refers to 4 related genes: *Myf5* (Braun, Buschhausen-Denker et al. 1989), *MyoD* (Davis, Weintraub et al. 1987), *myogenin* (Wright, Sassoon et al. 1989) and *MRF4* (Rhodes and Konieczny 1989) that are each capable of inducing a myogenic phenotype when force expressed in a wide variety of cell types and are expressed in a developmentally and temporally specific manner during embryogenesis as well as in postnatal and adult skeletal muscle (**Fig.4**). *Myf5* is the first MRF to be expressed during development (Delfini, Hirsinger et al. 2000), followed by *MyoD* (Sassoon, Lyons et al. 1989; Ott, Bober et al. 1991). Both Myf5 and MyoD are necessary to induce the proper timing of the expression of *myogenin* and *MRF4* (Rudnicki, Schnegelsberg et al. 1993). Embryonic muscle growth is the result of a balance between proliferation and differentiation. Myoblast proliferation is activated by a variety of growth factors including FGF (Marcelle, Wolf et al. 1995; Edom-Vovard, Bonnin et al. 2001), IGF (Mitchell, Johnson et al. 2002) and follistatin (Amthor, Christ et al. 2002) while HGF maintains the undifferentiated state (Scaal, Bonafede et al. 1999). In contrast, Notch, Twist, Noggin and myostatin are negative regulators of myoblast proliferation and differentiation (Kopan, Nye et al. 1994; Spicer, Rhee et al. 1996; McPherron, Lawler et al. 1997; Amthor, Christ et al. 1999). Decreases in growth factor concentration trigger myoblast cell cycle exit and differentiation. Cell cycle exit is achieved by the up-regulation of cyclin-dependent kinase (Cdk) inhibitors such as p21 and retinoblastoma protein (Rb), which is also necessary for the activation of the MRFs (Gu, Schneider et al. 1993; Halevy, Novitch et al. 1995; Andres and Walsh 1996). Committed myoblasts subsequently align, fuse into multinucleate

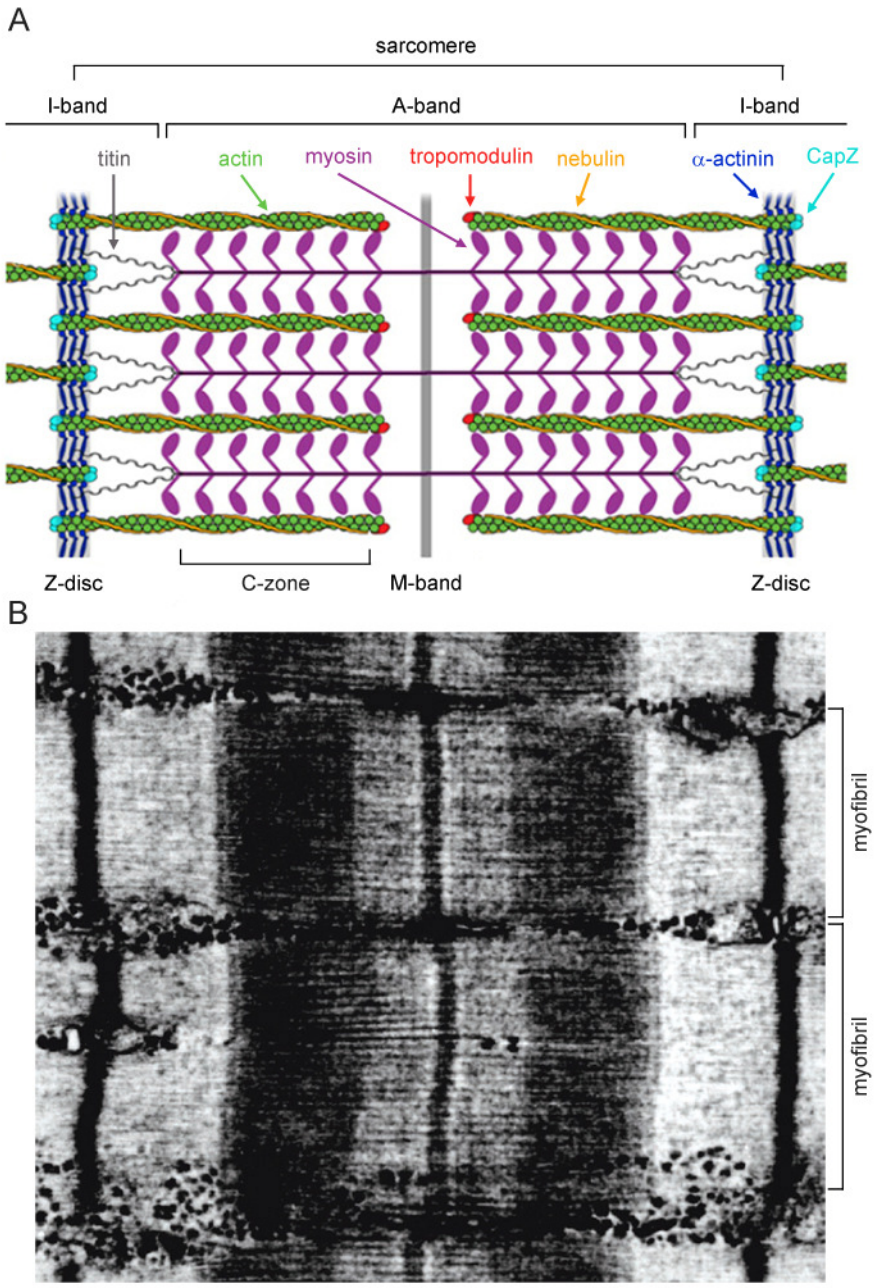

Figure 3. The skeletal muscle sarcomere. Schematic diagram (a) and electron micrograph (b) showing the organization of the vertebrate skeletal muscle sarcomere. (**a**) Elastic titin filaments (gray) extend from Z-disc to M-band to form a stable, yet flexible myofibril scaffold holding myosin thick filaments (purple). Actin thin filaments (green) are capped at the Z-disc by CapZ (cyan) and crosslinked to other actin filaments in adjacent sarcomeres by α-actinin (blue). They extend from the Z-disc, overlap myosin filaments at the C-zone, and are capped at their extremity by tropomodulin (red). Nebulin (orange) is anchored at the Z-disc and ensheaths actin filaments. (**b**) The myofilaments assemble into elementary contratile units, sarcomeres, which are concatenated to form myofibrils. *(adapted from (Littlefield and Fowler 2008) and (Tskhovrebova and Trinick 2003)).*

myotubes and initiate expression of muscle specific contractile proteins such as actin and myosin heavy chain (MHC) (Stockdale and Holtzer 1961; Ontell, Ontell et al. 1993).

Skeletal muscle formation is characterized by two temporally distinct waves of myoblast fusion referred to as primary myogenesis and secondary myogenesis. Primary myogenesis occurs between the 11th and 14th day of embryonic development (E11-14) in mice by fusion of embryonic myoblasts, forming primary fibers that traverse the future muscles from tendon to tendon and thus determine their location, shape and type (Ontell and Kozeka 1984). The muscle fibers formed by primary myoblasts constitute a scaffold for the secondary myogenesis, which occurs around E14-16 in mice coincident with innervation (Ontell and Kozeka 1984; Duxson, Usson et al. 1989). Secondary fibers begin to form near the site of innervation of primary fibers and rapidly increase in size and number (Duxson and Sheard 1995). Primary and secondary fibers can be distinguished by the expression of specific isoforms of myosin such that primary fibers are primarily slow twitch while secondary fibers are both fast and slow twitch and also display differences in mitochondrial content typical of postnatal muscles (Kelly and Rubinstein 1980; Ontell, Ontell et al. 1993; Van Swearingen and Lance-Jones 1995). A third population of myogenic cells, termed satellite cells, emerge at the end of fetal development around E12-13 in avian and E17,5 in mice (Cossu and Molinaro 1987; Hartley, Bandman et al. 1992). Satellite cells adhere to the surface of the myofibers and the basal lamina is deposited around the myofiber and satellite cells (Armand, Boutineau et al. 1983; Bischoff 1990). Embryonic progenitors giving rise to satellite cells express Pax3 and Pax7 but not the MRFs, and up-regulate Myf5 when reaching their sub-laminal position (Kassar-Duchossoy, Giacone et al. 2005; Relaix, Rocancourt et al. 2005). Unlike Pax3, Pax7 is not absolutely necessary for muscle formation but is required for satellite cell maintenance during the first few weeks of postnatal life (Bober, Franz et al. 1994; Seale, Sabourin et al. 2000; Oustanina, Hause et al. 2004).

Accumulated evidence demonstrates that satellite cells arise from dermomyotome somitic progenitors. Lineage tracing studies using Cre-stop-flox permanent fluorescent labeling as well as classic quail/chick grafting experiments demonstrated that cells

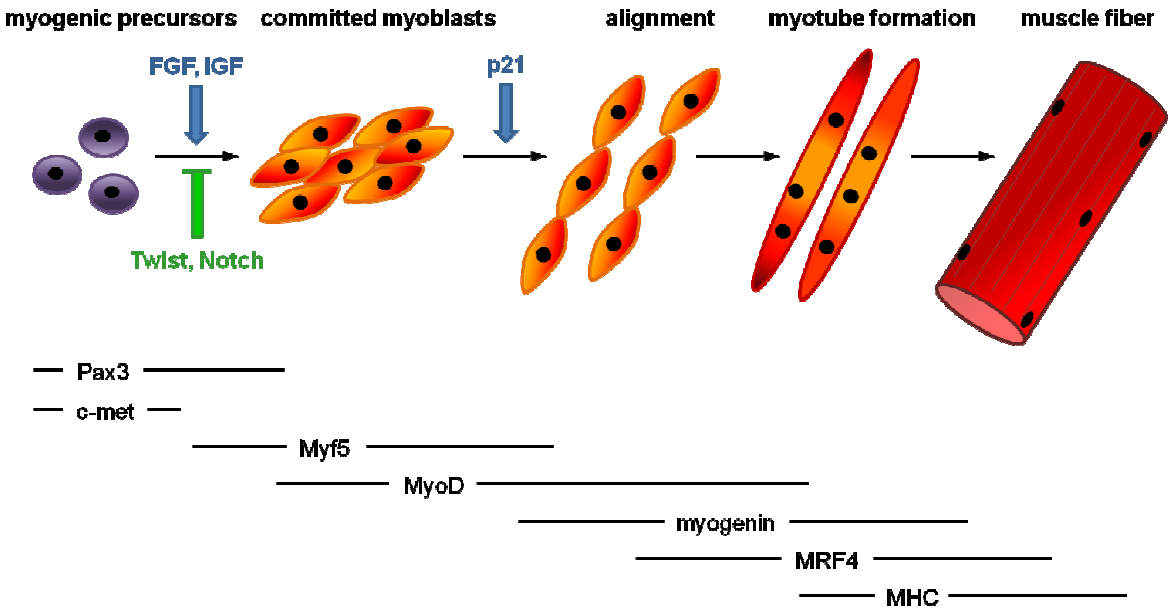

Figure 4. The myogenic lineage progression. Myogenic precursors from the dermomyotome migrate to the limb bud under the control of Pax3 and c-met. Cell proliferation is induced by Pax3 and growth factors present in the surrounding environment such as FGF and IGF, until commitment to the myogenic lineage through MRFs expression. Myoblasts then exit the cell cycle by up-regulation of p21, align and fuse to form myotubes. Myosin and other specific proteins expression lead to fiber maturation.

expressing Pax3 (Pax3+ cells) contribute to embryonic myoblasts and the endothelial lineage whereas Pax7+ cells from E16,5 contribute to fetal myoblasts, adult skeletal muscle and adult satellite cells (Gros, Manceau et al. 2005; Schienda, Engleka et al. 2006; Hutcheson, Zhao et al. 2009; Lepper and Fan 2010). These data suggest that adult satellite cells represent a lineage that arises from the embryonic Pax3+Pax7+MRF- progenitors (Kuang, Kuroda et al. 2007). However, other studies have demonstrated the presence of myogenic precursors within the embryonic dorsal aorta, displaying all the characteristics of adult satellite cells and that can be isolated even from *Pax3-null* mice that lack limb musculature (De Angelis, Berghella et al. 1999). This suggests that satellite cells may arise, at least in part, from an endothelial lineage. Taken together, these studies suggest that while satellite cells derive from somitic progenitors, other myogenic lineages exist that ultimately reside in the muscle mass (see section 2.2).

2. Postnatal skeletal muscle stem cells

2.1. The satellite cell

Since its discovery in 1961 (Mauro 1961), extensive work has been provided to understand the satellite cell biology and function (Mauro 1961; Wang and Rudnicki 2012). Located beneath the basal lamina of myofibers, satellite cells are in close relationship with the plasma membrane of the fiber (Mauro 1961). Their cytoplasmic volume is very small and thus they adopt the shape of their nucleus (Mauro 1961). Being quiescent in adult muscle under normal physiological conditions, satellite cells can re-enter the cell cycle and ultimately fuse to form new fibers in response to damage, exercise or disease (Schultz, Gibson et al. 1978; Dhawan and Rando 2005; Montarras, Morgan et al. 2005). As such, it is commonly accepted that satellite cells constitute the major source of progenitors for postnatal muscle growth, maintenance and repair.

During early postnatal development, satellite cells proliferate to produce enough material for providing additional myonuclei to fibers, allowing muscle growth through hypertrophic process (Moss and Leblond 1971; Schultz 1996; Davis and Fiorotto 2009).

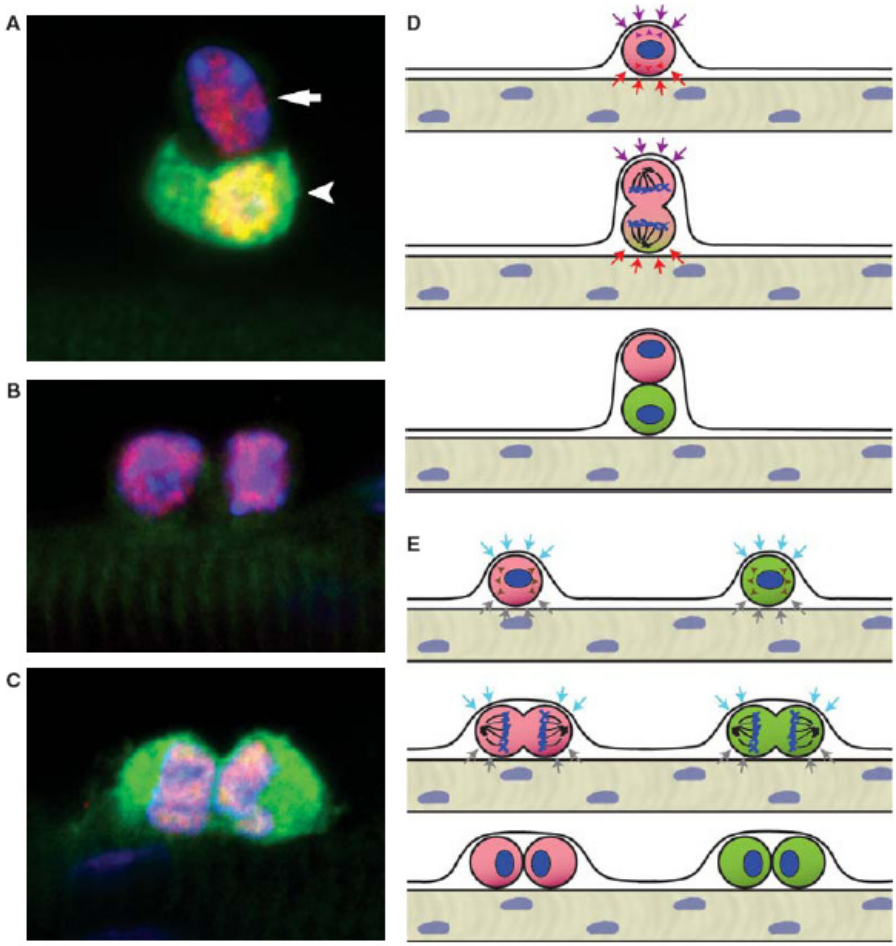

Figure 5. Asymmetric versus stochastic modes of satellite cell division. As determined by lineage tracing, 10% of adult satellite cells have never expressed Myf5 and are referred to as "satellite stem cells." (**a**) Satellite stem cells (arrow) undergo asymmetric division in an apical–basal orientation in which the daughter cell that is detached from the basal lamina up-regulates Myf5 and the fluorescent lineage tracer YFP (arrowhead). Pax7 (red); YFP (green); nuclei (blue). (**b,c**) In the stochastic mode of division, both types of satellite cells divide planar along the host fiber and give rise to two identical daughter cells. (**d**) Model of apical-basal divisions leading to an asymmetric outcome. Opposing signals from the basal lamina and the myofiber control the orientation of DNA spindles and the asymmetric cosegregation of proteins and DNA strands. Post-cytokinesis, daughter cells continue to be subjected to different signals leading to asymmetric cell fates. (**e**) Model of planar divisions leading to symmetric expansion of cells. Surrounding signals drive the planar orientation of DNA spindles. Daughter cells in this outcome remain attached to the host fiber and the basal lamina, thus receiving similar signals, and maintain identical cell fates. *(from (Bentzinger, Wang et al. 2012))*.

While representing nearly 30% of nuclei in newborn, the number of satellite cells rapidly decreases as the number of myonuclei per fiber is increasing and account for only 4% of nuclei in the adult (Allbrook, Han et al. 1971; White, Bierinx et al. 2010). At the end of postnatal growth, satellite cells enter quiescence under the control of Notch signaling, as demonstrated in mice lacking the two Notch target genes Hesr1 and Hesr3 that are unable to produce undifferentiated quiescent satellite cells (Fukada, Yamaguchi et al. 2011). Notch is moreover necessary to maintain the quiescent state of satellite cells in adult muscle (Bjornson, Cheung et al. 2012; Mourikis, Sambasivan et al. 2012). In resting muscle, satellite cells are characterized by the expression of a number of markers including Pax7 (Seale, Sabourin et al. 2000), α7-integrin (Burkin and Kaufman 1999), CD34 (Beauchamp, Heslop et al. 2000), syndecan 3 and 4 (Cornelison, Filla et al. 2001), M-cadherin (Irintchev, Zeschnigk et al. 1994) and CXCR4 (Pituch-Noworolska, Majka et al. 2003).

Upon injury, satellite cells are activated including distal satellite cells that migrate towards the injury site (Schultz, Jaryszak et al. 1985). In response to signals from the damaged environment, the majority of satellite cells re-enter the cell cycle and initiate expressing Myf5 and MyoD (Cornelison and Wold 1997; Cooper, Tajbakhsh et al. 1999; Zammit, Heslop et al. 2002). MyoD requirement for satellite cells to carry out regeneration was demonstrated in the *MyoD-null* mouse model, which displays poor regenerative capacity due to small number of newly formed myotubes and failure of satellite cells to enter differentiation (Megeney, Kablar et al. 1996; Sabourin, Girgis-Gabardo et al. 1999; Yablonka-Reuveni, Rudnicki et al. 1999; Cornelison, Olwin et al. 2000). Activated satellite cells rapidly expand and subsequently differentiate upon Pax7 down-regulation concomitant with myogenin and MHC up-regulation, exit the cell cycle and fuse into new myofibers or repaired fibers (Fuchtbauer and Westphal 1992; Yablonka-Reuveni and Rivera 1994; Cornelison and Wold 1997; Halevy, Piestun et al. 2004) (**Fig.4**). While the majority of proliferating satellite cells undergo differentiation, a small proportion of cells retains Pax7 expression and down-regulates MyoD and return to quiescence, allowing replenishment of the satellite cell pool (Olguin and Olwin 2004; Zammit, Golding et al. 2004).

Cell type	Markers	Proliferation *in vitro*	Systemic delivery	Repopulation of the satellite cell niche	Transplantation efficiency (number or % of positive myofiber, TA)	Improvement of muscle disease	References
Satellite cells	Pax7, CD34, c-met	high	no	yes	>500 fibers (i.m)	*mdx* mice	(Partridge, Morgan et al. 1989)
Side Population	Hoechst exclusion, CD45, Sca1	low	yes	yes	<50 (8%) (i.a.)	*mdx* mice	(Gussoni, Soneoka et al. 1999)
CD133+ cells	CD133, CD34, Thy1, CD45	low	yes	yes	50-100 fibers (i.a)	*mdx* mice	(Benchaouir, Meregalli et al. 2007)
Mesoangioblasts	CD34, c-kit, Flk1	high	yes	unknown	>20% (i.a)	α-sarco mice, dystrophic dogs	(Sampaolesi, Torrente et al. 2003) (Sampaolesi, Blot et al. 2006)
Pericytes	AP, NG-2 proteoglycan	high	yes	yes	300-500 (i.a) ; 20 (i.m)	*mdx* mice	(Dellavalle, Sampaolesi et al. 2007)
PICs	PW1	low	not tested	yes	60% (i.m)	not tested	(Mitchell, Pannerec et al. 2010)
integrinβ4+ cells	integrinβ4	high	not tested	unknown	50-100 (5-10%) (i.m)	*mdx* mice	(Liadaki, Casar et al. 2012)

i.m = intra-muscular injection ; i.a = intra-arterial injection

Table 1. Comparative potentials of myogenic progenitors. While satellite cells are very efficient to regenerate muscle, they cannot cross the endothelial barrier and have therefore been very disappointing in clinical trials. Reversely, Side Population and CD133+ cells can be delivered intra-arterially but are less efficient for muscle repair. Mesoangioblasts constitute a promising population for therapeutic use as they can be systemically delivered and display a high efficiency to regenerate muscle. Finally, other progenitors were recently identified but have not yet been tested in settings of clinical strategies.

In resting muscle, a minority of satellite cells is negative for CD34 and has never expressed Myf5 (Beauchamp, Heslop et al. 2000; Kuang, Kuroda et al. 2007). Lineage tracing study using a Myf5-Cre-stop-flox-YFP mouse model further revealed that Pax7+Myf5- satellite cells give rise to Pax7+Myf5+ satellite cells through apical-basal oriented divisions that asymmetrically generate a basal Pax7+Myf5- cell and an apical Pax7+Myf5+ cell (Kuang, Kuroda et al. 2007) (**Fig.5**). While Pax7+Myf5+ cells readily participate in fiber formation upon engraftment into damaged muscle, Pax7+Myf5- cells extensively contribute to the satellite cell reservoir. These data demonstrate that satellite cells are a heterogeneous population composed of stem cells and committed progenitors (Kuang, Kuroda et al. 2007). Asymmetric division can further be followed by DNA strand segregation in daughter cells (Shinin, Gayraud-Morel et al. 2006; Conboy, Karasov et al. 2007) as well as cytoplasmic separation of the cell fate determinant Numb (Shinin, Gayraud-Morel et al. 2006). Lastly, daughter cells inheriting the older template retained the more immature phenotype while daughter cells inheriting the newer template will ultimately acquire a more differentiated phenotype (Conboy, Karasov et al. 2007).

The self-renewing features of satellite cells combined with their extremely high efficiency to regenerate the muscle provide skeletal muscle tissue with a robust resident progenitor to support tissue integrity and maintenance throughout adult life. However, in the case of chronic muscular diseases such as Duchenne muscular dystrophy or during normal aging, the satellite cell pool loses regenerative capacity (Ervasti 2007; Vinciguerra, Musaro et al. 2010). In the previous 2 decades, trials using donor satellite cell engraftment into mouse dystrophic muscle led to a restoration of dystrophin expression generating hope for their clinical use in humans (Partridge, Morgan et al. 1989). Indeed, autologous myoblast transplantations in patients suffering from heart defects has yielded significant improvement of the heart function (Menasche 2007) but trials in boys suffering from Duchenne dystrophy failed due to very poor cell survival and lack of cell migration (Karpati, Ajdukovic et al. 1993; Mendell, Kissel et al. 1995; Fan, Maley et al. 1996; Miller, Sharma et al. 1997). In addition, brief periods of tissue culture prior to transplantation dramatically reduced satellite cell efficiency (Montarras, Morgan et al. 2005). Taken together, while

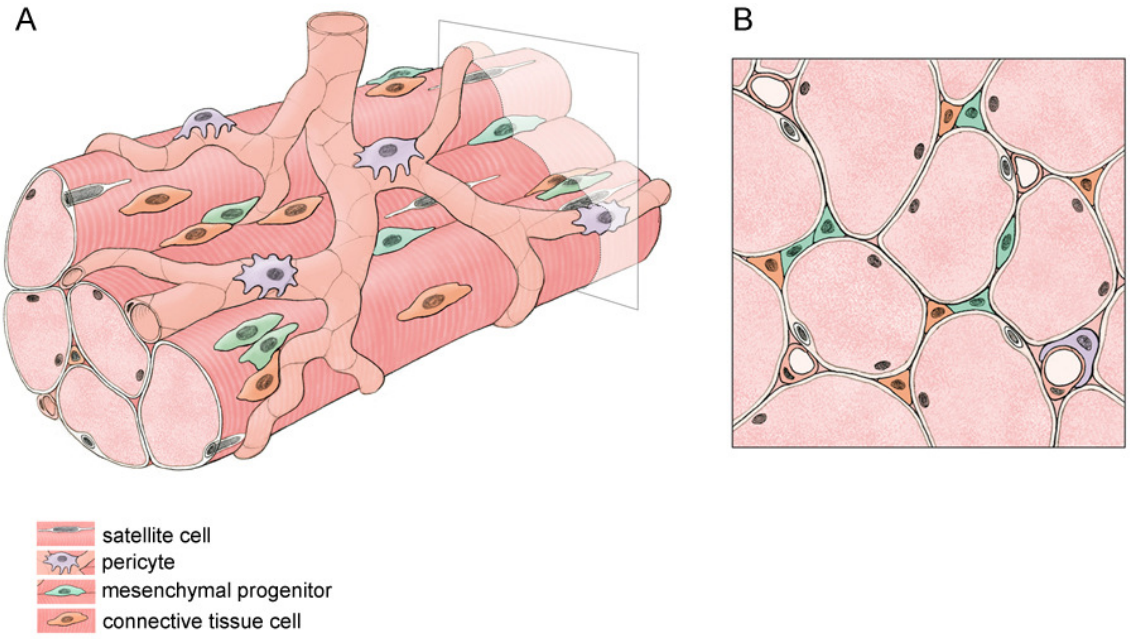

Figure 6. The skeletal muscle neighborhood. 3-dimension (**a**) or crossectional (**b**) view of adult skeletal muscle. Skeletal muscle is composed of myofibers, containing myonuclei, and satellite cells (white) that reside beneath the basal lamina and constitute the major muscle stem cell population. Blood vessels, composed of endothelial cells, permeate the interstitial space of the muscle fibers and in addition to providing a blood supply, the endothelial cells promote satellite cell proliferation through growth factors secretion and deliver circulating inflammatory cells. Pericytes (purple) actively contribute to postnatal muscle growth and regeneration. The interstitial space is occupied by mesenchymal progenitors (green) as well as connective tissue cells (orange).

satellite cells are the principal skeletal muscle progenitor, they have thus far failed to be of clinical use in muscle repair due to multiple limitations.

2.2. Non-satellite muscle progenitors

As described in the previous section, satellite cells constitute the major skeletal muscle stem cell. However, in the past 10 years, numerous non-satellite cell populations with myogenic capacity following engraftment into muscle tissue were reported (**Fig.6 and Table 1**).

Initial evidence for the existence of non-satellite cell myogenic populations came from experiments using bone marrow-derived cells transplantation into lethally irradiated mice, demonstrating the participation of these cells in skeletal muscle regeneration (Ferrari, Cusella-De Angelis et al. 1998; Gussoni, Soneoka et al. 1999). Further studies identified a sub-population of hematopoietic stem cells, termed side population (SP), isolated from bone marrow stem cells based on their ability to exclude Hoechst (Goodell, Brose et al. 1996). Following engraftment, SP cells reconstitute the hematopoietic lineage as well as give rise to new satellite cells and myofibers (Jackson, Mi et al. 1999; Asakura, Seale et al. 2002). Moreover, systemic injection into lethally irradiated *mdx* mice (a model for Duchenne Muscular Dystrophy) resulted in restoration of dystrophin expression and improved muscle function (Gussoni, Soneoka et al. 1999). More recently, it was demonstrated that SP cells transfected with the human micro-dystrophin gene and injected in the femoral artery of non-injured *mdx* mice incorporated into host muscle fibers and satellite cell compartment accompanied by transgene expression (Bachrach, Perez et al. 2006). However, the efficiency of SP cells participation in skeletal muscle formation is extremely low (**Table 1**). In humans, a sub-population of hematopoietic cells expressing the cell surface antigen CD133 displays myogenic capacity following co-culture with myoblasts (Torrente 2004). Intramuscular and intra-arterial injection of CD133+ cells into *scid/mdx* mice resulted in significant recovery of muscle morphology, function, and

dystrophin expression (Benchaouir, Meregalli et al. 2007) and is presently being tested in the clinic (Torrente, Belicchi et al. 2007).

An additional promising progenitor population for therapeutic use are mesoangioblasts (Mabs), which can be isolated from either embryonic (De Angelis, Berghella et al. 1999; Minasi 2002) or postnatal muscle vasculature (Tonlorenzi, Dellavalle et al. 2007) (**Fig.6**). Mabs display robust participation in muscle repair following engraftment or arterial delivery in both mice (Sampaolesi, Torrente et al. 2003) and dogs (Sampaolesi, Blot et al. 2006) and are presently in clinical trial. Although the anatomical location of adult Mabs is unclear, compelling data suggest they are derived from pericytes that also show robust myogenic capacity *in vitro* and *in vivo* (Dellavalle, Sampaolesi et al. 2007). Pericytes can be distinguished from endothelial cells by alkaline phosphatase (AP) expression, and do not express myogenic nor endothelial markers. Using lineage tracing, it was shown that pericytes contribute to post-natal muscle growth and regeneration *in situ* demonstrating a *bona fide* contribution to normal muscle growth by a non-satellite cell population during post-natal development (Dellavalle, Maroli et al. 2011).

We reported recently the identification of a muscle-resident population with myogenic potential located in the interstitial space and characterized by PW1/Peg3 expression, referred to as PICs (PW1+ Interstitial Cells) (**Appendix 1**) (Mitchell, Pannerec et al. 2010). PICs isolated from early postnatal muscle are able to generate new fibers, contribute to the satellite cell pool following engraftment into damaged muscle, and to give rise to more PICs (the latter property not shared by satellite cells). Lineage tracing experiments demonstrated that PICs and satellite cells do not share the same Pax3+ embryonic progenitor. Coupled with the observation that PICs can give rise to non-skeletal muscle lineages, we suggested that PICs constitute an upstream and/or more plastic stem population that plays a role during postnatal growth (Mitchell, Pannerec et al. 2010). Postnatal growth corresponds to a period of rapid muscle mass and nuclear accumulation (Young 1974) and thus, it will be important to determine the potential of PICs in the adult when stem cell properties may decline in general. A recent study defined another interstitial population of adult muscle stem cells characterized by β4-integrin expression,

which can participate in muscle repair following engraftment into *mdx* mice (Liadaki, Casar et al. 2012). Whether these β4-integrin+ cells are the same population as PICs and/or pericytes remains to be determined.

PW1/Peg3 is an imprinted gene that was identified in a differential screening approach to determine factors involved in skeletal muscle stem cell commitment (Relaix, Weng et al. 1996). Its expression initiates in early embryonic mesoderm and is down-regulated in tissues as they differentiate (Relaix, Weng et al. 1996). In postnatal skeletal muscle, PW1 expression is detected in PICs as well as satellite cells (Mitchell, Pannerec et al. 2010) and is markedly up-regulated during muscle regeneration (Nicolas, Marazzi et al. 2005; Schwarzkopf, Coletti et al. 2006). PW1 expression is also found in primary myoblasts and myogenic cell lines (Relaix, Weng et al. 1996; Nicolas, Marazzi et al. 2005; Schwarzkopf, Coletti et al. 2006). PW1 participates in TNF-NFκB signaling and, together with p53, participates in regulating intrinsic and extrinsic stress pathways in myoblasts (Relaix, Wei et al. 1998; Relaix, Wei et al. 2000; Coletti, Yang et al. 2002; Coletti, Moresi et al. 2005; Schwarzkopf, Coletti et al. 2006). Transgenic mice expressing a truncated form of PW1 (ΔPW1) that inhibits TNF and p53 signaling pathways are severely atrophic despite the presence of active Pax7+ cell clusters underneath the basal lamina, suggesting a role for PW1 in stem cell behavior and postnatal muscle growth (Nicolas, Marazzi et al. 2005). Analysis of a reporter mouse carrying a bac with the PW1/Peg3 locus driving ß-galactosidase revealed that both reporter and endogenous PW1 are co-expressed not only in muscle satellite cells and PICs, but also in stem/progenitor cells from a wide array of tissues including the gut, skin, CNS and early hematopoietic stem cells (Besson, Smeriglio et al. 2011).

Studies outlined above establish clearly that non-satellite cell progenitors are competent to contribute to muscle repair, and raise the possibility that multiple cell types support adult skeletal muscle regeneration and postnatal muscle growth. However, with the exception of pericytes, direct contribution of these progenitors to postnatal myogenesis and muscle repair has not been firmly demonstrated. Recently, two groups have created murine models in which satellite cells can be conditionally ablated to test

whether muscle regeneration is occurring in the absence of satellite cells (Lepper, Partridge et al. 2011; Sambasivan, Yao et al. 2011). In one case, the human diphteria toxin receptor was expressed under the control of the murine *Pax7* locus so that injection of diphteria toxin induces the death of cells expressing Pax7 (Sambasivan, Yao et al. 2011); and in the other study mice expressing the tamoxifen-inducible Cre under the control of Pax7 were crossed with mice expressing an inducible diphteria toxin, such that tamoxifen injection induces diphteria toxin expression in Pax7+ cells and subsequent loss of satellite cells (Lepper, Partridge et al. 2011). Both studies demonstrated that elimination of satellite cells in adult muscle markedly impairs regeneration capacity, leading to the conclusion that satellite cells are indispensable for muscle repair. It was further demonstrated that vasculature and innervation were not affected by diphteria toxin, and that other progenitors such as PICs and presumably pericytes were still present following satellite cell depletion. (Sambasivan, Yao et al. 2011). Based on these results, it would appear that non-satellite myogenic populations cannot give rise to new muscle fibers under normal physiological conditions. However, several *caveats* must be considered in the interpretation of these experiments. First, it is not clear whether the depletion of satellite cells is a neutral event. Specifically, depletion of satellite cells leads to a decrease in muscle mass in the absence of any apparent injury, thus it is possible that some stress or inflammatory processes disturb the muscle environment prior to damage. Secondly, the presence of satellite cells might be necessary to recruit non-satellite cell progenitors into the myogenic lineage through physical and/or molecular paracrine interactions. Taken together, these studies reveal that the skeletal muscle stem cell niche is complex and underline the importance of gaining a better understanding of the molecular and cellular events regulating muscle regeneration.

3. Influence of the niche on stem cell behavior

3.1. "External" signals: the action of inflammatory cells

Satellite cell behavior can be modulated significantly by the presence of growth factors, neighboring cells, and surrounding cellular matrix. It has been shown that even

brief periods of satellite cell growth *in vitro* leads to a marked reduction in their myogenic and self-renewal capacities as compared to freshly isolated satellite cells (Montarras, Morgan et al. 2005). Blau and colleagues further demonstrated that a more elastic cell substrate (in contrast to 'hard' plastic) increased primary myoblast self-renewal and regenerative capacity when grafted into injured muscle (Gilbert, Havenstrite et al. 2010).

In addition to the physical properties of the satellite cell environment, cytokines and other diffusible molecules produced by surrounding cells are important. Christov and colleagues observed that satellite cells are invariably located near small vessels, and that endothelial cells promote satellite cell proliferation through IGF-1, FGF, HGF and VEGF secretion (Christov, Chretien et al. 2007). In turn, differentiating myoblasts promote angiogenesis. They proposed that a positive feedback between these two cell types promotes faster and properly patterned tissue repair. Following muscle damage, pro-inflammatory monocytes accumulate at the site of injury and clear away cellular debris. This step is followed by a conversion of monocytes into anti-inflammatory macrophages, mediated by the p38-MKP1 balance, that stimulate myogenesis and fiber growth (**Fig.7**) (Arnold, Henry et al. 2007; Perdiguero, Sousa-Victor et al. 2011). When inflammatory monocytes are depleted at the time of injury, muscle regeneration is completely abrogated due to persistence of necrotic fibers (Arnold, Henry et al. 2007). These experiments demonstrated the crucial role of inflammatory cells in muscle repair and the importance of cellular debris removal. In addition to promote myogenesis, macrophages exert an anti-apoptotic effect on myoblasts and newly formed myotubes by direct cell contact through cell adhesion molecules associations such as VCAM-1/VLA-4 or PECAM-1/PECAM-1 (Sonnet, Lafuste et al. 2006). Finally, macrophages induce mesoangioblasts migration to the site of injury through HMGB1, TNFα and MMP9 secretion (Lolmede, Campana et al. 2009).

Taken together, these data demonstrate vascular-mediated paracrine effects on the stem cell niche that play a key role in coordinating stem cell responses, leading to the proposal of a transient vascular amplifying/differentiating niche in the regeneration process (Abou-Khalil, Le Grand et al. 2009; Mounier, Chretien et al. 2011).

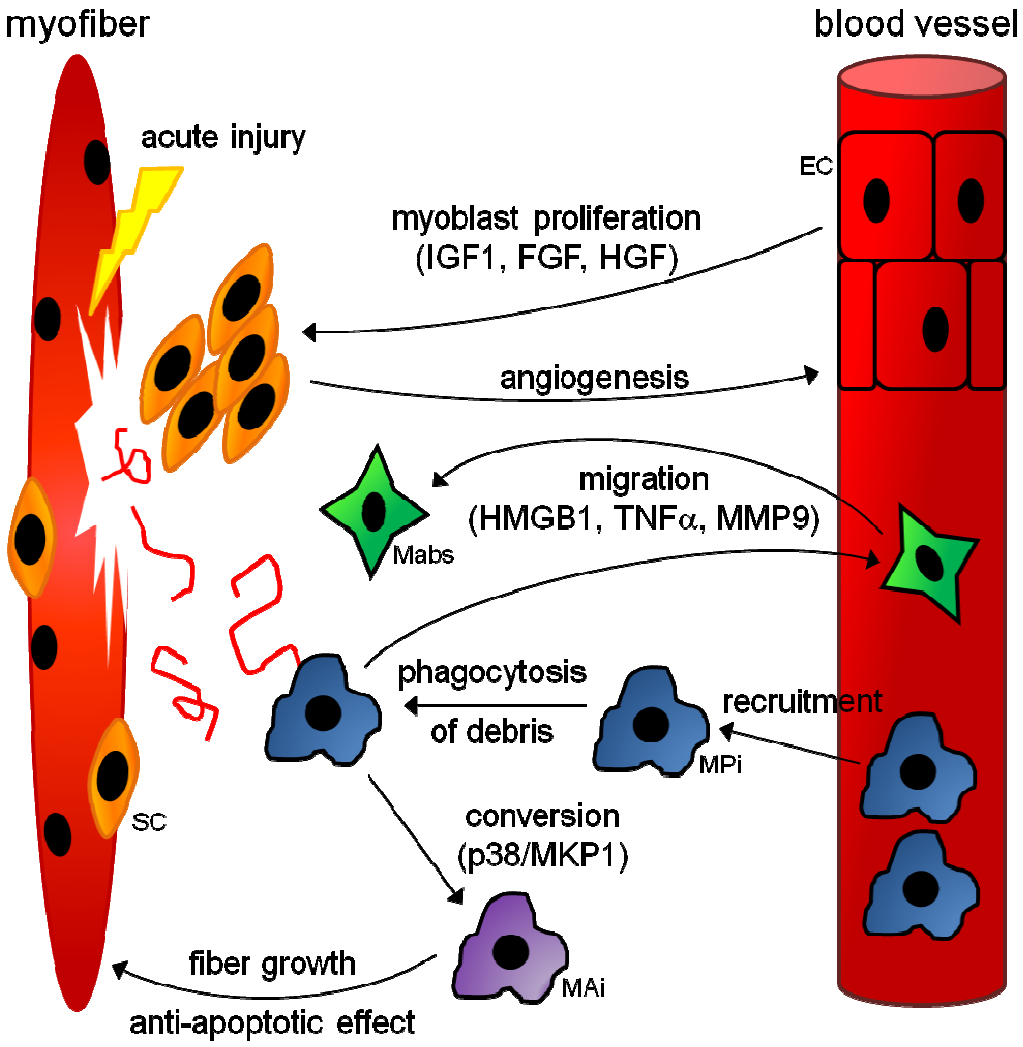

Figure 7. Interactions between inflammatory cells and muscle cells following injury. Acute muscle damage causes release of chemoattractant molecules that initially attract pro-inflammatory macrophages (MPi) into the muscle. MPi remove cellular debris by phagocytosis, attract mesoangioblasts (Mabs) to the site of injury, and convert to anti-inflammatory macrophages (MAi) through p38/MKP1 modulation. In the meantime, vascular endothelial cells (EC) activate satellite cells (SC) proliferation by producing growth factors such as IGF1. SC in turn promote angiogenesis for vascular repair, while MAi promote muscle repair by protecting newly formed fibers from apoptosis.

3.2. "Internal" signals: the myogenic/adipogenic balance

In addition to vascular cells, muscle-resident interstitial cells also influence muscle regeneration. First, connective tissue fibroblasts identified by Tcf4 expression proliferate in close proximity to satellite cells following injury (Murphy, Lawson et al. 2011). Conditional ablation of Tcf4+ cells prior to muscle damage leads to smaller regenerated fibers with fewer satellite cells due to premature differentiation, suggesting that fibroblasts normally provide signals that regulate satellite cell expansion during regeneration (Murphy, Lawson et al. 2011). This was further confirmed by *in vitro* experiments in which myoblasts cultured in the presence of Tcf4+ fibroblasts form larger myotubes containing more nuclei (Mathew, Hansen et al. 2011), and by the observation that C2C12 form more mature myotubes with better survival when cultured on a fibroblast feeder-layer (Cooper, Maxwell et al. 2004). In turn, satellite cells ablation not only leads to a complete loss of muscle regeneration, as observed by others, but also to a defect in fibroblast recruitment at the onset of regeneration followed by connective tissue expansion (Murphy, Lawson et al. 2011). Taken together, these results demonstrate that satellite cells and fibroblasts reciprocally regulate the expansion of each other to ensure efficient muscle repair. It was further proposed that fibroblasts and atypical myogenic stem cells are recruited to the site of injury by factors secreted by satellite cells (Wang and Rudnicki 2012).

Adipogenic progenitors also participate in muscle homeostasis and regeneration. Recently, two populations with similar characteristics have been identified in the interstitial space of skeletal muscle. Mesenchymal progenitors are characterized by PDGFRα expression (Uezumi, Fukada et al. 2010), and fibro/adipogenic progenitors (FAPs) have been isolated upon Sca1 expression (Joe, Yi et al. 2010). These cells display a strong adipogenic potential *in vitro* and differentiate into adipocytes in models favoring fat deposition. In addition, FAPs become activated upon injury and promote myoblast differentiation through cell-cell signaling (Joe, Yi et al. 2010) whereas the adipogenic fate of PDGFRα+ cells is strongly inhibited by myotubes (Uezumi, Fukada et al. 2010). It is tempting to speculate that mesenchymal PDGFRα+ cells and FAPs are overlapping

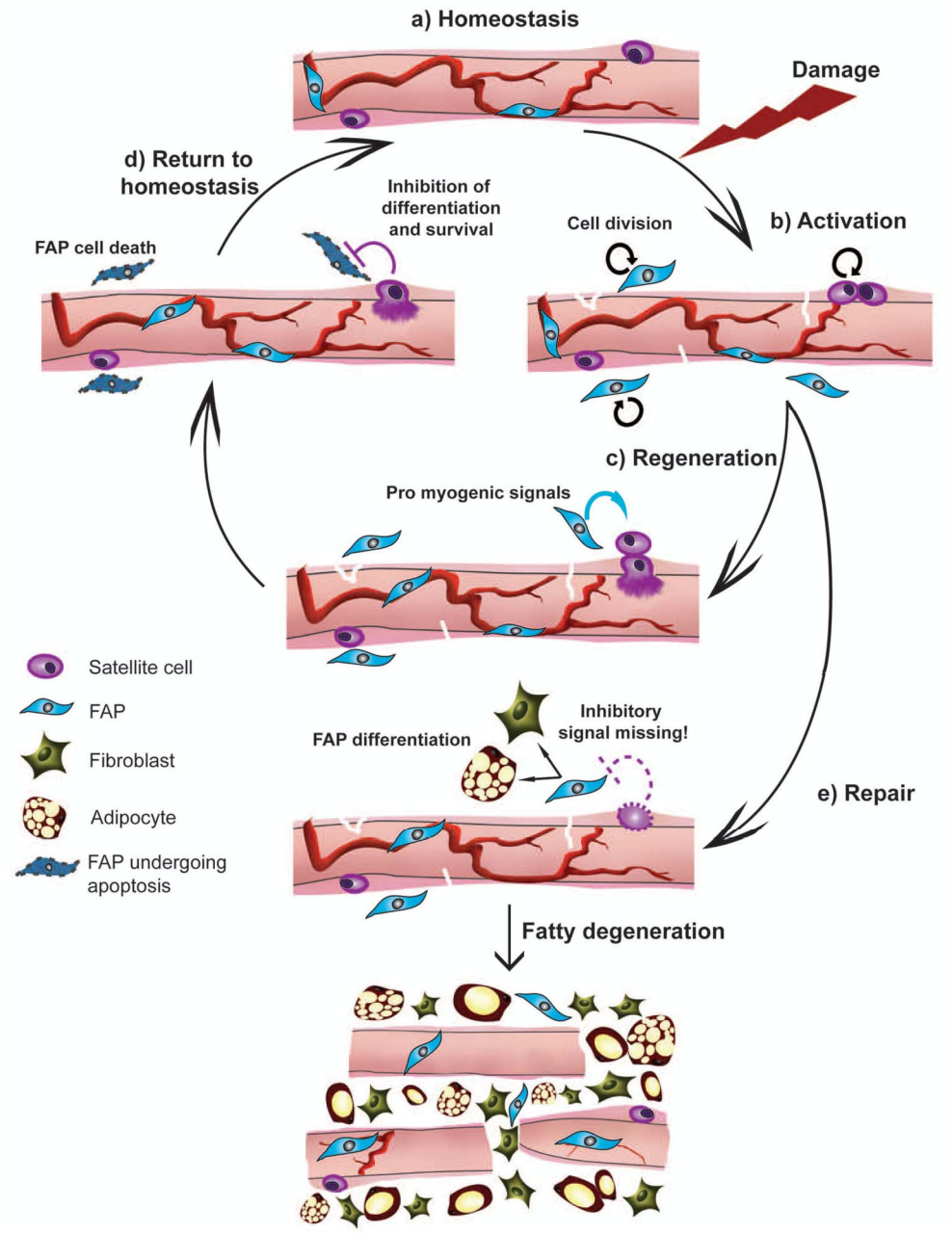

Figure 8. Multiple roles of FAPs during muscle regeneration and repair. (**a**) At muscle homeostasis, both satellite cells and FAPs are quiescent. (**b**) Following injury, both satellite cells and FAPs are activated and start to proliferate. (**c**) FAPs provide trophic factors to support satellite cell differentiation. (**d**) Following successful regeneration, satellite cells induce FAPs apoptosis and muscle returns to quiescence. (**e**) Following unsuccessful regeneration, FAPs differentiate into adipocytes and muscle becomes infiltrated with fat. *(adapted from (Natarajan, Lemos et al. 2010)).*

populations, nonetheless, these adipogenic progenitors share the property of adopting different fates depending on the surrounding environment as well as promote differentiation of neighboring myogenic progenitors (Joe, Yi et al. 2010). The authors propose that a balance between satellite cell-dependent myogenesis and PDGFRα+ cells/FAPs dependent adipogenesis regulates muscle homeostasis and regeneration (**Fig.8**). Another recent study demonstrated that the combined injection of fetal CD34+ myogenic, adipogenic, and angiogenic progenitors into damaged muscle leads to improved regeneration capacity compared to the injection of the myogenic fraction alone (Dupas, Rouaud et al. 2011). Taken together, these data provide a clear demonstration of interactions between multiple resident cell populations that promote activation of muscle progenitors.

In conclusion, a more complete picture is emerging in which efficient muscle regeneration requires the coordinated interactions of multiple cell types. Satellite cells, atypical myogenic progenitors and non-myogenic populations provide transient supportive niches that promote muscle repair by producing trophic signals favoring myoblast specification and tissue reconstitution. However, the relationship between these diverse cell types remains to be further clarified.

CHAPTER 2. MECHANISMS REGULATING SKELETAL MUSCLE MASS

1. **Changes in skeletal muscle mass throughout life**
    - Muscle hypertrophy during postnatal growth and physical training
    - Age and disease-related loss of muscle mass

2. **The major signaling pathways regulating muscle size**
    - Myostatin, a potent inhibitor of muscle mass
    - Myostatin non-canonical pathways
    - A myostatin endogenous antagonist: follistatin

3. **Pharmaceutical approaches to induce skeletal muscle hypertrophy**
    - Targeting muscle mass via myostatin inhibition
    - Satellite cells and muscle hypertrophy

1. Changes in muscle mass throughout life

1.1. Muscle hypertrophy during postnatal growth and physical training

Upon birth all skeletal muscle tissues are formed and the number of myofibers is set, such that postnatal growth occurs by a process of fiber hypertrophy. Some exceptions notably exist in several lower vertebrates, such as fish, that undergo continuous postnatal growth through both hypertrophy and hyperplasia processes (Mommsen 2001; Steinbacher, Haslett et al. 2007). Organ growth occurs when the rate of protein synthesis is higher than the rate of protein degradation. In skeletal muscle, accretion of progenitors is more rapid compared to other tissues and therefore the proportion of muscle proteins relatively to total body proteins is significantly increased (about 45% in rats) (**Fig.9**) (Davis and Fiorotto 2009). Skeletal muscle postnatal growth is allowed by intense proliferation and differentiation of satellite cells which provide additional nuclei to myofibers (Shafiq, Gorycki et al. 1968; Moss and Leblond 1971; Schultz 1996; Davis and Fiorotto 2009). The dependence of neonatal muscle hypertrophy on satellite cells has been demonstrated by the study of *Pax7-null* mice, which possess a normal pool of satellite cells at birth but their number rapidly declines leading to severely impaired muscle growth and survival (Seale, Sabourin et al. 2000; Oustanina, Hause et al. 2004).

Postnatal myofiber hypertrophy occurs in 2 phases. In the first phase, between birth and 3 weeks old, the number of myonuclei per fiber rapidly increases by fusion of satellite cells and the myofiber volume is multiplied by 5 (White, Bierinx et al. 2010). About 80% of satellite cells in neonatal muscle are proliferating (Schultz 1996; White, Bierinx et al. 2010), mostly under the activation of IGF-1 (Adams 1998; Shavlakadze, Chai et al. 2010). IGF-1 enhancing effect on muscle growth has been demonstrated in mice over-expressing IGF-1, which display enhanced postnatal muscle growth and protein accretion leading to significantly bigger muscles (Fiorotto, Schwartz et al. 2003). Before 9 days old, the number of satellite cells per fiber is similar to the rate of myonuclear addition per day (~13 cells) (White, Bierinx et al. 2010). Since the satellite cell number was found to be relatively stable over this period, the authors propose that most satellite cells are undergoing asymmetric

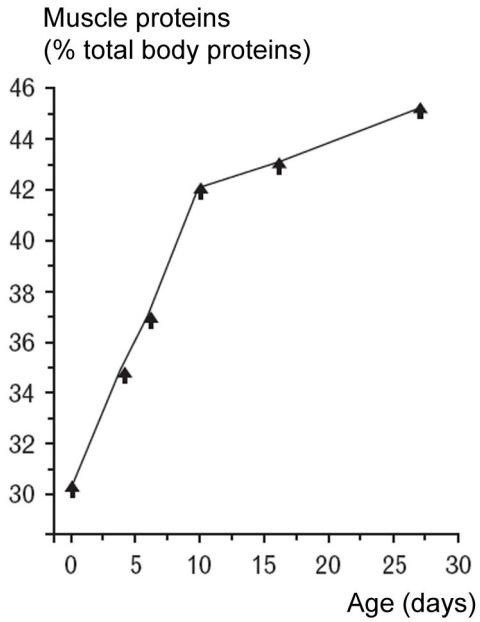

Figure 9. Relative changes in the proportion of whole-body protein mass attributable to skeletal muscle protein in the rat between birth and weaning. The more rapid accretion of muscle proteins than other tissue proteins results in a substantial increase in the proportion of the body protein pool that is muscle protein. *(From (Davis and Fiorotto 2009)).*

divisions, each giving rise to both one myonucleus and one new satellite cell, as first suggested by Moss and Leblond in 1971 (Moss and Leblond 1971). Between 2 and 3 weeks old, the available number of satellite cells decreases until the adult configuration is established. As such, by 3 weeks of age, the adult number of myonuclei and satellite cells is already established in the mouse muscle. In the second phase of postnatal myofiber hypertrophy, from 3 weeks old to adulthood, the myofiber volume is multiplied by 3 through expansion of the myonuclear domain without addition of new myonuclei (Mantilla, Sill et al. 2008; White, Bierinx et al. 2010). Down-regulation of IGF-1 and HGF (Alexandrides, Moses et al. 1989; Jennische, Ekberg et al. 1993) concomitant with the up-regulation of inhibitors such as myostatin (Suryawan, Frank et al. 2006) induce satellite cells to exit the cell cycle and enter quiescence.

In adult muscle, fiber hypertrophy does not spontaneously occur but can be activated by physical exercise. In humans, muscle fiber size significantly increases following 3 months of training but quickly goes back to its original size when the individual stops any physical activity (Kadi, Schjerling et al. 2004). Even though activated satellite cells can be detected in skeletal muscle as soon as one day following the onset of physical exercise (Walker, Fry et al. 2012), increase in the number of myonuclei per fiber is observed only following several months of physical training (Kadi, Schjerling et al. 2004; Petrella, Kim et al. 2008). These data demonstrate that myofibers possess a high plasticity in the short-term, but prolonged training is needed to achieve sustainable fiber hypertrophy. Physical exercise-induced satellite cell proliferation is activated by a splice-variant of IGF-1 termed mechano-growth factor (MGF), which expression appears a few days after the training started (Goldspink 2005). MGF functions as an early autocrine growth factor to activate satellite cell proliferation, but not differentiation (Yang and Goldspink 2002). After initial splicing leading to MGF formation, the IGF-1 gene product undergoes further splicing generating the IGF-1EA isoform which stimulates satellite cell differentiation and fusion (**Fig.10**) (Hill, Wernig et al. 2003; Jacquemin, Furling et al. 2004). In addition to IGF-1, HGF and nitric oxide are also capable to activate resting satellite cells (Wozniak, Kong et al. 2005) while FGF stimulates their proliferation (Kastner, Elias et al. 2000).

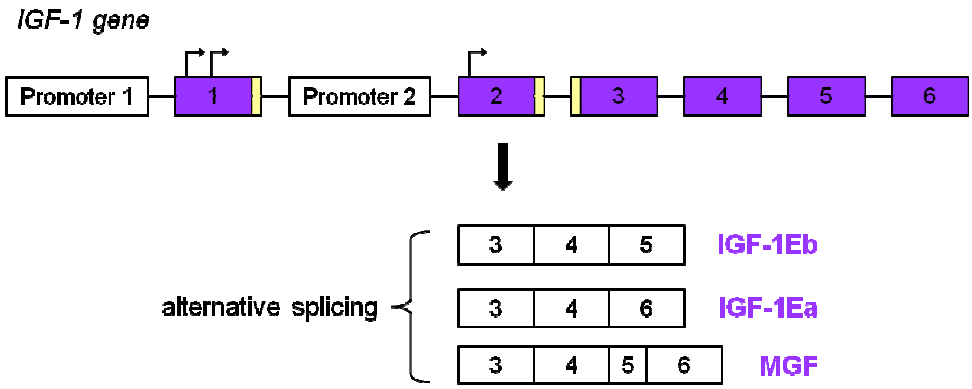

Figure 10. Schematic representation of the rodent IGF-1 gene. IGF-1 gene is composed of six exons (purple boxes), separated by introns (black lines). Multiple transcription start sites are present on exons 1 and 2. Exons 1, 2 and 3 code for the signal peptide of precursor IGF-1. Exons 5 and 6 each encode either IGF-1Eb or IGF-1Eb, respectively. *(Adapted from (Lee 2007)).*

1.2. Age and disease-related loss of muscle mass

Increasing skeletal muscle mass is a hallmark of postnatal growth or physical training. The reverse phenomenon, i.e a loss of muscle mass, occurs following long-term inactivity, disease, or as the organism is aging. The most significant cases of muscle atrophy are associated with muscular dystrophies, which are characterized by a progressive muscle weakness due to genetic mutations in sarcolemmal proteins (dystrophin, sarcoglycans and dysferlin), nuclear proteins (emerin and lamin A/C) or extracellular proteins (collagen-6 and alpha-2 laminin) (**Fig.11**) (Cohn and Campbell 2000). Most of these diseases result in weakened myofiber integrity followed by fiber degeneration, triggering repeated cycles of degeneration/regeneration. These repeated cycles of regeneration are proposed to eventually result in an exhaustion of the stem cell pool leading to a myopathic presentation in which the muscle tissue is infiltrated with fibrotic and fat tissue. Chronic stem cell activation has been linked with telomere shortening and has been proposed to induce mutations in key regulatory genes required for proper self-renewal and myogenic competence (Sacco, Mourkioti et al. 2010). While stem cell dysfunction only occurs in genetic disorders, skeletal muscle atrophy is more generally characterized by the over-expression of two E3-ubiquitin ligases, the Muscle Atrophy F-box (MAFbx) and Muscle Ring Finger 1 (MuRF1) (Bodine, Latres et al. 2001). MuRF1 acts on sarcomeres by degrading proteins of the thick filament (myosins) (Clarke, Drujan et al. 2007; Cohen, Brault et al. 2009), while MAFbx is thought to act on substrates that have been previously phosphorylated (Jackson and Eldridge 2002). MAFbx transcription is activated by Foxo3, which activity is repressed under normal physiological conditions but increases in case of muscle wasting (Lecker, Jagoe et al. 2004; Sandri, Sandri et al. 2004). Then MAFbx targets the eukaryotic initiation factor 3 subunit 5 (eIF3-f), normally inducing the expression of muscle structural proteins, for ubiquitination and degradation by the proteasome (Lagirand-Cantaloube, Offner et al. 2008). Some reports also demonstrated MAFbx interaction with MyoD, suggesting that its degradation also accounts for muscle atrophy (Tintignac, Lagirand et al. 2005; Lagirand-Cantaloube, Cornille et al. 2009).

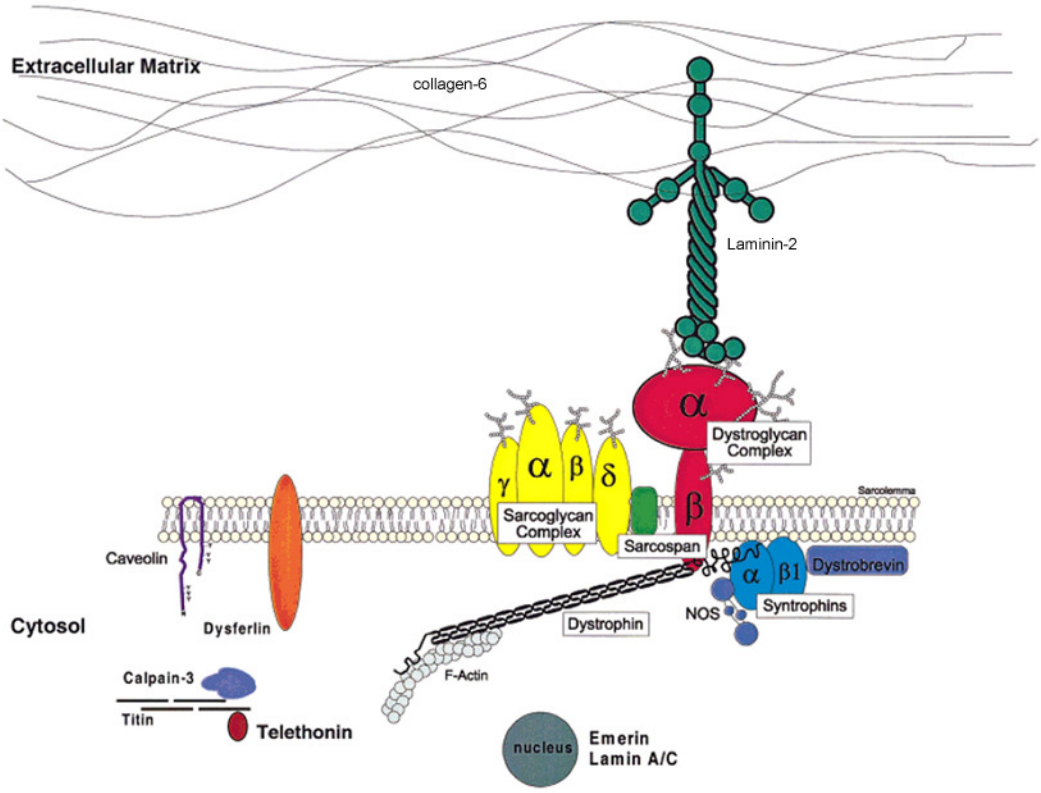

Figure 11. The molecular organization of integral and peripheral components of the dystroglycan complex. Muscular dystrophies are characterized by progressive muscle weakness due to mutations in the sarcolemmal proteins (dystrophin, sarcoglycans, dysferlin), nuclear proteins (Emerin, lamin A/C) or extracellular proteins (collagen-6, alpha-2 laminin) *(Adapted from (Cohn and Campbell 2000))*.

Age-related loss of muscle mass, termed sarcopenia, is characterized by a reduction of the myofiber volume and a progressive expansion of fat and fibrous tissue. As stated previously, the number of satellite cells represents about 30% of myonuclei in the neonate, declines to 4% in the adult, and represents only 2% of nuclei in the old mouse (Shefer, Van de Mark et al. 2006). Collins and colleagues demonstrated that aging affects satellite cells intrinsically, with a massive loss of Pax7 expression leading to a loss of myogenicity accompanied with a high susceptibility to undergo apoptosis (Collins, Zammit et al. 2007). Our lab has demonstrated recently that satellite cells undergo pronounced telomere shortening with age suggesting that even in the absence of repeated stem cell activation, changes occur at the level of the muscle stem cells (Didier, Hourdé et al, in press). Still, several studies have demonstrated that aged satellite cells retain myogenic and self-renewing capacities comparable to young cells, suggesting that the overall myogenic potential is not lost with age but that systemic factors account for these changes (Shefer, Van de Mark et al. 2006; Carlson and Conboy 2007; Collins, Zammit et al. 2007). Indeed, an age-related delay in the early inflammatory response following muscle injury was reported in mice, although regeneration still occurs presumably as this delay is not very long (Smythe, Shavlakadze et al. 2008). *In vivo*, satellite cell proliferative and regenerative capacities are restored in old mice when exposed to circulating factors from young mice (**Fig.12**) (Conboy, Conboy et al. 2005). It has been reported that activation of the canonical Wnt pathway in aged satellite cells elicits a conversion to a fibrogenic lineage. Following injection into young mice, serum obtained from old mice provokes an 'old age' regenerative phenotype in muscle which has been attributed to the presence of Wnt molecules, activating downstream targets such as Axin2 and β-catenin (Brack, Conboy et al. 2007). Conversely, canonical Wnt signaling inhibition by Frizzled-related protein 3 or DKK1 in old mice restores the regenerative potential (Brack, Conboy et al. 2007). Wnt molecules may be secreted by tissue-resident endothelial precursors in old muscle. Specifically, endothelial precursors are activated during neo-angiogenesis following muscle injury and inhibit myoblast proliferation through Wnt3a production (Grenier, Scime et al. 2007; Trensz, Haroun et al. 2010). In addition, impaired muscle regeneration in aged animals has been linked to a decline in Notch signaling (Conboy, Conboy et al. 2003). Insufficient up-

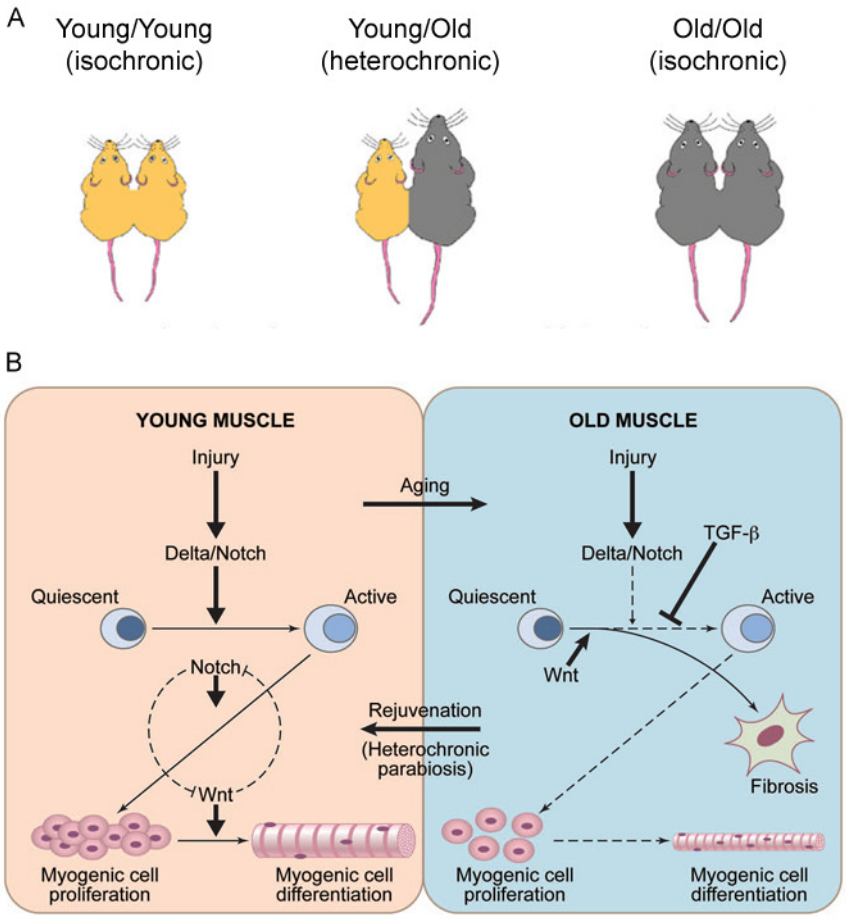

Figure 12. Aging and rejuvenation of adult myogenesis. (**a**) Parabiotic pairings (i.e shared circulatory system) between two young, two old, or one young and one old mouse allowed Conboy and colleagues to demonstrate the importance of the age of the environment on stem cell function (**b**) Comparison between young and old myogenesis. In young muscle, injury induces an up-regulation of the Notch ligand Delta-1, which activates Notch signaling. Active Notch promotes a G_0 to G_1 transition in muscle stem cells. Interplay between Notch and Wnt controls myogenic cell proliferation and differentiation. In old muscle insufficient activation of Notch pathway following injury, combined with an excess of TGFβ and Wnt signaling, leads to a suppression of myogenesis and a promotion of fibrosis. Notch, Wnt and likely other signaling pathways are regulated by systemic factors, such that in the aged environment, systemic signals contribute to the activities that inhibit myogenesis and promote fibrosis. By contrast, in the setting of heterochronic parabiosis (a), young systemic factors restore more youthful states and are able to rejuvenate the aged stem cells and stem cell niches to promote enhanced muscle regeneration. *(Adapted from (Kirk, Oldham et al. 2000) and (Spiller, Kambadur et al. 2002)).*

regulation of the Notch ligand Delta-1 in satellite cells following injury in old animals leads to a decrease in their myogenic capacities, which can be restored by forced activation of Notch (**Fig.12**). In turn, *in vivo* inhibition of Notch signaling inhibits muscle regeneration in young mice and results in a phenotype similar to that of old muscle (Conboy, Conboy et al. 2003). In addition, Notch signaling has been recently shown to be necessary for satellite cell maintenance, such that satellite cells undergo accelerated terminal differentiation without self-renewal in the absence of Notch signaling, resulting in satellite cell depletion (Bjornson, Cheung et al. 2012). Loss of Notch activation in aged muscle has been associated with high levels of TGFβ-1 and Cdk inhibitors in satellite cells (Carlson, Conboy et al. 2009). Abnormally high levels of phospho-Smad3 in aged satellite cells impair regenerative capacity, whereas regenerative competence can be restored *in vivo* by pSmad-3 shRNA blockade. Activation of Notch blocks the phospho-Smad3 mediated up-regulation of the Cdks p15, p16, p21 and p27, whereas inhibition of Notch induces these Cdks (Carlson, Hsu et al. 2008; Carlson, Conboy et al. 2009). Therefore, endogenous Notch and phospho-Smad3 antagonize each other in the control of satellite cell proliferation, and deregulation of this balance in old animals leads to regenerative potential decrease. Taken together, these data reveal that sarcopenia is primarily due to a progressive change in the stem cell environment rather than intrinsic loss of myogenic cells competence although some intrinsic changes are observed.

2. The major signaling pathways regulating muscle size

2.1. Myostatin, a potent inhibitor of muscle mass

Myostatin, also called GDF-8, is one of the most potent muscle mass inhibitors identified to date (McPherron, Lawler et al. 1997). Myostatin belongs to the TGFβ superfamily of secreted molecules regulating cell proliferation, differentiation, migration, adhesion and apoptosis (Kollias and McDermott 2008). The TGFβ family consists of over 50 structurally related ligands, which can be divided into 3 categories: TGFβs, Bone Morphogenic Proteins (BMPs) and activin/inhibins. As seen for other TGFβ family members, myostatin is secreted as a latent form (McPherron, Lawler et al. 1997). Following removal of

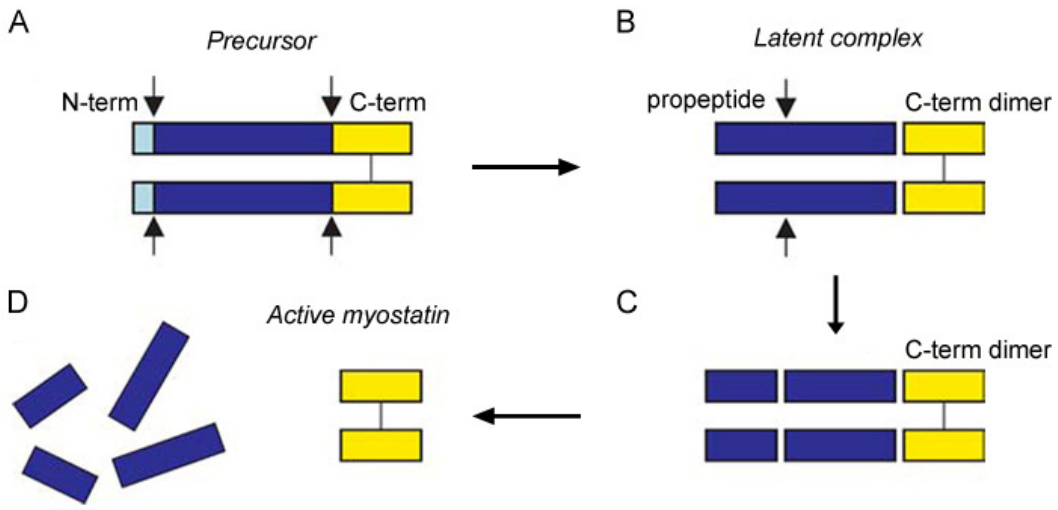

Figure 13. Processing of the myostatin protein. (**a**) Myostatin is synthesized as a precursor form that undergoes two proteolytic processing events; one removes the N-terminal signal sequence (light blue), and the second generates the C-terminal fragment (yellow), which possesses receptor binding activity. (**b**) Following proteolytic processing, the propeptide (blue) and the disulfide-linked C-terminal dimer (yellow) remain bound non-covalently in a latent complex. (**c,d**) Activation of latent myostatin occurs by proteolytic cleavage of the propeptide by members of the BMP-1/tolloid family of metalloproteinases, which causes dissociation of the latent complex. *(Adapted from (Sandri, Sandri et al. 2004)).*

the N-terminal propeptide, myostatin forms disulfide-linked dimers that represent the only active form of the protein (**Fig.13**). However the majority of myostatin circulating in the serum is bound to its propeptide as part of a latent complex (Hill, Davies et al. 2002).

Myostatin expression initiates in the somites of mouse embryo, and becomes restricted to cardiac and skeletal muscle in the adult but it can also be detected in adipose tissue and mammary gland (McPherron, Lawler et al. 1997; Ji, Losinski et al. 1998; Sharma, Kambadur et al. 1999). Its inhibitory effect on skeletal muscle growth was initially reported by Lee and colleagues, who observed that *myostatin-null* ($Mst^{-/-}$) mice are about 30% larger than their wildtype (WT) littermates due to a 2-3 fold increase in skeletal muscle mass (**Fig.14**) (McPherron, Lawler et al. 1997). Numerous species ranging from mouse to humans display a massive increase in muscle mass and size in response to a loss of function of the myostatin gene (Grobet, Martin et al. 1997; Kambadur, Sharma et al. 1997; Schuelke, Wagner et al. 2004; Clop, Marcq et al. 2006; Shelton and Engvall 2007). Histological analyses of muscles of $Mst^{-/-}$ mice showed that this increase in muscle mass results from a combination of increased muscle fiber number (hyperplasia) and increased fiber size (hypertrophy) (McPherron, Lawler et al. 1997). Complementary studies demonstrated that myostatin plays a role in regulating skeletal muscle mass in the embryo and in the adult. It was shown that $Mst^{-/-}$ primary myotubes number and size are significantly increased compared to WT at E14.5, and the secondary to primary fiber ratio is increased at E18.5 so that $Mst^{-/-}$ embryos harbor 87% of their adult fiber number compared to only 73% in WT embryos (Matsakas, Otto et al. 2010). This accelerated myogenic program is due to an expansion of Pax7+ progenitors at E14.5, demonstrating myostatin role in embryogenesis (Matsakas, Otto et al. 2010). In adult mice, conditional inactivation of myostatin leads to a generalized muscular hypertrophy of the same magnitude as that observed for constitutive $Mst^{-/-}$ mice (Grobet, Pirottin et al. 2003), while myostatin over-expression leads to a massive decline in body weight due a skeletal muscle atrophy and loss of adipose tissue, demonstrating myostatin role in controlling adult muscle homeostasis (Zimmers, Davies et al. 2002; Reisz-Porszasz, Bhasin et al. 2003).

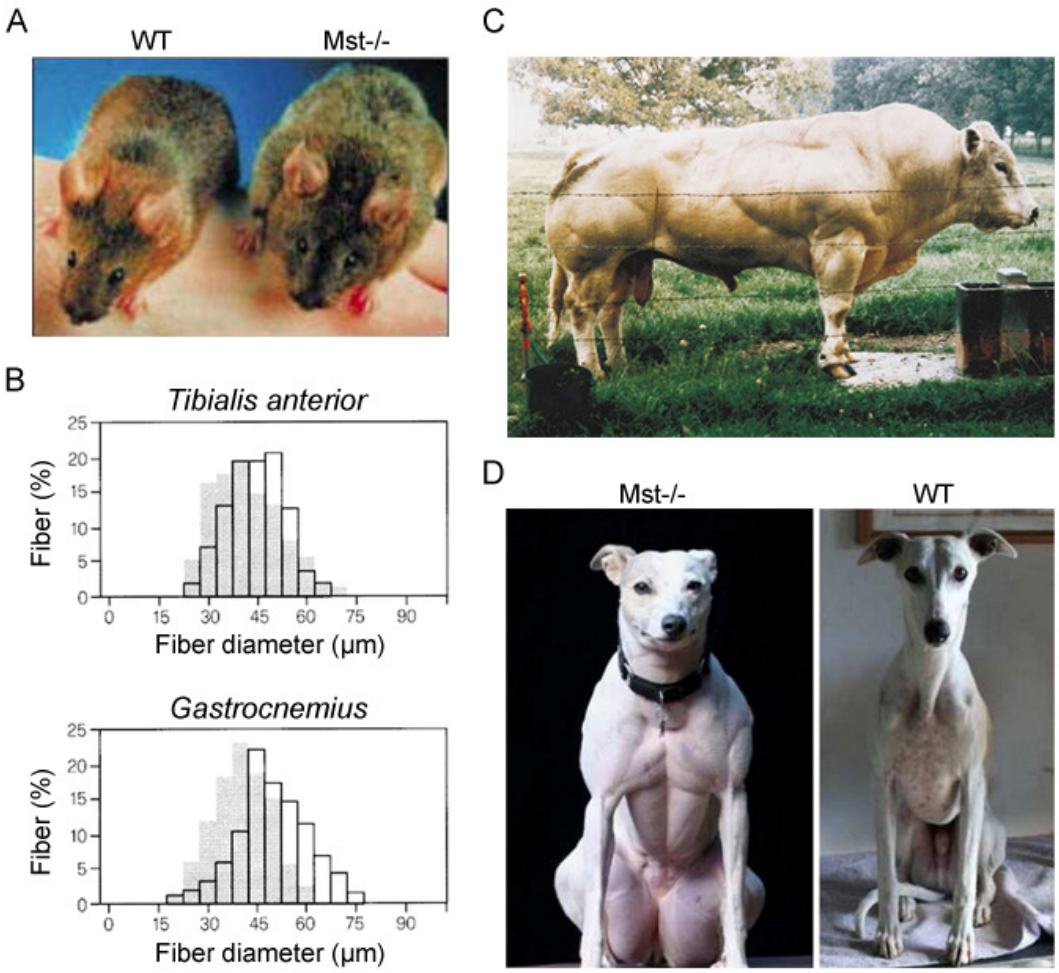

Figure 14. Myostatin mutation leads to hyper-muscled phenotype. Mice in which the myostatin gene has been genetically knocked out ($Mst^{-/-}$) are twice as big as their wildtype (WT) littermates (**a**) due to increased skeletal myofiber size (**b**). Natural mutations occurring in the myostatin gene have been reported in several species including cattle (**c**) and dog (**d**) *(Adapted from (Shelton and Engvall 2007), (Lecker, Jagoe et al. 2004), (McPherron and Lee 1997) and (McPherron, Lawler et al. 1997)).*

As observed for other TGFβ molecules, myostatin acts through a canonical signaling pathway which initiates by its binding to a type 2 transmembrane serine/threonine kinase receptor (Schmierer and Hill 2007). Myostatin binding to its specific activin receptor type 2a (ActR2a) or 2b (ActR2b) leads to the association with its corresponding type 1 receptor, Alk4 or Alk5 (Lee and McPherron 2001). This activated heterotetrameric receptor complex transphosphorylates the type 1 receptor, activating the latent kinase activity of the receptor complex. The signal is propagated inside the cell when type 1 receptors phosphorylate Smad2 and Smad3 proteins, which then form heteromeric complexes with co-Smads such as Smad4 and translocate to the nucleus to control gene expression (**Fig.15**) (Zhu, Topouzis et al. 2004). This intracellular signaling is inhibited by Smurf1 and Smad7, the latter being activated by myostatin itself presumably to terminate the signaling through a negative regulatory feedback (Zhu, Topouzis et al. 2004).

To date, published studies have focused upon the effect of myostatin on the myogenic C2C12 cell line, as well as murine and bovine myoblasts obtained from pre-plating or fluorescent activated cell sorting (FACS) techniques. It is well accepted that myostatin inhibits myoblast proliferation and differentiation *in vitro*, for concentrations ranging from 2 to 10µg/mL (Thomas, Langley et al. 2000; Taylor, Bhasin et al. 2001; Langley, Thomas et al. 2002). Myostatin inhibits the progression of myoblasts from the G_1 to S phase of the cell cycle by up-regulating the Cdk inhibitor p21 and reducing Cdk2 activity (Thomas, Langley et al. 2000; Joulia, Bernardi et al. 2003), resulting in a suppression of retinoblastoma protein (Rb) phosphorylation and concurrent cell cycle arrest in G_1 (Thomas, Langley et al. 2000). When differentiating myoblasts are exposed to myostatin, MyoD, Myf5 and myogenin are inhibited resulting in a block in myogenic progression (Langley, Thomas et al. 2002; Rios, Carneiro et al. 2002; Joulia, Bernardi et al. 2003). MyoD inhibition is mediated through Smad3, presumably by direct interaction between Smad3 and the *MyoD* promoter leading to transcriptional repression, as previously reported for TGFβ signaling (Liu, Black et al. 2001; Langley, Thomas et al. 2002; McFarlane, Plummer et al. 2006). However myostatin inhibitory effects on myoblasts are reversible upon removal

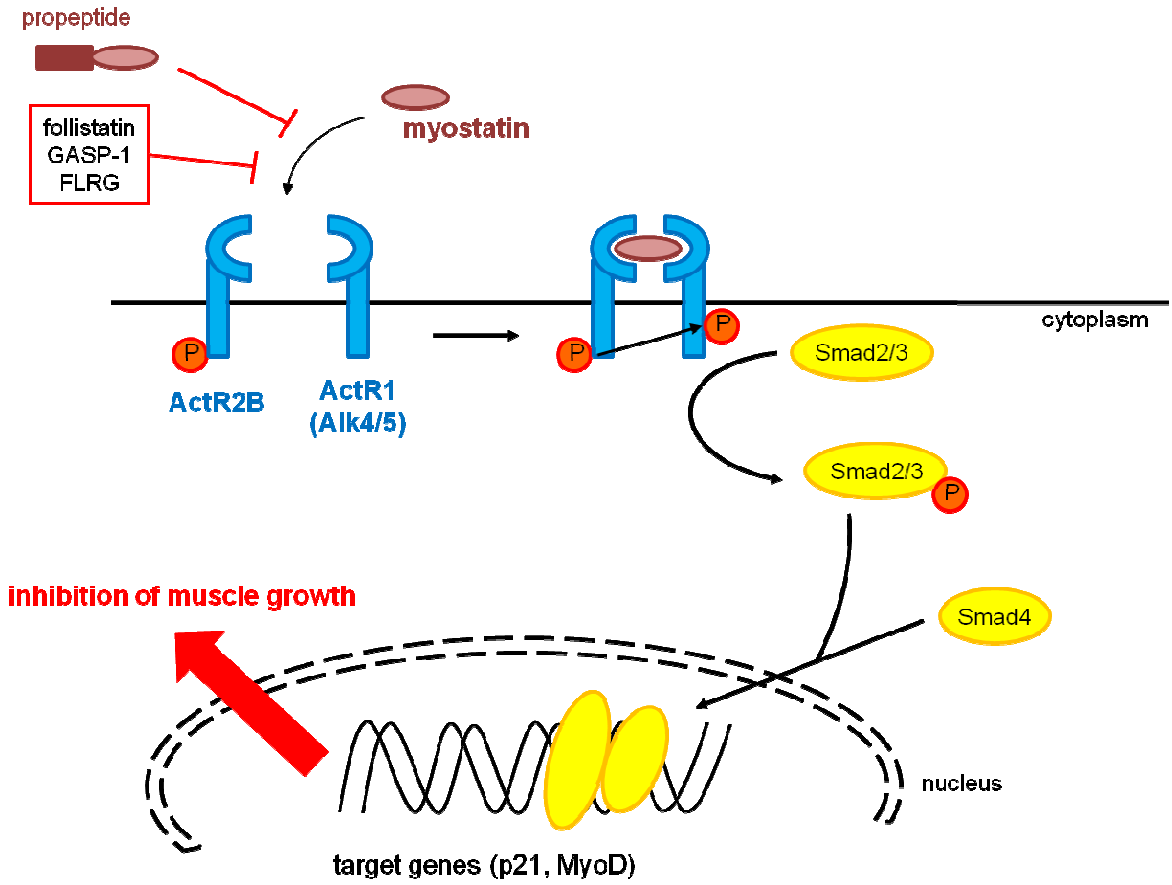

Figure 15. Myostatin canonical signaling pathway. Myostatin binding to its transmembrane receptor ActR2b leads to heterodimerization with type 1 receptor (Alk4 or Alk5). Transphosphorylation of ActR1 by ActR2 stimulates the serine/threonine kinase activity of ActR1, inducing phosphorylation of Smad2 and Smad3. Smad proteins form a complex which co-Smads such as Smad4 and translocate to the nucleus to regulate transcription of genes such as p21 and MyoD. Myostatin binding is inhibited by antagonists such as follistatin or its own propeptide.

from the culture medium (Thomas, Langley et al. 2000; Langley, Thomas et al. 2002; Rios, Carneiro et al. 2002). Taken together, these results show that myostatin inhibits myogenesis by impairing cell cycle progression and myogenic commitment. It is of interest that satellite cells and myoblasts express myostatin (Thomas, Langley et al. 2000; McCroskery, Thomas et al. 2003) which presumably acts in an autocrine manner to regulate their proliferation, as myoblasts isolated from $Mst^{-/-}$ skeletal and cardiac muscle proliferate faster *in vitro* but lose this capacity when exogenous myostatin is added to the medium (McCroskery, Thomas et al. 2003; Morissette, Cook et al. 2006). The inhibitory effects of myostatin on myoblasts were confirmed *in vivo* by studies on $Mst^{-/-}$ muscle regenerative capacities. Upon damage, $Mst^{-/-}$ muscles display an increased number of macrophages and proliferating myoblasts at the site of injury (McCroskery, Thomas et al. 2005). MyoD was expressed earlier than in WT muscle, newly formed fibers were bigger and regenerated muscle showed reduced fibrosis (McCroskery, Thomas et al. 2005; Wagner, Liu et al. 2005). Taken together, $Mst^{-/-}$ muscles regenerate faster due to accelerated migration of inflammatory cells and enhanced activation of myogenic precursors.

2.2. Myostatin non-canonical pathways

In addition to the canonical ActR2b/Smad signaling pathway, myostatin has been shown to interact with the IGF-1/Akt/mTOR signaling which promotes skeletal muscle growth by activating satellite cells (Adams 1998; Musaro, McCullagh et al. 2001). IGF-1 binding to its receptor activates the phosphatidylinositol-3 kinase (PI3K)/Akt signaling which in turn activates the mammalian target of rapamycin (mTOR), resulting in the downstream activation of targets that induce protein synthesis (**Fig.16**) (Bodine, Stitt et al. 2001; Rommel, Bodine et al. 2001). In the meantime, activated Akt inhibits the protein degradation pathways mediated by MuRF1 and MAFbx by blocking the transcription factor FoxO1 (Stitt, Drujan et al. 2004; Latres, Amini et al. 2005). Thus, IGF-1 promotes muscle growth by simultaneously activating protein synthesis and inhibiting protein degradation. It was demonstrated that activation of Akt is sufficient to induce muscle hypertrophy in transgenic mice expressing a conditional form of constitutively active Akt

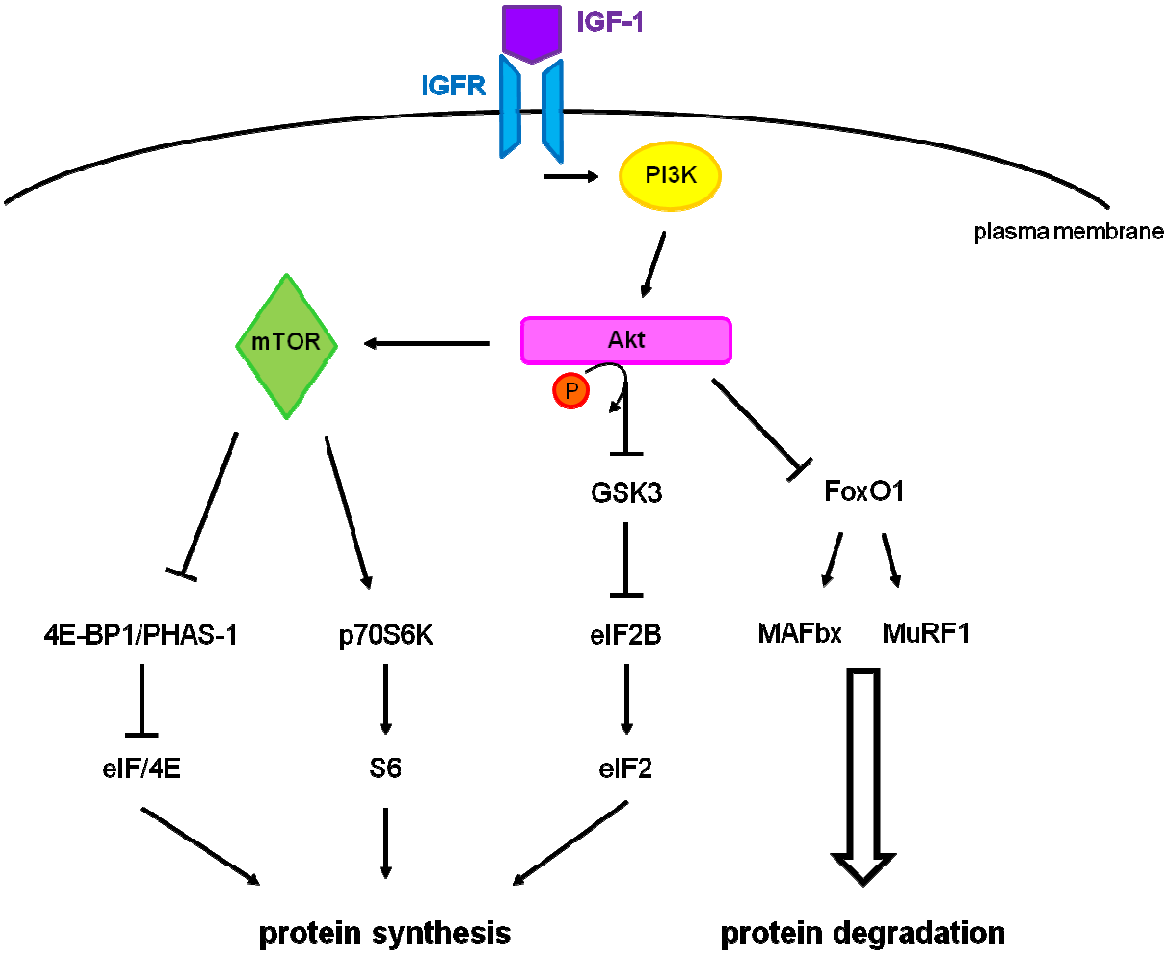

Figure 16. IGF-1 signaling pathway. IGF-1 binding to its receptor (IGFR) leads to the activation of PI3K, in turn activating Akt. The subsequent activation of mammalian target of rapamycin (mTOR) by Akt modulates sub-pathways including p70S6K and 4E-BP1/PHAS-1, which induce protein synthesis. In the meantime, Akt phosphorylation leads to glycogen synthase kinase 3 (GSK3) and FoxO1 repression, thus blocking protein degradation by proteasome. Overall, IGF-1 promotes synthesis of muscle proteins through Akt/mTOR mediated signaling pathways.

(Lai, Gonzalez et al. 2004). Following 2 weeks of induction, the animals showed a double-muscled phenotype due to increased protein synthesis mediated by the Akt downstream target p70S6K (Lai, Gonzalez et al. 2004).

Conversely, Akt down-regulation is associated with skeletal muscle atrophy (Peng, Xu et al. 2003; Sugita, Kaneki et al. 2005). McFarlane and colleagues demonstrated that myostatin inhibits Akt phosphorylation, thereby blocking the PI3K/Akt pathway and leading to an increase in active FoxO1 level (Sandri, Sandri et al. 2004; McFarlane, Plummer et al. 2006). In turn, FoxO1 induces MuRF1 and MAFbx gene expression and thus promotes muscle atrophy (McFarlane, Plummer et al. 2006). It was recently demonstrated that Akt inhibition by myostatin is mediated through Smad2/3 (Sartori, Milan et al. 2009; Lokireddy, McFarlane et al. 2011) but this process can be reverted by addition of IGF-1 to the culture medium, indicating that IGF-1/Akt signaling is dominant over myostatin/Smad/Akt inhibition (Trendelenburg, Meyer et al. 2009). Taken together, these data demonstrate that myostatin has 2 different mechanisms of action dependent upon Smad2/3: the classic canonical pathway leading to direct gene regulation, and indirect activation of the proteasome through inhibition of the Akt/mTOR pathway (**Fig.17**). Lastly, myostatin has been reported to act through a Smad-independent mitogen-activated protein kinase (MAPK) pathway. Both p38 MAPK and c-Jun N-terminal kinase are activated by myostatin through the TGFβ-activated kinase 1 (TAK1), and in turn phosphorylate downstream targets participating in the inhibition of cell proliferation (Philip, Lu et al. 2005; Huang, Chen et al. 2007; Bhatnagar, Kumar et al. 2010). For example, downstream of p38 is ATF-2 which complexes with Smad3/4 to up-regulate p21 expression (Philip, Lu et al. 2005).

2.3. A myostatin endogenous antagonist: follistatin

Follistatin is a highly conserved glycoprotein that was named after its suppressing effect on follicle-stimulating hormone (FSH) synthesis and secretion (Esch, Shimasaki et al. 1987). *Follistatin-null* mice die within hours after birth due to severe defects in muscle, skin, skeletal and growth development, suggesting that follistatin exerts many important roles in development (Matzuk, Lu et al. 1995; Lee, Lee et al. 2010). Alternative splicing of the

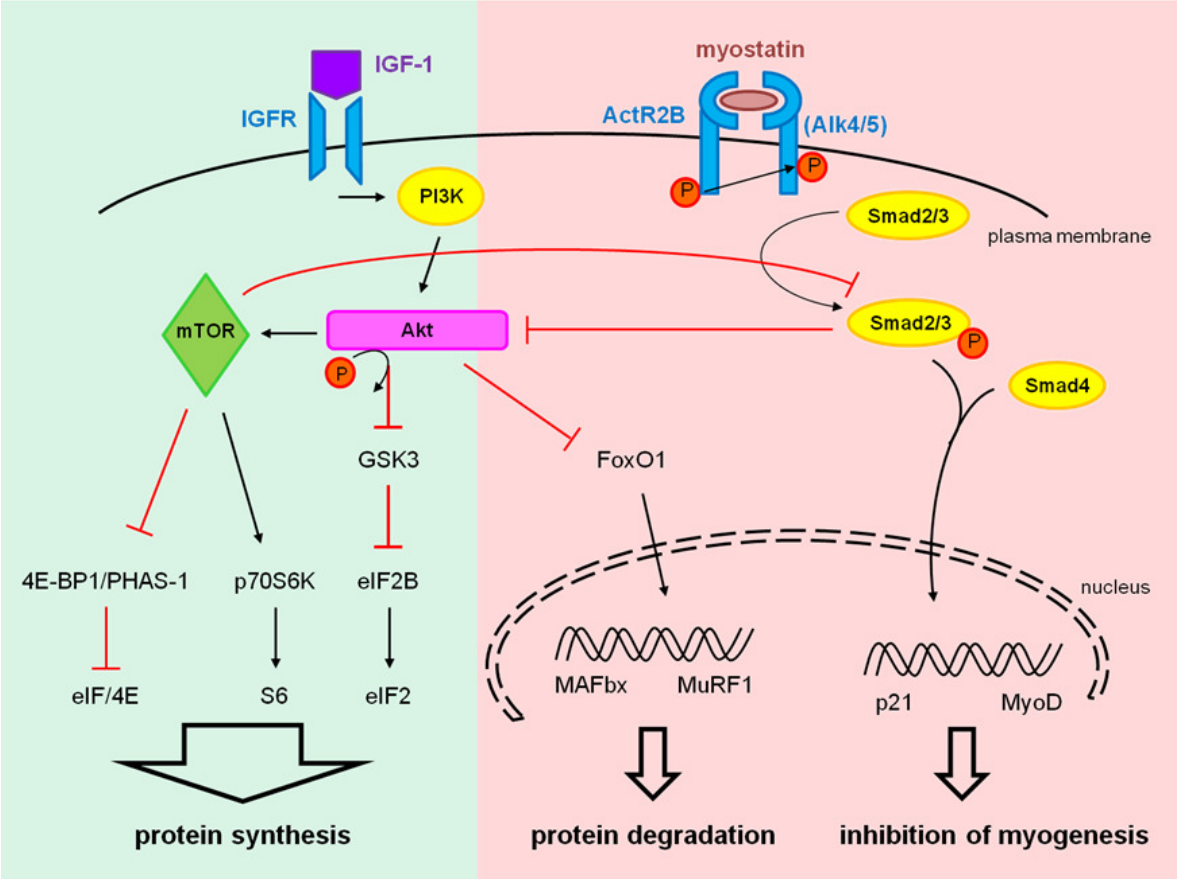

Figure 17. Interactions between myostatin and IGF-1 signaling pathways involved in the control of muscle mass. IGF-1 binding to its receptor (IGFR) induces activation of Akt through phosphatidylinositol-3 kinase (PI3K). Direct downstream targets of Akt include glycogen synthase kinase 3 (GSK3) and Forkhead box O (FoxO1) transcription factors. Inhibition of GSK3 by Akt relieves inhibition onto the initiation factor eIF2B, and thereby increases protein synthesis. Inhibition of FoxO1 decreases the expression of the E3 ubiquitin ligases Muscle Atrophy F-box (MAFbx) and Muscle Ring Finger1 (MuRF1), therefore inhibiting protein degradation. Akt also activates the mammalian target of rapamycin (mTOR) which in turn induces p70S6K and 4E-BP1/PHAS-1, promoting protein synthesis. Myostatin acts via the activin receptors (ActR2b and Alk3/4), leading to the activation of Smad2/3. Activated Smads complex with Smad4 and translocate to the nucleus to inhibit myogenic genes expression. In parallel Smads inhibit Akt, leading to FoxO1 accumulation and subsequent activation of MAFbx and MuRF1. Inhibition of mTOR contributes to myostatin's inhibitory effects by increasing the myostatin-induced Smad phosphorylation and establishing a feed-forward mechanism: myostatin activates Smad2, which inhibits Akt and its downstream target mTOR, which in turn potentiates myostatin's activation of Smad2. *(Adapted from (Kota, Handy et al. 2009)).*

follistatin gene generates 3 different protein isoforms Fst288, Fst303 and Fst315 that all need signal peptide removal to be activated (**Fig.18**) (Shimasaki, Koga et al. 1988; Sugino, Kurosawa et al. 1993). The predominant form of follistatin is the circulating Fst315, widely distributed in adult tissues such as brain, ovary, bone marrow, pancreas or liver (Patel 1998). Activated follistatin binds to its target molecules, neutralizing their biological function. Initially reported to be activin-binding protein, thus inhibiting FSH release (Nakamura, Takio et al. 1990), follistatin can also bind BMP-2, BMP-4, BMP-7 (Yamashita, ten Dijke et al. 1995; Iemura, Yamamoto et al. 1998), and myostatin (Amthor, Nicholas et al. 2004).

Experiments in chick embryos demonstrated that follistatin potentiates muscle cells development by inducing Pax3 and Myf5 expression (Link and Nishi 1997; Amthor, Christ et al. 2002), and rescues the myostatin-induced impairment of myogenesis both *in vitro* and *in vivo* (Amthor, Nicholas et al. 2004). In mouse, it was shown that follistatin up-regulation is required for myoblast fusion. In normal culture conditions, follistatin expression is induced by nitric oxide (Pisconti, Brunelli et al. 2006) but it can also be activated by a deacetylase inhibitor (TSA) treatment (Iezzi, Di Padova et al. 2004). This group reported an increase in follistatin expression following muscle injury or disease, which is further increased by TSA treatment. Satellite cells isolated from *mdx* mice treated with TSA differentiated better than those from non-treated mice, with higher levels of follistatin and decreased levels of myostatin mRNA (Minetti, Colussi et al. 2006). Follistatin beneficial effect on myoblast differentiation and fusion was also demonstrated in human myoblasts, in which follistatin over-expression allowed better expansion *in vitro* and ameliorated engraftment *in vivo* (Benabdallah, Bouchentouf et al. 2009).

Mice over-expressing follistatin under the control of the myosin light chain promoter show an increase in muscle mass superior to that observed in $Mst^{-/-}$ animals (Lee and McPherron 2001), and a similar phenotype was also recently reported in non-human primates (Kota, Handy et al. 2009). Crossing of the two mouse strains results in a quadruple-muscled phenotype reflecting the additive effect of follistatin over-expression and myostatin loss and demonstrating that follistatin acts on other ligands than myostatin

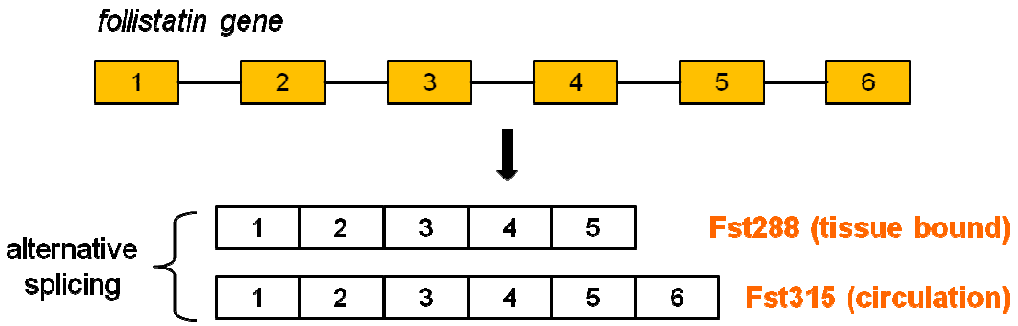

Figure 18. Follistatin alternative splicing. The follistatin gene consists of 6 exons. Alternative splicing occurs between exon 5 and exon 6, generating two isoforms. Post-translational modifications result in two polypeptides, the tissue-bound Fst288 (minor form) and the circulating Fst315 (major form) *(Adapted from (Rodino-Klapac, Haidet et al. 2009)).*

(Lee 2007). Indeed, follistatin electroporation into skeletal muscle leads to a 30% increase in muscle weight while electroporation of a mutated form which does not bind activin only leads to a 15% increase in muscle weight, demonstrating that activin acts in concert with myostatin to inhibit muscle growth (Gilson, Schakman et al. 2009). Interestingly, activin can regulate its own bioavailability by inducing follistatin expression through FoxL2 and Smad3 interactions (Blount, Schmidt et al. 2009). In addition to activins, BMP-11 was found to signal through ActR2b to inhibit myoblast differentiation and could therefore constitute another ligand to follistatin (Souza, Chen et al. 2008).

Finally, it was recently demonstrated that a follistatin-mediated increase in muscle mass is abrogated when IGF-1 and its subsequent pathways are blocked (Kalista, Schakman et al. 2012). All together, these data indicate that negative (myostatin) and positive (IGF-1, follistatin) factors are regulating muscle mass through overlapping signaling pathways.

3. Pharmaceutical approaches to induce skeletal muscle hypertrophy

3.1. Targeting muscle mass via myostatin inhibition

Muscle atrophy and wasting are common features of most muscular dystrophies but muscle loss is a more common phenomenon that is found in many other conditions such as cachexia, long-term bed rest and sarcopenia. As such, there is significant therapeutic interest in developing pharmaceutical products that promote muscle growth. The identification of myostatin has provided a conceptual framework for which a variety of approaches have been developed to block its biological function by systemic delivery of antagonists, or by insertion of genes via adeno-associated viruses (AAV) (**Table 2**).

Crossing the $Mst^{-/-}$ mice with the *mdx* model of Duchenne dystrophy or the α-$sarcoglycan^{-/-}$ model of limb-girdle dystrophy attenuated the disease severity such that muscles are larger, stronger, and contain less fibrosis (Wagner, McPherron et al. 2002; Parsons, Millay et al. 2006). Similarly, one month of treatment with the myostatin neutralizing antibody JA16 significantly increases muscle mass and grip strength in WT mice (Whittemore, Song et al. 2003). When administered to *mdx* mice, JA16 allows

Model	Blocking agent	Muscle hypertrophy (muscle mass increase compared to control)	References
Genetic models	$Mst^{-/-}$ mice	+100%	(McPherron, Lawler et al. 1997)
	propeptide ov.exp.	+50%	(Lee and McPherron 2001)
	FLRG ov.exp.	+120%	(Lee 2007)
	Fst ov.exp.	+200%	(Lee and McPherron 2001), (Zhu, Li et al. 2011)
	$Mst^{-/-}$ X Fst ov.exp.	+350%	(Lee 2007)
	ActR2b dominant negative	+100%	(Lee and McPherron 2001)
Gene delivery	AAV-propeptide	+20%	(Qiao, Li et al. 2008)
	AAV-FLRG	+20%	(Haidet, Rizo et al. 2008)
	AAV-GASP1	+30%	(Haidet, Rizo et al. 2008)
	AAV-Fst	+100%	(Haidet, Rizo et al. 2008)
Injection of antagonists	JA16 neutralizing antibody	+25%	(Bogdanovich, Krag et al. 2002), (Whittemore, Song et al. 2003)
	propeptide	+30%	(Bogdanovich, Perkins et al. 2005)
	Fst I-I	+25%	(Nakatani, Takehara et al. 2008)
	soluble ActR2b	+40% low dose ; +60% high dose	(Lee, Reed et al. 2005), (Cadena, Tomkinson et al. 2010)

$Mst^{-/-}$ = *myostatin-null* ; Fst = follistatin ; Fst I-I = follistatin derivative which does not bind activin ; ov.exp. = over-expression

Table 2. Comparative potentials of techniques used to inhibit myostatin signaling. While genetic models display marked increase in their muscle mass, pharmaceutical approaches developed to inhibit myostatin lead to less significant fiber hypertrophy with the exception of follistatin and ActR2b.

improvement of muscle functional capacity so that dystrophic mice perform similarly to WT animals in endurance tests (Bogdanovich, Krag et al. 2002). Senescent $Mst^{-/-}$ mice still display increased muscle mass and strength with strong regenerative capacities compared to their WT counterparts, suggesting that loss of myostatin function provides long-term beneficial effects (Wagner, Liu et al. 2005). Indeed, long-term treatment of aged mice with a myostatin-blocking antibody prevented sarcopenia by attenuating muscle loss and reducing apoptosis (Murphy, Koopman et al. 2010). However myostatin loss in a much more severe dystrophic mouse model, the laminin α2-deficient dy^{w}/dy^{w} mice, did not ameliorate muscle pathology (Li, Shelton et al. 2005). Similarly, JA16 treatment in late stage dystrophic α-sarcoglycan$^{-/-}$ mice was not efficient in rescuing the muscles from the disease, suggesting that myostatin inhibition may be ineffective in severely affected or late-stage diseases (Parsons, Millay et al. 2006).

Another approach consists of delivering the soluble or AVV-mediated form of natural antagonists to myostatin. The majority of myostatin circulating in the serum is part of a latent complex containing its propeptide and/or the follistatin-related gene (FLRG) (Hill, Davies et al. 2002; Zimmers, Davies et al. 2002). *Mdx* mice injected with the soluble or AAV form of myostatin propeptide display improved pathophysiology and increased muscle mass, but to a lower level than animals genetically over-expressing the propeptide (**Table 2**) (Lee and McPherron 2001; Bogdanovich, Perkins et al. 2005; Qiao, Li et al. 2008). FLRG, together with growth and differentiation factor-associated serum protein-1 (GASP-1) were demonstrated to inhibit the biological activity of mature myostatin (Hill, Davies et al. 2002; Hill, Qiu et al. 2003). Intramuscular delivery of AAV encoding these antagonists resulted in enhanced muscle mass accompanied with long-term improvement of strength in WT and *mdx* mice (Haidet, Rizo et al. 2008). But the greatest effects were observed following injection of AAV-follistatin, providing functional long-term improvement of the muscle pathology (Haidet, Rizo et al. 2008). Mice over-expressing follistatin display muscles weighting 3 times more than those of WT animals (Lee and McPherron 2001), and undergo more efficient skeletal muscle regeneration while developing less fibrosis (Zhu, Li et al. 2011). Crossing of these mice with the $Mst^{-/-}$ strain leads to a quadrupled muscle mass,

corresponding to the most dramatic increase reported so far (**Table 2**) (Lee and McPherron 2001). Since follistatin can bind a variety of molecules for which expression is not restricted to skeletal muscle, such as activins, its use is not suitable for therapeutic application due to possible undesirable side effects. Nakatani and colleagues recently developed a follistatin derivative, Fst I-I, which does not affect the activin bioactivity (Nakatani, Takehara et al. 2008). Transgenic mice expressing Fst I-I under the myosin light chain promoter showed increased skeletal muscle mass and strength, and the same features were observed when crossed with *mdx* mice (Nakatani, Takehara et al. 2008).

Finally, mice over-expressing a dominant-negative form of ActR2b display a doubled muscle mass similar to that of $Mst^{-/-}$ animals (Lee and McPherron 2001). Mice injected with a soluble form of ActR2 attain a 50% increase in muscle mass after only 2-4 weeks of treatment (**Table 2**) (Lee, Reed et al. 2005). Moreover ActR2b injection in $Mst^{-/-}$ mice enhanced the effect of myostatin blockade, demonstrating that it targets multiple negative regulators and therefore constitutes a more suitable compound to promote muscle mass than the JA16 blocking antibody (Lee, Reed et al. 2005). In addition, a recent study reported that skeletal muscle hypertrophy obtained with ActR2b injection is independent of fiber-type (Cadena, Tomkinson et al. 2010), in contrast to $Mst^{-/-}$ animals which display a switch from slow to fast oxidative fibers and are therefore more susceptible to fatigue (Girgenrath, Song et al. 2005; Hennebry, Berry et al. 2009).

Taken together these different methods of myostatin blockade demonstrate unequivocally that inhibition of this signaling has beneficial effects in a wide range of disease settings, by reducing fibrosis and enhancing muscle regeneration.

3.2. Satellite cells and muscle hypertrophy

Pharmaceutical strategies described above allow a rapid increase in skeletal muscle mass due to fiber hypertrophy rather than fiber hyperplasia (Whittemore, Song et al. 2003; Lee, Reed et al. 2005; Haidet, Rizo et al. 2008). However these studies did not investigate

whether fiber size increase is the result of myonuclei number augmentation, myonuclear domain expansion, or both. Satellite cells are the major source of muscle progenitors for postnatal growth and regeneration and their proliferation is inhibited by myostatin *in vitro*, suggesting that blocking myostatin will lead to satellite cell differentiation and fusion supporting myofiber growth. In accordance with this hypothesis, McCroskery and colleagues observed that $Mst^{-/-}$ mice display a ~50% increase in the number of activated satellite cells as compared to WT animals (McCroskery, Thomas et al. 2003). Furthermore, a 5-fold increase in satellite cell numbers is observed following a single injection of the soluble ActR2b (Zhou, Wang et al. 2010). In contrast, other groups have provided evidence that muscle hypertrophy induced by myostatin inhibition does not involve myonuclei addition nor require satellite cells but is simply the result of an increase in myofiber protein content (Amthor, Otto et al. 2009; Blaauw, Canato et al. 2009; Wang and McPherron 2012). One explanation for this discrepancy comes from a recent study demonstrating that myostatin inhibition by the soluble ActR2b leads to an initial increase in fiber size followed later by satellite cell activation and myonuclear addition (Wang and McPherron 2012).

Taken together, these studies imply that a fiber size increase is a two-step process that may require nuclear addition to sustain the increase in fiber size. In addition, the potential roles of other muscle resident progenitor cells outlined in chapter 1 have not yet been investigated and while their role(s) in muscle homeostasis and regeneration are demonstrated, they may also play a critical role in the myostatin control of muscle mass.

RATIONALE. Introduction at a glance and objectives of the thesis

Skeletal muscle is composed of multinucleated fibers that are established during embryogenesis and undergo extensive growth during postnatal life (White, Bierinx et al. 2010). A population of resident progenitor cells, referred to as satellite cells, are found underneath the muscle fiber basal lamina and closely juxtaposed to the muscle fiber plasma membrane (Mauro 1961). Satellite cells are quiescent in adult muscle under normal physiological conditions, but can be quickly reactivated upon injury to support muscle repair (Cornelison and Wold 1997; Halevy, Piestun et al. 2004). While the majority of satellite cells differentiate and fuse with newly formed fibers, a small proportion of cells exit the cell cycle and replace the satellite cell pool in a self-renewing process providing a mechanism to support multiple rounds of injury (Olguin and Olwin 2004). It is generally accepted that satellite cells constitute the major skeletal muscle stem cell underlying muscle regeneration.

However, the past ten years have seen the identification of a variety of non-satellite cell skeletal muscle progenitors such as bone-marrow derived Side Population, vessel-associated mesoangioblasts, and interstitial β4-integrin+ cells (De Angelis, Berghella et al. 1999; Gussoni, Soneoka et al. 1999; Liadaki, Casar et al. 2012). All these populations display robust myogenic capacities *in vitro* and *in vivo*, and some are presently in clinical trial to repair human muscular dystrophy. While these studies clearly establish that non-satellite cell progenitors are competent to participate in muscle repair, direct contribution of these progenitors to postnatal myogenesis and regeneration has not been firmly demonstrated. Recently, two laboratories have created murine models in which satellite cells can be conditionally depleted in order to test whether muscle regeneration is possible in the absence of satellite cells (Lepper, Partridge et al. 2011; Sambasivan, Yao et al. 2011). Both

studies demonstrated that elimination of the satellite cells in adult muscle leads to a severely impaired regenerative response following injury, leading to the conclusion that satellite cells are indispensable for muscle repair. Based on these results, it would appear that non-satellite myogenic populations do not play a role in the generation of new muscle fibers under normal physiological conditions. Alternatively, the presence of satellite cells may be necessary in order to successfully recruit non-satellite cell progenitors into the myogenic lineage and, as such, their elimination does not constitute a neutral event but rather has a profound impact on the skeletal muscle stem cell niche.

While it remains unclear whether satellite cells are involved in recruiting other populations into the muscle lineage, recent evidence reveals that neighboring non-myogenic cells secrete crucial factors that promote myoblast recruitment and proliferation during muscle regeneration. For instance inflammatory cells clear away cellular debris, attract mesoangioblasts to the site of injury, and protect newly formed fibers from apoptosis. Importantly, two cell populations with strong adipogenic potential have been recently identified in the interstitial space of the muscle. Mesenchymal progenitors are characterized by PDGFRα expression (Uezumi, Fukada et al. 2010), while fibro/adipogenic progenitors (FAPs) have been isolated upon Sca1 expression (Joe, Yi et al. 2010). Both of these populations are suggested to play an important role in regulating the myogenic/adipogenic balance during muscle homeostasis and regeneration. Taken together, there is an increasing body of research supporting a dynamic model in which interactions between the myogenic and non-myogenic cell populations of the skeletal muscle stem cell niche promote and direct muscle growth and repair.

We identified recently a muscle-resident stem cell population located in the interstitial space and characterized by PW1/Peg3 expression, referred to as PICs (PW1+ Interstitial Cells) (Mitchell, Pannerec et al. 2010). PICs isolated from neonatal muscle can participate in myofiber formation following engraftment into damaged muscle, as well as give rise to satellite cells and replenish their own pool. However PICs do not express satellite cell markers nor do they share the same embryonic lineage, suggesting they

constitute an upstream stem cell population that play important roles in postnatal growth and regeneration. **The first part of the thesis focuses upon the characterization of adult PICs that had not been formally studied in our previous efforts. We evaluated their lineage potentials and explored their interactions with satellite cells.** This study was conceptually guided by work in our lab that defined PW1 as a universal marker for stem /progenitor populations in all adult tissues examined (Besson, Smeriglio et al. 2011). Since PW1 is expressed by PICs and satellite cells, a general hypothesis for this work was that all PW1 expressing cells in muscle are progenitor cells although their lineage (muscle, fat, etc.) remained to be defined. In this context, it was important to determine the interactions of PICs with satellite cells as well as to unravel the relationship between PICs and newly discovered resident progenitor populations in muscle tissue. **Using PW1 as a stem cell marker, we sought to better understand the hierarchy between the diverse cell types of the skeletal muscle niche.**

An understanding of skeletal muscle stem cells is not only of key interest for fundamental skeletal muscle biology, but also serves as a solid basis upon which therapeutic approaches to muscle disease can be designed. Loss of muscle mass is a common feature of muscular diseases and aging, as well as cachexia which is a key symptom in response to cancer that can threaten the life of a patient more than the primary tumor. Significant strides have been made in developing pharmaceutical agents that induce muscle hypertrophy and thereby preserve muscle mass in diseased patients. The major inhibitor of muscle mass is myostatin, a member of the TGFβ superfamily which is predominantly expressed in skeletal muscle and acts through the activin transmembrane receptor type 2b (ActR2b) (McPherron, Lawler et al. 1997; Lee and McPherron 2001). Animals carrying a constitutive loss of function for the myostatin gene display a hyper-muscled phenotype due to a marked increase in skeletal muscle mass (Grobet, Martin et al. 1997; Schuelke, Wagner et al. 2004; Clop, Marcq et al. 2006). Muscle mass increase, i.e hypertrophy, can be achieved by the over-expression of the myostatin antagonist follistatin, or by injecting myostatin inhibitors such as the soluble form of ActR2b (Lee, Reed et al. 2005; Cadena, Tomkinson et al. 2010). Even though these treatments lead to a

rapid increase in skeletal muscle size due to fiber hypertrophy, the participation of muscle progenitors during this process is not firmly established and the molecular mechanisms involved are not yet understood. **In the second part of the thesis, we explored the role of PICs and satellite cells in skeletal muscle hypertrophy induced by soluble ActR2b treatment and the potential for myostatin blockade as a means to activate the muscle stem cell niche.**

CHAPTER 3. DEFINING THE POSITION OF PICS IN THE SKELETAL MUSCLE STEM CELL HIERARCHY.

This part of the project focuses upon the characterization of PICs in adult muscle and their relationship with the other cell types of the niche. As described in our previous study, PICs isolated from neonatal (7 to 10 days old) mouse muscle display a pronounced myogenic capacity *in vitro* and *in vivo* (Mitchell, Pannerec et al. 2010). Although they do not share a common Pax3/7 progenitor with satellite cells, PICs up-regulate Pax3 and Pax7 when grown in myogenic conditions and give rise to satellite cells when engrafted into damaged muscle (Mitchell, Pannerec et al. 2010). Coupled with the observation that PICs spontaneously differentiate into smooth and skeletal muscle *in vitro*, these results suggested that PICs constitute a more plastic population compared to satellite cells. To gain further insights into PICs, we compared their gene expression profile with that of satellite cells using micro-array analyses and tested their capacity to differentiate into several lineages *in vitro*.

Secondly, in order to evaluate the potential of adult PICs and to compare this with juvenile PICs, we established a flow cytometric (FACS) technique to purify PICs from adult muscle. PICs isolation from young skeletal muscle was described in our initial study and is based upon the expression of the cell surface markers CD34 and Sca1 (Mitchell, Pannerec et al. 2010). While the CD34+Sca1- population constitutes the satellite cell fraction, PICs are found in the CD34+Sca1+ population that is divided into 2 sub-populations based on their level of Sca1 expression. Cells expressing intermediate levels of Sca1, referred to as $Sca1_{MED}$, are >90% pure for PW1 expression; and cells expressing high levels of Sca1, referred to as $Sca1_{HIGH}$, are 50% pure for PW1 expression. Due to our inability to further purify the $Sca1_{HIGH}$ fraction, we had limited our previous analyses on the $Sca1_{MED}$ fraction (Mitchell, Pannerec et al. 2010). To enrich the $Sca1_{HIGH}$ population for PW1, we used the $PW1^{nLacZ/+}$ mouse model developed in our laboratory. The $PW1^{nLacZ/+}$ mouse expresses the reporter gene β-galactosidase under the control of the PW1/Peg3 cis-regulatory sequences and shows >98% co-localization of PW1 protein and reporter gene expression (Besson, Smeriglio et al. 2011). This model had allowed us to more directly and rapidly analyze PW1 expression in various tissues leading to the unexpected conclusion that PW1 marks stem cells in all adult tissues examined (Besson, Smeriglio et al. 2011). Using single

cell preparations from $PW1^{nLacZ/+}$ mice and a fluorescent substrate to β-galactosidase, we successfully purified PW1+ cells in the $Sca1_{HIGH}$ fraction allowing us to subsequently test their fates *in vitro*. Our results demonstrate that both young and adult PICs can differentiate into smooth muscle, skeletal muscle and adipocytes while satellite cells only adopt a skeletal muscle phenotype.

Two recent studies suggest that adipogenic progenitors located in the muscle interstitium and characterized by Sca1 and PDGFRα expression regulate muscle homeostasis by interacting with myogenic cells (Joe, Yi et al. 2010; Uezumi, Fukada et al. 2010). To gain insight into the relationship between these adipogenic progenitors and PICs, we looked at PDGFRα and PW1 expression in sorted cells. Our results demonstrate that almost all the PDGFRα+ cells reside in the PIC population whereas a smaller PDGFRα-PW1+ fraction is myogenic. Therefore, as seen for other tissues, all PW1+ cells in skeletal muscle are progenitor cells possessing different lineage potentials.

The results presented in this section provide new insights in the organization of the skeletal muscle stem cell niche. We can now distinguish the various progenitors in muscle tissue based upon the expression of PW1 coupled with a specific marker. Specifically, satellite cells are PW1+/Pax7+ while adipogenic progenitors are PW1+/PDGFRα+. It is of keen interest that there remains a myogenic sub-population of PICs that is PW1+/PDGFRα- but for which no specific marker has yet been found. We are testing presently the expression of β4-integrin, which was recently reported to mark an interstitial cell population with strong myogenic potential (Liadaki, Casar et al. 2012).

Defining skeletal muscle resident progenitors and their cell fate potentials.

Alice Pannérec, Luigi Formicola, Vanessa Besson, Giovanna Marazzi and David A. Sassoon

The satellite cell is considered to be the major tissue-resident stem cell underlying muscle regeneration, however, the past decade has seen the identification of multiple non-satellite cell myogenic progenitors as well as non-myogenic cells that support the muscle regenerative process. PW1/Peg3 expression characterizes both satellite cells and interstitial stem cells, termed PICs (PW1+ Interstitial Cells), which readily participate in myofiber formation following engraftment into damaged muscle. Here, we investigated the relationship between PICs and the other muscle resident cells. Micro-array profiling revealed that PICs express a broad range of genes typical of mesenchymal stem cells while satellite cells display a profile consistent with committed myogenic progenitors, suggesting that PICs constitute an upstream and less committed cell type. This hypothesis was confirmed by the observation that satellite cells only adopt a skeletal muscle cell fate *in vitro*, while PICs can differentiate into smooth and skeletal muscle as well as adipocytes. We further demonstrate that the adipogenic potential of PICs correlates with the PW1 positive (PW1+) sub-population that also expresses PDGFRα, while its myogenic potential resides in the PW1+ sub-population that do not express PDGFRα. Since almost all PDGFRα+ cells are found in the PW1+/Sca1+ population, we conclude that mesenchymal fibro/adipogenic population constitute a sub-population of PICs. These results provide new insights in the skeletal muscle stem cell niche.

Key words: skeletal muscle, PW1/Peg3, stem cell, PDGFRα.

INTRODUCTION

Since their initial identification, extensive work has demonstrated that satellite cells constitute the major source of postnatal skeletal muscle progenitors (Mauro 1961; Montarras, Morgan et al. 2005; Relaix, Rocancourt et al. 2005; Zammit, Relaix et al. 2006). However multiple non-satellite cell populations possessing myogenic capacity that are either freely circulating or resident in the muscle interstitium and vessels have been described (Gussoni, Soneoka et al. 1999; Sampaolesi, Torrente et al. 2003; Sampaolesi, Blot et al. 2006; Benchaouir, Meregalli et al. 2007; Dellavalle, Sampaolesi et al. 2007; Mitchell, Pannerec et al. 2010; Liadaki, Casar et al. 2012). While these progenitors can adopt a skeletal myogenic fate following engraftment into damaged or diseased muscle, their direct contribution to postnatal muscle growth and regeneration is unknown or extremely limited *in vivo* (Dellavalle, Maroli et al. 2011). Two recent studies reported that muscle regeneration is completely abrogated when the satellite cell population is depleted suggesting that either the contribution of non-satellite cell progenitors is minor, or that satellite cells are required to recruit and/or induce other populations to the myogenic lineage (Lepper, Partridge et al. 2011; Sambasivan, Yao et al. 2011).

Purified satellite cells display robust myogenic differentiation *in vitro* (Konigsberg 1961; Bischoff 1975) suggesting a strong cell fate commitment to the myogenic program, however specific interactions between satellite cells and other muscle resident cell populations have been demonstrated to occur *in vivo*. Specifically, resident interstitial cells can regulate the balance between myogesis, adipogenesis and fibrogenesis during regeneration (Joe, Yi et al. 2010; Uezumi, Fukada et al. 2010; Murphy, Lawson et al. 2011). Connective tissue fibroblasts, identified by Tcf4 expression, proliferate in close proximity to satellite cells following injury (Murphy, Lawson et al. 2011) and depletion of Tcf4+ cells prior to muscle damage leads to poor regeneration and a reduced replacement of satellite cells due to premature differentiation (Murphy, Lawson et al. 2011). Two adipogenic progenitor populations have also been identified in the muscle interstitium. These two populations have been isolated either by PDGFRα expression (Uezumi, Fukada et al. 2010) or from a Sca1 positive (Sca1+) population (Joe, Yi et al. 2010). While it remains unclear whether these cell populations are overlapping or not, both populations

display a strong adipogenic potential *in vitro* and differentiate into fat when engrafted in a model of fatty infiltration, but not in healthy muscle, suggesting that the surrounding environment controls their fate. Fibro/adipogenic progenitors (FAPs) are markedly activated upon injury and promote myoblast differentiation through cell-cell signaling, although they do not originate from a myogenic lineage nor do they display myogenic differentiation (Joe, Yi et al. 2010). In turn, adipogenesis of PDGFRα+ cells is strongly inhibited by the presence of myotubes (Uezumi, Fukada et al. 2010). These data suggest that muscle homeostasis and regeneration is dependent upon a balance between satellite cell-dependent myogenesis and PDGFRα+ cells/FAPs dependent adipogenesis.

We identified previously a non-satellite muscle resident cell population characterized by PW1/Peg3 expression referred to as PICs (PW1+ Interstitial Cells), that display myogenic potential *in vitro* and participate in the generation of new myofibers, satellite cells, and PICs following engraftment into damaged muscle (Mitchell, Pannerec et al. 2010). Whereas lineage-tracing studies demonstrated that PICs and satellite cells do not share a common Pax3/7 expressing progenitor during development, PICs express both Pax3 and Pax7 as they acquire a myogenic fate (Mitchell, Pannerec et al. 2010). Coupled with the observation that PICs spontaneously form skeletal muscle and smooth muscle *in vitro*, we suggested that they represent a more plastic muscle resident progenitor. While we had characterized the lineage potential of PICs in juvenile mouse muscle (<3 weeks postnatal), we did not characterize adult PICs due to the inability to sufficiently purify the PW1+ expressing cells using the available markers. More recently, we have generated a PW1 reporter transgenic mouse carrying a bac with the PW1/Peg3 locus driving the ß-galactosidase (Besson, Smeriglio et al. 2011). Analyses of adult $PW1^{nlacZ/+}$ mice revealed that both reporter and endogenous PW1/Peg3 are co-expressed not only in muscle satellite cells and PICs, but also in stem/progenitor cells from a wide array of tissues including the gut, skin, CNS and early hematopoietic stem cells (Besson, Smeriglio et al. 2011). Based upon these observations, we suggested that all PW1+ cell populations in adult muscle are stem cells although this had not been demonstrated. As seen in early postnatal muscle (Mitchell, Pannerec et al. 2010), we observed PW1/Peg3 expression in satellite cells as well as in a sub-population of interstitial cells in the adult indicating that PICs persist throughout postnatal life (Besson, Smeriglio et al. 2011).

In this study, we took advantage of the PW1/Peg3 reporter mouse in order to characterize adult PICs and their relationship to the other progenitor populations thus far identified in skeletal muscle. We demonstrate that PICs are multipotent progenitors in both young and adult mice and provide evidence that a sub-population of PICs, defined by PDGFRα expression, constitutes the source of fibro/adipogenic progenitors whereas the remaining population constitutes a latent source of resident non-satellite myogenic cells thus redefining the specific populations of stem cells in muscle tissue.

MATERIALS AND METHODS

Mice. The animal models used were the *PW1*^{IRESnLacZ} transgenic mice (PW1$^{nlacZ/+}$), in which a nuclear operon lactose gene is expressed under the control of the Pw1 promoter (Besson, Smeriglio et al. 2011); and C57Bl6J mice (Elevage Janvier). All work with mice was carried out in adherence to French government guidelines.

FACS analysis. For fluorescence-activated cell sorting, limb muscles from 1-7 week old PW1$^{nlacZ/+}$ or C57Bl6J mice were minced and digested in HBSS (Hank's Balanced Salt Solution, GIBCO) containing 2μg.ml^{-1} collagenase A (Roche), 2.4U.ml^{-1} dispase I (Roche), 10ng.ml^{-1} DNAse I (Roche), 0.4mM $CaCl_2$ and 5mM $MgCl_2$ for 90-120 min at 37°C with agitation every 15 min. Three successive cycles of washing and filtration were performed before re-suspension of the cell pellet in HBSS containing 0.2% (w/v) BSA (bovine serum albumin, Sigma), 1% (v/v) penicillin-streptomycin, 10ng.ml^{-1} DNAse I and 10% (v/v) mouse serum (Jackson Immunoresearch). Cells were incubated for 5 min on ice before adding the following primary antibodies at a concentration of 10ng.ml^{-1}: rat anti-mouse CD45-APC (BD Biosciences), rat anti-mouse Ter119-APC (BD Biosciences), rat anti-mouse CD34-E450 (eBiosciences), rat anti-mouse Sca1-A700 (eBiosciences), and rat anti-mouse PDGFRα-PE (CD104a, eBiosciences). Cells were incubated for 30 min on ice. Cell pellets were washed twice before incubation with 5-Dodecanoylaminofluorescein Di-β-D-Galactopyranoside (C_{12}FDG, 60μM, Life Technologies) 1h at 37°C. Cells were finally washed once with ice-cold HBSS, filtered and re-suspended in HBSS containing 0.2% (w/v) BSA, 1% (v/v) penicillin-streptomycin and 10ng.ml^{-1} DNAse I. Flow cytometry analysis and cell sorting were performed on a FACSAria (Becton Dickinson), with appropriate isotype matching controls. Ter119+ and CD45+ cells were gated and excluded by negative selection. The remaining cells were gated based on their CD34 and Sca1 expression, and PW1 expression was then assessed by green

fluorescence in the $Sca1_{HIGH}$ fraction. PDGFRα positive or negative cells were sorted in the $Sca1_{HIGH}$/PW1+ and $Sca1_{HIGH}$/PW1- fractions. Purified cell populations were cultured as described below. For immunocytochemical analyses, freshly sorted cells (4,000 cells per well) were immediately centrifuged in 96-well plate (Becton Dickinson) coated with 0.1% porcine gelatin (Sigma) and subsequently reacted with X-Gal as described previously (Gross and Morgan 1999) for 2h at 37°C. For immunofluorescence analyses, freshly sorted cells (10,000 cells per well) were immediately centrifuged onto 8-well chamber glass slides (Thermo Scientific) for 5 min at 100g and immunostained for PW1 and Pax7 or PDGFRα, as described below. Quantitative analyses were performed by counting the number of positive cells out of a minimum of 300 cells in randomly chosen fields for each of three independent experiments.

Primary cell culture. Primary skeletal muscle cells from limb muscles of 1-7 week old mice were obtained as described above. Immediately after sorting, cells were plated on gelatin-coated dishes at a density of 2,000 cells per cm^2 for myogenic differentiation and at a density of 6,000 cells per cm^2 for adipogenic differentiation. Cells were grown for one week in high-glucose Dulbecco's modified eagle medium (DMEM, Gibco) supplemented with 2.5ng.ml^{-1} bFGF (Invitrogen), 20% heat-inactivated FBS (Invitrogen), 10% heat-inactivated horse serum (Gibco), 1% (v/v) penicillin-streptomycin (Gibco), 1% (v/v) L-Glutamine (Gibco) and 1% (v/v) Na-pyruvate (Gibco). Medium was changed every 2 days. For myogenic differentiation, cells were transferred to differentiation medium (DM) for 2 days: DMEM containing 5% (v/v) horse serum and 1% (v/v) penicillin-streptomycin. For adipogenic differentiation, cells were transferred to adipogenic differentiation medium (ADM) for 5 days: DMEM containing 20% (v/v) FBS, 1% (v/v) penicillin-streptomycin, 0.25μM dexamethasone (Sigma), 0.5mM isobutylmethylxanthine (Sigma), 1μg.ml^{-1} insulin (Sigma) and 5μM troglitazone (Sigma). Medium was changed every 2 days.

Histological analyses. Cryosections (10μm) from 7-week old mice *tibialis anterior* snap frozen in liquid nitrogen-cooled isopentane, cultured cells and cytospin preparations were fixed in 4% (w/v) paraformaldehyde and processed for immunostaining as described previously (Mitchell, Pannerec et al. 2010). The following primary antibodies were used: PW1 ((Relaix, Weng et al. 1996) 1:3,000), Pax7 (Developmental Studies Hybridoma Bank, 1:15), laminin (Sigma, 1:100), MyoD (BD Biosciences, 1:200), alpha-smooth muscle actin (Sigma; 1:200), MF20 (Developmental Studies Hybridoma Bank, 1:100), and PDGFRα-biotin (R&D systems, 2.5ng.mL^{-1}). Antibody binding was revealed using species-specific secondary antibodies coupled to Alexa Fluor 488 (Molecular Probes), Cy3 or Cy5 (Jackson Immunoresearch). Nuclei were counterstained with DAPI (Sigma). To stain lipids, cells were fixed in 10% formalin (Sigma) for 5 min at 4°C, rinsed in water and then

100% propylene glycol (Sigma) for 10 min, stained with Oil red O (Sigma) for 10 min at 60°C, placed in 85% propylene glycol for 2 min and rinsed in water. Nuclei were counterstained with Mayer's Hematoxylin Solution (Sigma). For quantitative analyses of cells in culture, at least 300 cells from randomly chosen fields for each of three independent experiments were counted. Myogenic potential was quantified by counting the number of nuclei in MF20+ cells per total number of nuclei. Adipogenic potential was quantified by counting the number of nuclei in Oil red O+ cells per total number of nuclei.

Micro-array analysis. Freshly isolated PICs and satellite cells from 9-day old C57Bl6 limb muscles from 3 different experiments were subjected to RNA extraction (RNeasy microkit, Qiagen), and Affymetrix micro-array analysis (GeneChip MOE 430 2.0, Affymetrix) was performed by PartnerChip (Evry). Statistical analysis was performed using a Fold Change threshold=1 and p-value=0,01. Subsequent analyses were performed using the Gene Ontology database.

Statistical analysis. All statistics were performed using an unpaired Student's t-test in the StatView software. Values represent the mean ± s.em. *p<0.05, **p<0.01 and ***p<0.001.

RESULTS

PICs and satellite cells are distinct myogenic progenitors

We performed transcriptome analyses (Affymetrix) using RNA from wildtype PICs and satellite cells freshly isolated from 9 days old limb muscles. At this stage, we observed distinct transcriptome signatures with 1345 and 1233 genes expressed specifically in PICs and satellite cells respectively (**Fig.1a**). Gene Ontology database-based analyses revealed that satellite cell-specific genes are primarily related to the skeletal muscle lineage (c-met, desmin). As expected, we observed the expression of myogenic markers (Pax7, MRFs, CXCR4, integrin-α7, FGFR4) only in satellite cells while PW1 is expressed by both cell types (**Fig.1c**). In contrast, PICs expressed genes that have been implicated in a wide variety of processes such as FoxC2 and ephrins (mesenchymal stem cells), VEGF and angiogenin (angiogenesis), and numerous genes governing cell interaction and cell migration (**Fig.1b-e**). We noted that specific growth factors and their cognate receptors were reciprocally expressed by PICs and satellite cells suggesting a paracrine relationship

between these two cell types (**supplementary tables 1 and 2**). These results, combined with our previous analyses demonstrating that PICs do not arise from a Pax3 lineage (Mitchell, Pannerec et al. 2010), lend further support to the hypothesis that PICs and satellite cells are distinct progenitor populations and that PICs represent a less committed progenitor compared to satellite cells.

Adult PICs are found in the $Sca1_{HIGH}$ fraction

We reported previously that PICs can be isolated from young muscle (1-2 weeks of age) using the cell surface markers CD34 and Sca1 (Mitchell, Pannerec et al. 2010). In young muscle, we observed 2 populations highly enriched for PW1 expression that can be distinguished by levels of Sca1 expression (medium versus high) termed $Sca1_{MED}$ and $Sca1_{HIGH}$. $Sca1_{MED}$ cells isolated from 1-week old muscle are >90% pure for PW1 expression, while the $Sca1_{HIGH}$ fraction only contains 60% of PW1+ cells (**supplementary Fig.1**). Previously, we did not analyze the $Sca1_{HIGH}$ fraction further due to the inability to purify the PW1 population above 50% (Mitchell, Pannerec et al. 2010). In order to systematically isolate and test the lineage potential of PW1+ cells from both Sca1 expressing fractions throughout postnatal development, we used the $PW1^{nLacZ/+}$ reporter mice that express the β-galactosidase gene (βgal) under the control of the PW1 locus (Besson, Smeriglio et al. 2011). These analyses revealed that there is a progressive decline of the $Sca1_{MED}$ fraction during the first 3 weeks of life and it is no longer detectable by 5 weeks after birth (**Fig.2a,b**). In contrast, the proportion of the $Sca1_{HIGH}$ fraction increased during postnatal life to reach ~70% of CD45-Ter119- muscle cells in the adult where it contains ~20% of PW1+ cells (**Fig.2b and supplementary Fig.1**). βgal+ (i.e PW1+) cells in the $Sca1_{HIGH}$ fraction were FACS purified using a fluorescent substrate to βgal, $C_{12}FDG$, and the expression of PW1 was verified on freshly sorted cytospun cells (**Fig.2c,d**). In parallel, we immunostained cytospun cells for PW1 and Pax7 to check for the purity of the satellite cell fraction (**Fig.2d**). These data reveal that the $Sca1_{MED}$ population of PICs is restricted to the first 3 weeks of postnatal life raising the question as to what stem cell potential, if any, is present in the $Sca1_{HIGH}$ population.

Adult PICs are multipotent progenitors

We showed previously that $Sca1_{MED}$ cells from juvenile mouse muscle are capable of differentiating into smooth and skeletal muscle (Mitchell, Pannerec et al. 2010). In order to test all the PW1+ populations (satellite cells, $Sca1_{MED}$ and $Sca1_{HIGH}$/PW1+), we purified these fractions the $PW1^{nLacZ/+}$ reporter mice and tested their myogenic and adipogenic potentials (**Fig.3**). Consistent with previous results (Mitchell, Pannerec et al. 2010), we observed that satellite cells isolated from 1-week old muscles are highly myogenic (fusion index >80%) and do not display adipogenic capacity whereas $Sca1_{MED}$ cells gave rise to myogenic (fusion index=40%) and smooth muscle cells. In addition, the $Sca1_{MED}$ cells also gave rise to adipocytes (adipogenic potential=40%) (**Fig.3a,c**). The $Sca1_{HIGH}$/PW1+ cells also displayed myogenic potential (fusion index=20%), as well as formed smooth muscle cells and adipocytes (adipogenic potential=30%). These observations suggest that the $Sca1_{MED}$ and $Sca1_{HIGH}$/PW1+ cells possess a high degree of lineage plasticity. As both $Sca1_{HIGH}$/PW1+ and $Sca1_{MED}$ cells are present in the interstitium, we refer to both populations collectively as PICs (PW1+ Interstitial Cells). In contrast to the PICs, we found that $Sca1_{HIGH}$/PW1- cells adopt a smooth muscle phenotype and do not display myogenic nor adipogenic potential (**Fig. 3a,c**). In order to test the potential of PICs at later stages of postnatal life, we extended these analyses to include 2, 3, 5 and 7 weeks old muscle (**Fig.3b,d and supplementary Fig.2**). As expected, satellite cells were highly myogenic at all stages tested (fusion index>70%) and did not form smooth muscle cells nor adipocytes. PICs retained the capacity to generate skeletal muscle (fusion index=20%) and smooth muscle, as well as fat (adipogenic potential=45%) (**Fig.3b,d and supplementary Fig.2**). In contrast, the $Sca1_{HIGH}$/PW1- cells did not show any myogenic nor adipogenic potential but adopted a smooth muscle like phenotype instead. Taken together, these results demonstrate that adult PICs constitute a multipotential population resident in skeletal muscle.

Interstitial adipogenic progenitors constitute a sub-population of PICs

Previous reports demonstrated that adult skeletal muscle contains resident progenitor cells that can adopt an adipogenic fate under pathological conditions (Joe, Yi et al. 2010; Uezumi, Fukada et al. 2010). These adipogenic progenitors can be isolated either on the basis of Sca1 expression (Joe, Yi et al. 2010), or by a combination of Sca1 and PDGFRα expression (Uezumi, Fukada et al. 2010), following removal of blood cells and satellite

cells. The potential overlap of these populations has not been elucidated, so we refer to them collectively as interstitial adipogenic progenitors (IAPs). In order to determine whether PICs and IAPs represent distinct or overlapping populations, we first performed histological analyses on wildtype 7-weeks old mouse *tibialis anterior* and observed that PW1 was co-expressed with PDGFRα in interstitial cells (**Fig.4a**). We next isolated PDGFRα positive and negative cells from the $Sca1_{HIGH}$ fraction to assess their level of PW1 expression (**Fig.4b**). Immunostaining for PW1 and PDGFRα on freshly cytospun cells revealed that all the PDGFRα+ cells of the $Sca1_{HIGH}$ fraction were also PW1+, whereas the $Sca1_{HIGH}$/PDGFRα- fraction contained a small proportion of cells expressing PW1 (12% ±0,9) (**Fig.4c**). We note that co-expression of PDGFRα and PW1 was found even in the small numbers of cells found in all fractions consistent with the conclusion that all the PDGFRα+ cells express PW1 and therefore constitute a sub-population of PICs (**Fig.4c**). We next tested each population for myogenic and adipogenic potential and observed that only the $Sca1_{HIGH}$/PDGFRα+ cells formed adipocytes (**Fig.4d**) consistent with previous results (Uezumi, Fukada et al. 2010). Furthermore, while PDGFRα+ cells displayed no myogenic potential, the PDGFRα- fraction was weakly myogenic. To assess whether this myogenicity was due to the PW1+ cells contained in the $Sca1_{HIGH}$/PDGFRα- fraction, we separated the PDGFRα- from the PDGFRα+ cells in the $Sca1_{HIGH}$/PW1+ fraction (i.e PICs) and cultured them in myogenic or adipogenic conditions (**Fig.5**). As expected, the PDGFRα+ PICs did not form any myogenic colonies but were strongly adipogenic (adipogenic potential=60%). In contrast, the PDGFRα- PICs were strongly myogenic (fusion index=50%) but not adipogenic (**Fig.5b,c**). Taken together these results demonstrate that PICs can be separated on the basis of PDGFRα expression. The majority of PICs express PDGFRα and constitute committed adipogenic progenitors, while a smaller subset of PICs constitute a myogenic progenitor population.

DISCUSSION

In this study, we provide evidence that PICs and satellite cells represent two distinct progenitors in skeletal muscle. As expected, satellite cells are strongly committed to the

myogenic program as reflected by their gene expression profile. In contrast, PICs display a more stem-like profile consisting of genes from a wide range of mesenchymal lineages (**Fig.1 and supplementary tables**). In particular we find that PICs differ from satellite cells not only by their anatomical location (satellite cells are sublaminal and PICs are interstitial) but also by the expression of an array of genes that are commonly associated with angiogenic progenitors including VEGF, angiogenin or the pericyte marker NG2. This finding raises the possibility that PICs and pericytes are derived from a common vessel-related embryonic progenitor. In addition, PICs also express genes that are associated with cell migration and cell-matrix adhesion. Lastly, a comparison between gene expression profiles of PICs and satellite cells reveals a reciprocal expression of growth factors and their cognate receptors suggesting crosstalk between the two cell populations (**supplementary tables**).

Our initial study of PICs was confined to a period of postnatal growth (Mitchell, Pannerec et al. 2010). Thus, an understanding of the existent progenitor populations in adult muscle was undertaken as well as how these populations change during postnatal development. In contrast to quiescent adult muscle, postnatal muscle growth is characterized by high rates of protein synthesis and cell proliferation (Moss and Leblond 1971; Schultz 1996; Davis and Fiorotto 2009). As such, the cellular characteristics and interactions found in juvenile muscle might not reflect the situation in adult muscle maintenance or regeneration. We note that the population of PICs used for transcriptome analyses in this study, and for which the myogenic behavior was established in our initial study, came from 1-2 weeks old postnatal muscle corresponding to this period of intense postnatal muscle growth (Mitchell, Pannerec et al. 2010). We reported previously that PICs were primarily present in 2 cell fractions corresponding to medium and high levels of Sca1 expression, however due to limitations in appropriate markers allowing further enrichment, we could not further address the behavior of the $Sca1_{HIGH}$ population (Mitchell, Pannerec et al. 2010). In order to address this limitation, we took advantage of the $PW1^{nlacZ/+}$ reporter mouse line that accurately reflects the spatiotemporal expression of PW1 (Besson, Smeriglio et al. 2011). Our previous analyses of the PW1 reporter mouse revealed the unexpected finding that PW1 was not only expressed in skeletal muscle satellite cells and PICs as expected, but also in every stem/progenitor cell niche examined in the adult including skin, CNS, bone and gut

(Besson, Smeriglio et al. 2011). Here, we used the β-galactosidase green fluorescent substrate C_{12}FDG to purify PW1+ cells from the $Sca1_{HIGH}$ fraction in both young and adult mouse muscle as well as to analyze whether their cell fate potential changed during the first 7 weeks of postnatal muscle development (**Fig.2**). The time course analysis revealed that PICs are present in the $Sca1_{MED}$ and $Sca1_{HIGH}$ fractions in juvenile muscle while the $Sca1_{MED}$ fraction is no longer present by 5 weeks old such that PICs are only found in the $Sca1_{HIGH}$ fraction in the adult. Testing their fate potential *in vitro*, we find that PICs from both juvenile and adult $Sca1_{MED}$ and $Sca1_{HIGH}$ fractions can differentiate into smooth muscle, skeletal muscle and fat while satellite cells can only adopt a skeletal muscle fate (**Fig.3 and supplementary Fig.2**). These results demonstrate that PICs are multipotent progenitors in both young and adult muscle. However, we noticed that $Sca1_{HIGH}$ PICs are less myogenic (~25%) than $Sca1_{MED}$ PICs (~50%), suggesting that $Sca1_{MED}$ PICs play a specific role in postnatal muscle growth. This hypothesis is further supported by the observation that $Sca1_{MED}$ fraction progressively loses its potentiality at 2 and 3 weeks old and is no longer present by 5 weeks old. These data suggest that the transient postnatal $Sca1_{MED}$ PIC fraction plays a role only during the first weeks of postnatal muscle growth, while the $Sca1_{HIGH}$ PIC fraction is present throughout life and therefore might reflect the $Sca1_{MED}$ cells in the adult. A recent study reported that adult satellite cells do not need Pax7 expression to allow muscle regeneration contrary to juvenile satellite cells, and that this switch from a Pax7-dependent to a Pax7-independent state occurred around 3 weeks of age (Lepper, Conway et al. 2009). As such, radical changes in stem cell genetic requirements occurring at the end of the juvenile period might induce the quiescent adult state. All together, we postulate that the level of Sca1 expression in PICs correlates with their activation state. $Sca1_{MED}$ PICs would thus constitute a transient highly myogenic population that participates in postnatal fiber growth either directly or by sending trophic signals to satellite cells. Between 1 and 3 weeks old $Sca1_{MED}$ PICs fuse with fibers or progressively up-regulate Sca1, so that this population is no longer present by the end of postnatal growth.

While juvenile PICs display similar myogenic and adipogenic potentials (~50%), we noticed that adult PICs are primarily adipogenic (adipogenic potential 60=% and myogenic potential=25%). Two recent studies identified adipogenic progenitors present in the

interstitial space of skeletal muscle (IAPs), that play important roles in regulating the myogenic/adipogenic balance (Joe, Yi et al. 2010; Uezumi, Fukada et al. 2010). Similar FACS isolation techniques based upon Sca1 expression following blood and satellite cells removal, as well as similar behavior *in vitro* and *in vivo*, strongly suggests that IAPs are overlapping populations. In the present work, we demonstrate that the PDGFRα+ cells described by Uezumi *et al*. constitute a sub-population of PICs (**Fig.4**). Our micro-array analyses revealed that PICs highly express PDGFRα in contrast to satellite cells in juvenile muscle (**supplementary table 1**). In adult muscle, both histological and cytospin analyses revealed that all PDGFRα+ cells are located in the interstitial space of the muscle and co-express PW1. Moreover, *in vitro* studies confirmed that only the $Sca1_{HIGH}$/PDGFRα+ cells can differentiate into adipocytes while the other fractions (satellite cells, $Sca1_{HIGH}$/PDGFRα- cells, and double negative cells) did not display any adipogenic capacity. These findings are in agreement with Uezumi *et al*., who reported adipogenic differentiation only in a PDGFRα+ fraction isolated from adult muscle (Uezumi, Fukada et al. 2010). Joe *et al*. isolated FAPs upon CD34 and Sca1 expression, following removal of CD45+ and CD31+ cells (Joe, Yi et al. 2010). Similar to us, they find satellite cells with strong myogenic potential but no adipogenic potential in the CD34+Sca1- fraction. In contrast, CD34+Sca1+ cells, corresponding to our $Sca1_{HIGH}$ fraction, displayed strong adipogenic potential but no myogenic potential (Joe, Yi et al. 2010). We note that in our study, all PDGFRα+ cells were restricted to the $Sca1_{HIGH}$ fraction that also expresses CD34 (**Fig.4**). The isolation of FAPs relied exclusively upon the expression of Sca1, which marks multiple progenitors although with variable reliability (Sherwood, Christensen et al. 2004; Mitchell, Mills et al. 2005). We observed that the $Sca1_{HIGH}$/PDGFRα- population display poor myogenic capacity (fusion index=10%), whereas additional sorting based upon PW1 expression ($Sca1_{HIGH}$/PW1+/PDGFRα-) allowed us to easily detect myogenic capacity (fusion index=50%) (**Fig.5**). Taken together, these results reveal that Sca1+ cells can be divided into 3 distinct populations: the PW1- cells, which adopt a smooth or fibroblast phenotype ; the PW1+/PDGFRα- cells (i.e PDGFRα- PICs), constituting the myogenic fraction ; and the PW1+/PDGFRα+ cells (i.e PDGFRα+ PICs) which represent the adipogenic progenitors (**Fig.6**). These data reveal that PW1 expression in adult muscle identifies at least 3 progenitor populations which can be further distinguished by the

expression of specific markers. We note however that specific genes or epigenetic state for the myogenic fraction of the PICs remain to be determined.

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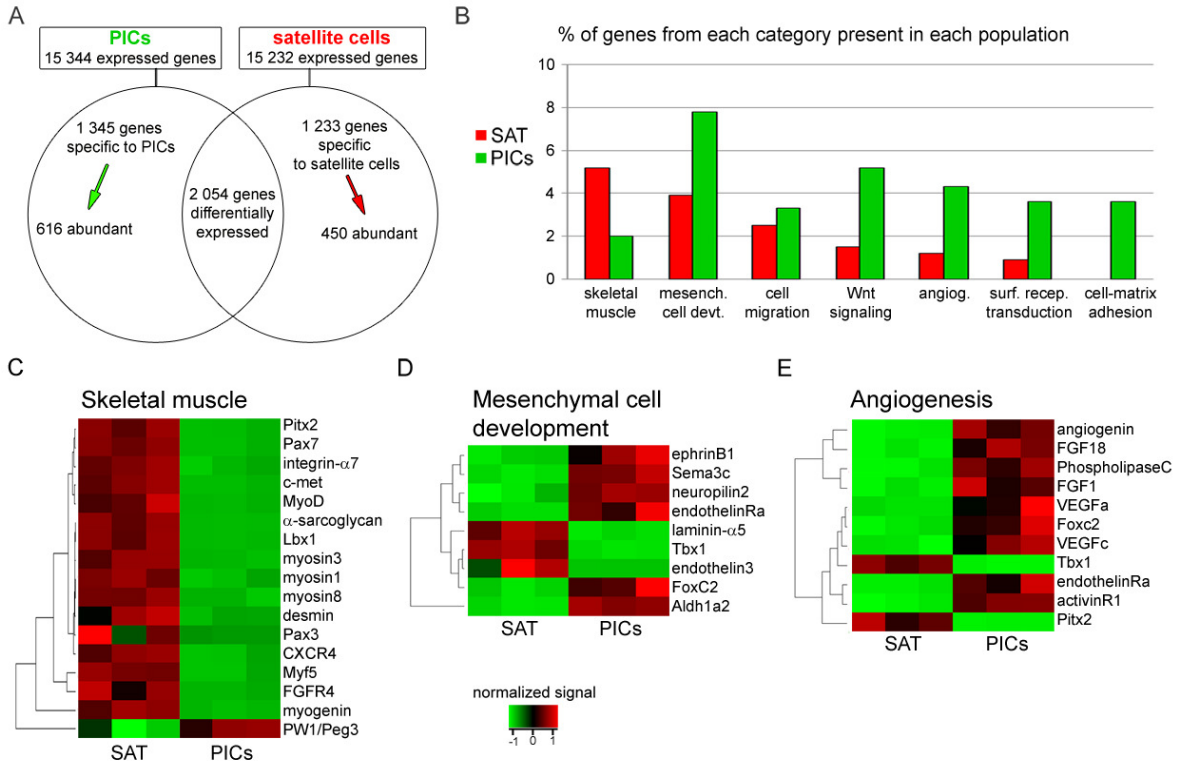


Figure 1. PICs and satellite cells are distinct progenitors which show complementarities in TGFβ pathway genes. (**a**) Venn diagram presenting gene distribution in PICs and satellite cells. Data were obtained from Affymetrix micro-array analysis of 9-day old C57bl6 mice with n=3 for each population, Fold Change threshold=1, p-value=0,01. (**b**) Gene Ontology analysis of "abundant" genes reveals metabolic categories specific to each population. Graph presents percentage of genes from each category expressed by each population. *Mesench. cell devt.=mesenchymal cell development ; angiog.=angiogenesis ; surf. recep. transduction=cell surface receptor linked signal transduction.* (**c-e**) Gene Ontology clustering analysis of "abundant" genes for Skeletal muscle (c), Mesenchymal cell development (d) and Angiogenesis (e) categories. The gene tree is shown on the left, and gene colouring was based on normalized signals as shown in the colour bar.

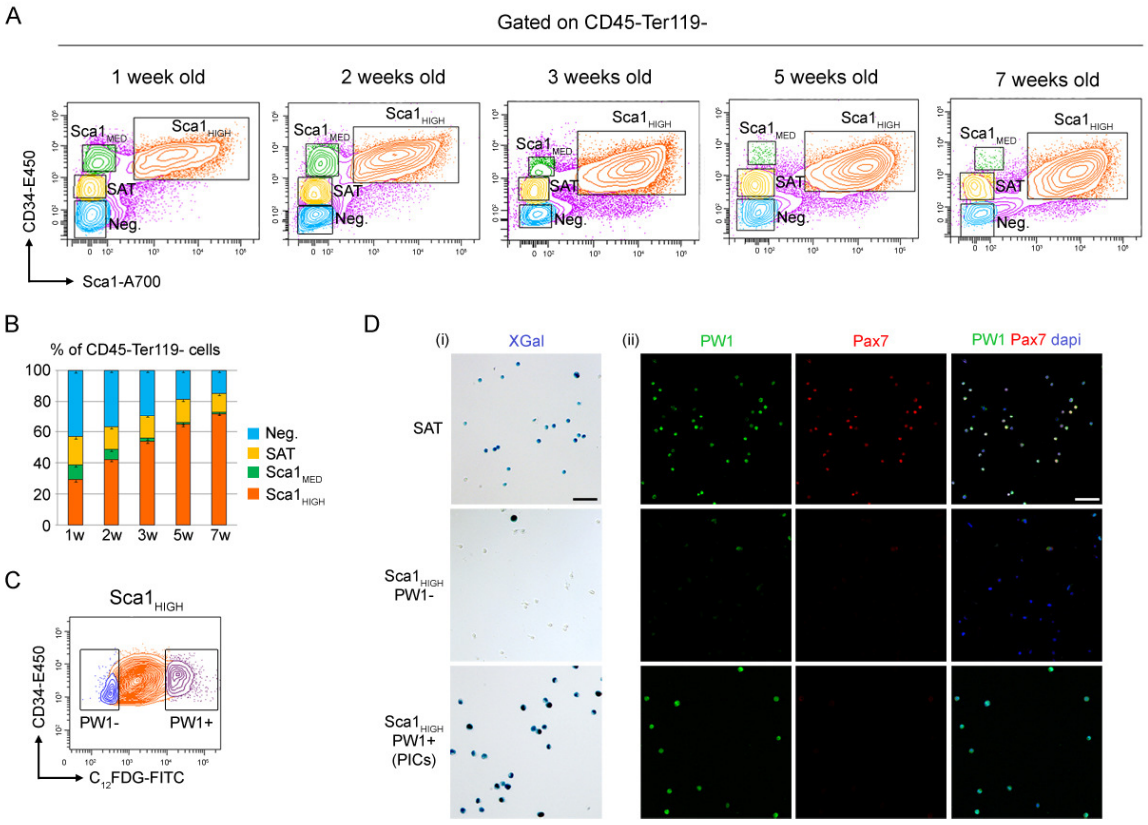


Figure 2. PICs isolation from adult muscle. (**a**) Flow cytometric analyses of single cells from 1-week to 7-week old $PW1^{nLacZ/+}$ mice hindlimb muscles, stained for CD45, Ter119, CD34 and Sca1. The gates used to isolate CD34-Sca1- (Neg.), CD34+Sca1- (SAT), CD34+$Sca1_{MED}$ ($Sca1_{MED}$) and CD34+$Sca1_{HIGH}$ ($Sca1_{HIGH}$) cells are shown. (**b**) Repartition of cell populations described in (a) presented as the mean percentage ± s.e.m. of CD45-Ter119- cells from at least 3 independent experiments. (**c**) Separation of PW1+ (C_{12}FDG+) and PW1- (C_{12}FDG-) cells from the $Sca1_{HIGH}$ fraction using the β-galactosidase green fluorescent substrate C_{12}FDG. (**d**) X-Gal reaction (i) and cytospin analysis (ii) of freshly isolated cells stained for PW1 (green), Pax7 (red) and dapi (blue) to assess purity in each population. Scale bars, 30µm.

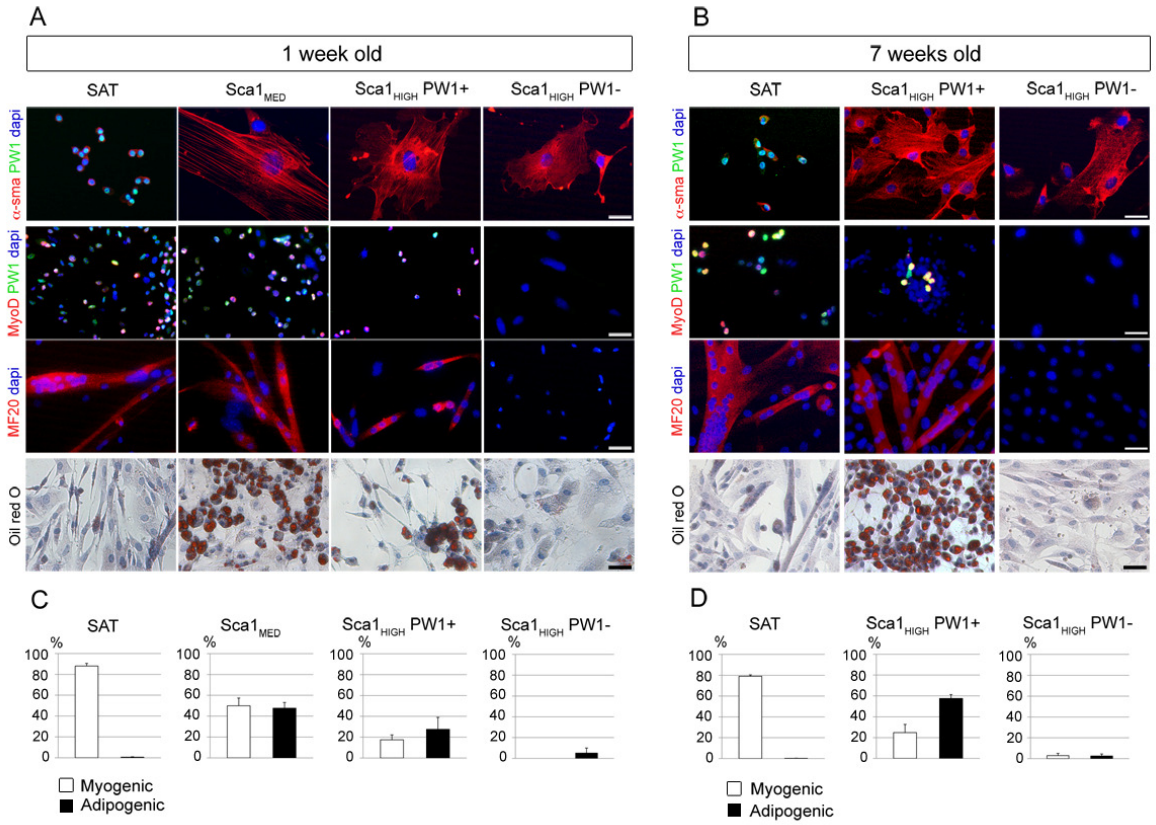


Figure 3. PICs are multipotent progenitors. (**a,b**) Representative images of FACS isolated populations from 1-week (a) and 7-week old (b) $PW1^{nLacZ/+}$ mice immunostained for smooth muscle marker (α-sma, top panel), myogenic markers (MyoD, PW1, MF20, middle panels), or adipogenic marker (Oil red O, bottom panel). Scale bars, 20µm. (**c,d**) *In vitro* myogenic and adipogenic potential of FACS isolated cell populations from 1-week (c) and 7-week old (d) $PW1^{nLacZ/+}$ mice. Myogenic potential was assessed by fusion index after 1 week in GM followed by 2 days in DM, and adipogenic potential was assessed by the percentage of adipocytes after 1 week in GM followed by 5 days in ADM. Values are presented as percentage of positive cells ± s.e.m. from at least n=3 experiments.

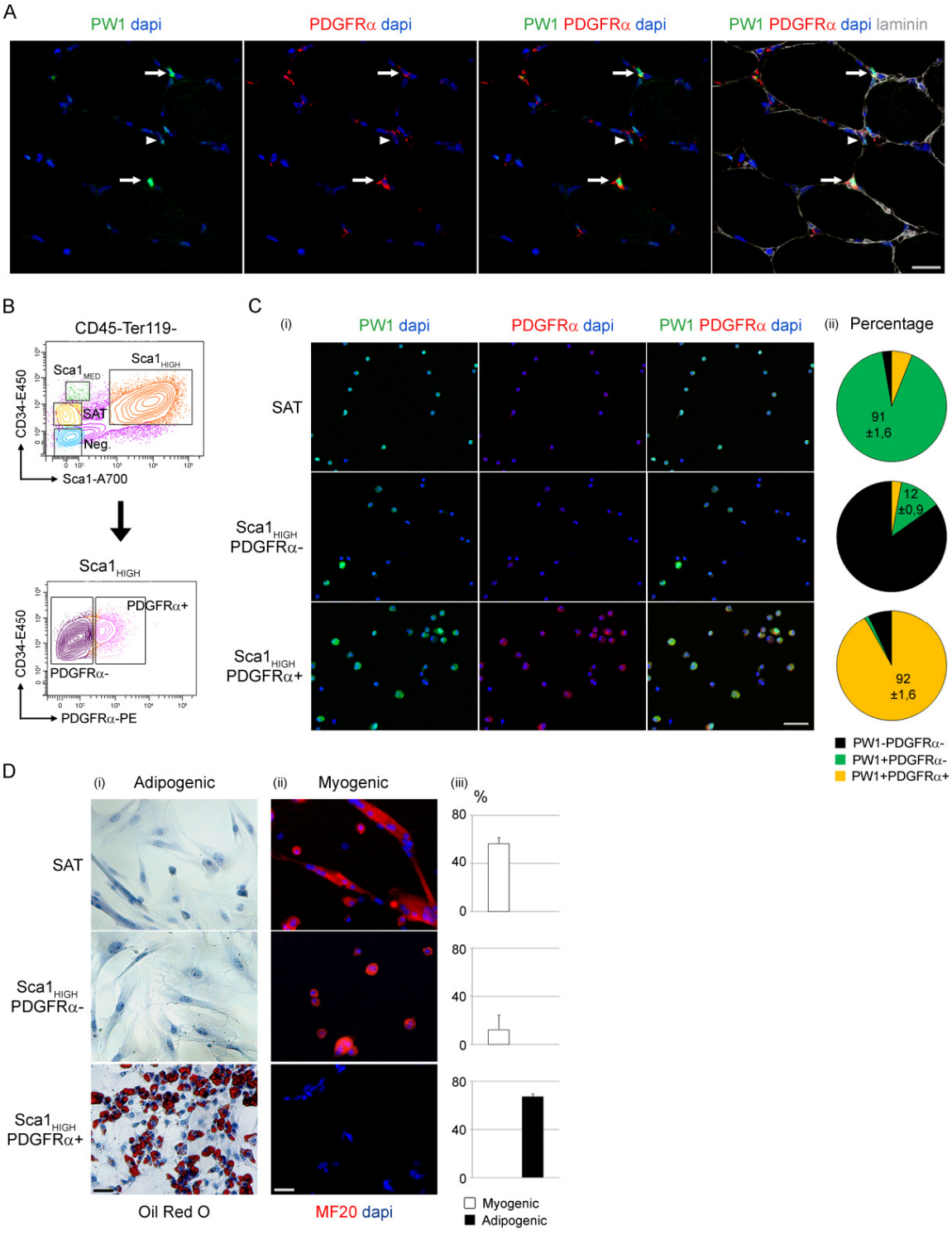


Figure 4. PDGFRα+ cells constitute a sub-population of PICs. (**a**) *Tibialis anterior* cross-section from a 7-week old mouse immunostained for PW1 (green), PDGFRα (red), laminin (grey) and dapi (blue). PDGFRα+ cells are designated with arrows and PICs which do not express PDGFRα are designated with arrowheads. Scale bar, 20µm. (**b**) FACS isolation of PDGFRα+ cells from 7-week old $Sca1_{HIGH}$ cells. (**c**) Cytospin analysis of freshly isolated cells stained for PW1 (green), PDGFRα

(red) and dapi (blue) (i). For each population, the percentage ± s.e.m. of positive cells from 3 independent experiments is indicated in the pie chart (ii). (**d**) Adipogenic and myogenic potentials of the same populations as in (c). For each population, representative images of adipogenic (i) and myogenic (ii) differentiation are shown, as well as the percentage of differentiated cells calculated from 3 independent experiments (iii). Scale bars, 20µm.

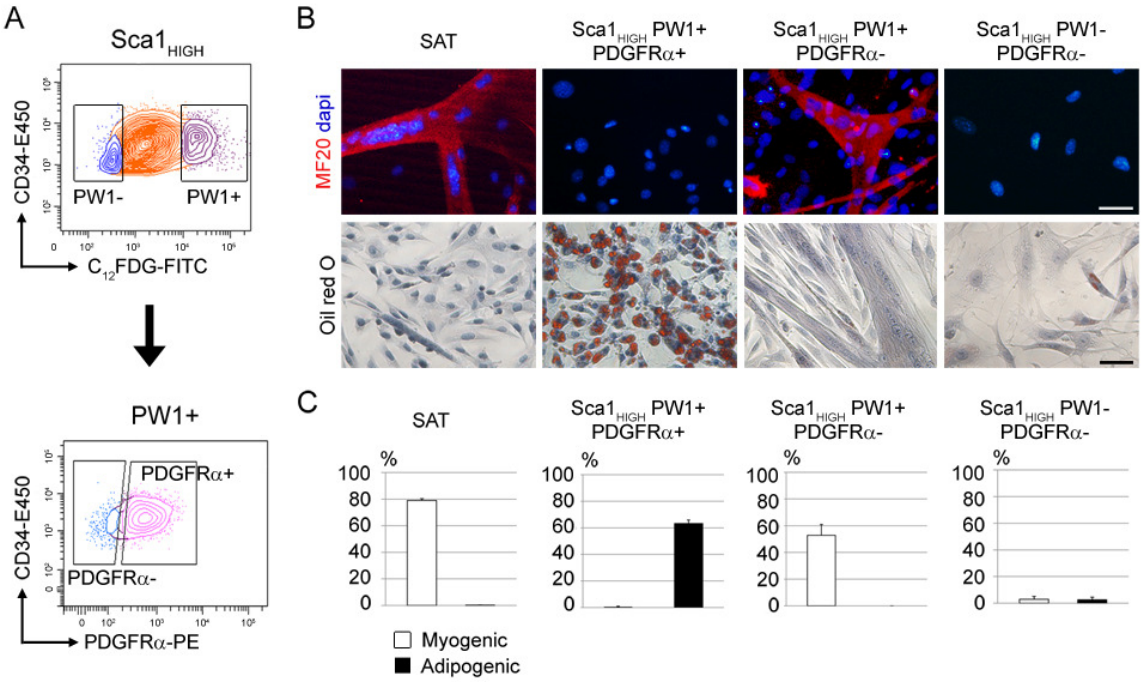


Figure 5. PICs potentials can be distinguished upon PDGFRα expression. (**a**) FACS isolation of PDGFRα+ and PDGFRα- cells from 7-week old $Sca1_{HIGH}$/PW1+ fraction. (**b**) Representative images of FACS isolated populations presented in (a) grown in myogenic (top panel) or adipogenic (bottom panel) conditions. Scale bars, 20µm. (**c**) Quantification of myogenic and adipogenic potentials of cells presented in (b). Myogenic potential was assessed by the fusion index after 1 week in GM followed by 2 days in DM, and adipogenic potential was assessed by the percentage of adipocytes after 1 week in GM followed by 5 days in ADM. Values are presented as percentage of positive cells ± s.e.m. from at least 3 independent experiments.

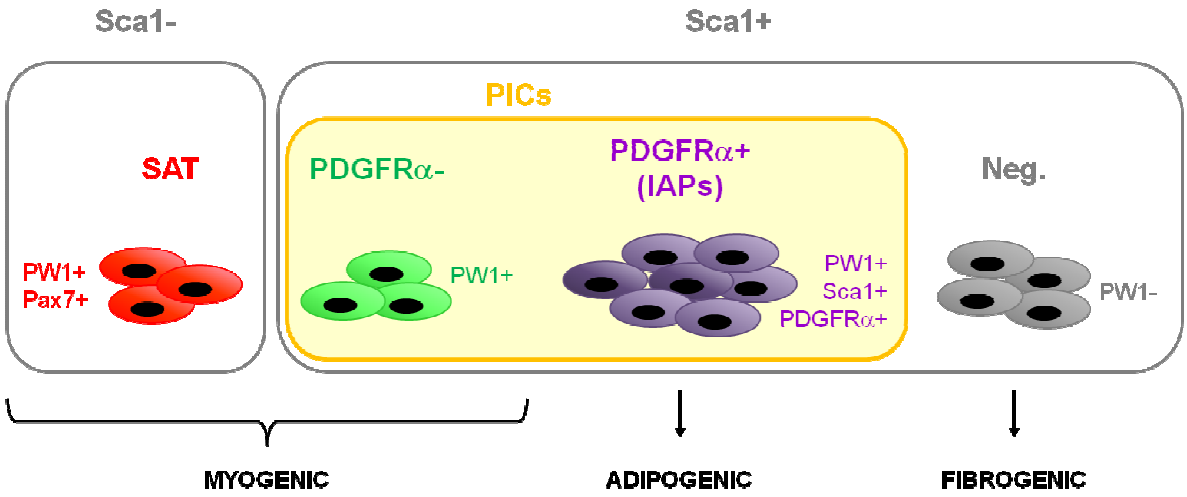
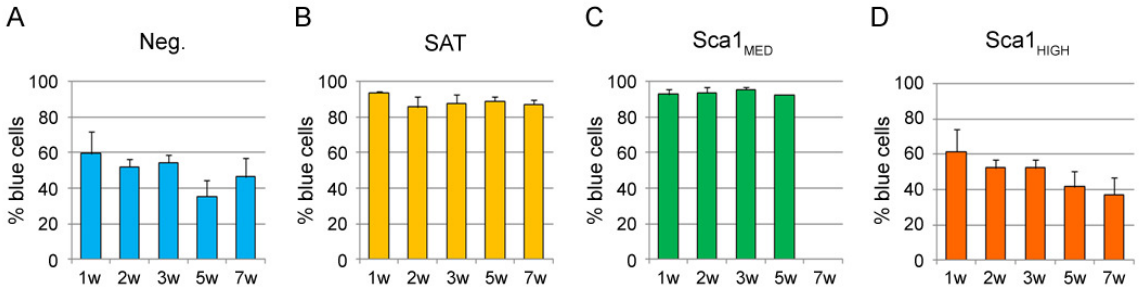
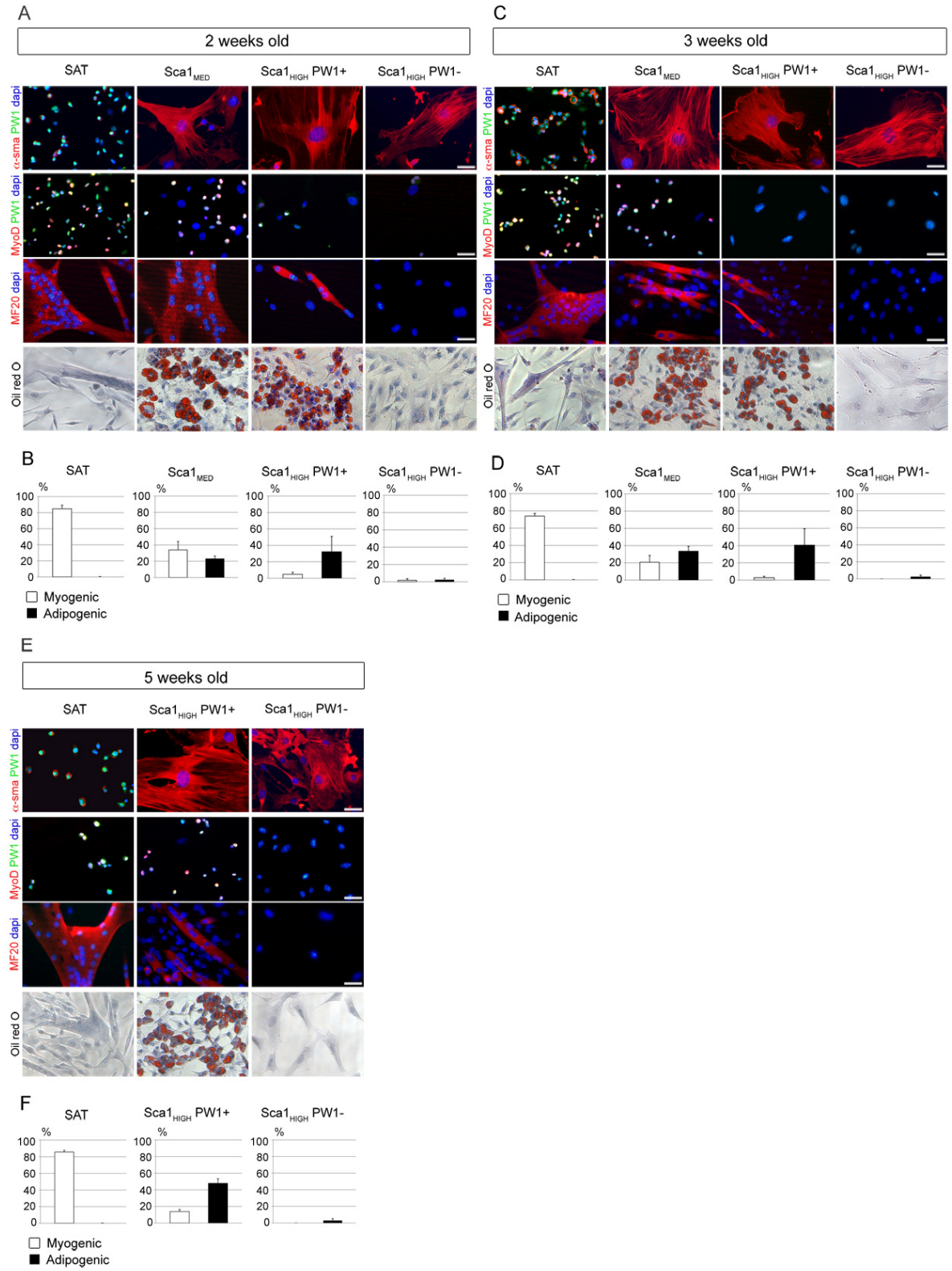


Figure 6. Redefinition of the skeletal muscle stem cell hierarchy. Model presenting stem cell hierarchy upon PW1 expression, in which each progenitor population expresses PW1 and a specific marker. Myogenic potential resides in satellite cells and a sub-population of PICs, while adipogenic potential arises from the IAP fraction of PICs. Cells which do not express PW1 give rise to fibroblasts. *IAPs=interstitial adipogenic progenitors; Neg.=PW1-/PDGFRα- cells.*



Supplementary Figure 1. Purity of FACS sorted muscle populations for PW1 throughout life. FACS isolated cell fractions from PW1$^{nLacZ/+}$ mice were reacted with the β-galactosidase substrate X-Gal to assess PW1 purity. (**a**) CD34-Sca1- cells (Neg.), (**b**) CD34+Sca1- cells (SAT), (**c**) CD34+Sca1$_{MED}$ cells (Sca1$_{MED}$), (**d**) CD34+Sca1$_{HIGH}$ cells (Sca1$_{HIGH}$).



Supplementary Figure 2. PICs are multipotent progenitors during postnatal growth (**a,c,e**) Representative images of FACS isolated populations from 2-week old (a), 3-week old (c) and 5-week old (e) $PW1^{nLacZ/+}$ mice immunostained for smooth muscle marker (α-sma, top panel),

myogenic markers (MyoD, PW1, MF20, middle panels), or adipogenic marker (Oil red O, bottom panel). Scale bars, 20µm. (**b,d,f**) *In vitro* myogenic and adipogenic potential of cells presented in (a,c,e). Myogenic potential was assessed by fusion index after 1 week in GM followed by 2 days in DM, and adipogenic potential was assessed by the percentage of adipocytes after 1 week in GM followed by 5 days in ADM. Values are presented as percentage of positive cells ± s.e.m. from at least n=3 experiments.

Gene symbol	Gene name	Average intensity		Fold Change	p-value
		PICs	SAT		
Qprt	quinolinate phosphoribosyltransferase	793	14	-62,7	0,0027
P2ry14	purinergic receptor P2Y, G-protein coupled, 14	1477	32	-46,5	0,0005
Itgb3	integrin beta 3	518	14	-41,4	0,0019
Apod	apolipoprotein D	1403	39	-39,1	0,0077
Gpr133	G protein-coupled receptor 133	438	13	-35,6	0,0025
Aldh1a2	aldehyde dehydrogenase family 1, subfamily A2	1444	49	-35,4	0,0048
Myoc	myocilin	1549	50	-35,2	0,0035
Adam33	a disintegrin and metallopeptidase domain 33	398	13	-34,8	0,0043
Fst	follistatin	381	11	-32,8	0,0073
Foxd1	forkhead box D1	724	21	-32,4	0,0025
Ptch2	patched homolog 2	840	35	-31,8	0,0094
Il1rl2	interleukin 1 receptor-like 2	499	19	-29,3	0,0027
Pdgfra	platelet derived growth factor receptor, alpha polypeptide	897	124	-28,3	0,0016
Ebf2	early B-cell factor 2	224	8	-28,1	0,0005
Gjb2	gap junction membrane channel protein beta 2	950	35	-27,3	0,0013
Vtn	vitronectin	1201	49	-26,2	0,0023
Il15	interleukin 15	204	8	-25,6	0,0005
Cd55	CD55 antigen	438	18	-25,2	0,0015
Mmp16	matrix metallopeptidase 16	892	44	-23,3	0,0066
Arhgap20	Rho GTPase activating protein 20	473	24	-22,8	0,0041
Dnm1	dynamin 1	1517	91	-19,8	0,0084
Avpr1a	arginine vasopressin receptor 1A	331	20	-19,4	0,0088
Adra2a	adrenergic receptor, alpha 2a	369	21	-18,8	0,0040
Bmp7	bone morphogenetic protein 7	123	6	-18,2	0,0025
Wnt5a	wingless-related MMTV integration site 5A	1293	75	-18,1	0,0018
Ednra	endothelin receptor type A	261	15	-17,7	0,0026
Ephb2	ephrin receptor B2	300	19	-16,7	0,0030
Pi15	Peptidase inhibitor 15	171	12	-16,6	0,0083
Fgf1	fibroblast growth factor 1	462	31	-16,6	0,0060
Twist1	twist gene homolog 1 (Drosophila)	523	33	-16,5	0,0018
Negr1	neuronal growth regulator 1	136	8	-16,4	0,0012
Lepr	leptin receptor	173	11	-16,2	0,0013
Twist2	twist homolog 2 (Drosophila)	114	7	-16,1	0,0013
Efnb1	ephrin B1	315	46	-6,7	0,0013
NG2	chondroitin sulfate proteoglycan 2	11930	1991	-6,3	0,0044
Pdgfrb	platelet derived growth factor receptor, beta polypeptide	4916	1050	-4,8	0,0089
Tgfbr2	transforming growth factor, beta receptor II	9019	2521	-3,6	0,0023

Supplementary table 1. Genes preferentially expressed by PICs. For each gene, the average expression intensity was calculated from Affymetrix analysis. Fold change and p-value are indicated.

Gene symbol	Gene name	Average intensity		Fold Change	p-value
		PICs	SAT		
Gpr50	G-protein-coupled receptor 50	10	1012	117,6	0,0016
Fgfr4	fibroblast growth factor receptor 4	13	1032	86,5	0,0048
Ryr1	ryanodine receptor 1, skeletal muscle	8	640	80,8	0,0019
Chrna1	cholinergic receptor, nicotinic, alpha polypeptide 1 (muscle)	75	4851	74,8	0,0028
Pitx3	paired-like homeodomain transcription factor 3	4	310	68,9	0,0005
Myod1	myogenic differentiation 1	47	2275	57,1	0,0036
Sgca	sarcoglycan, alpha (dystrophin-associated glycoprotein)	6	350	55,6	0,0002
Cdh15	cadherin 15	59	2648	55,3	0,0040
Lbx1	ladybird homeobox homolog 1 (Drosophila)	7	332	48,4	0,0007
Tnnt2	troponin T2, cardiac	23	989	44,5	0,0014
Pitx2	paired-like homeodomain transcription factor 2	8	329	42,6	0,0007
Traf3ip3	TRAF3 interacting protein 3	21	891	41,6	0,0004
Lama5	laminin, alpha 5	13	364	33,8	0,0090
Pde1c	phosphodiesterase 1C	6	166	26,6	0,0005
Arpp21	cyclic AMP-regulated phosphoprotein, 21	13	349	26,1	0,0005
Atp2a1	ATPase, Ca++ transporting, cardiac muscle, fast twitch 1	64	1481	22,4	0,0022
Tnik	TRAF2 and NCK interacting kinase	28	593	22,0	0,0041
Myh3	myosin, heavy polypeptide 3, skeletal muscle, embryonic	18	378	21,3	0,0016
Neb	nebulin	7	155	21,1	0,0002
Dcx	doublecortin	10	194	19,5	0,0046
Ephb1	ephrin receptor B1	8	124	16,0	0,0016
Chrng	cholinergic receptor, nicotinic, gamma polypeptide	8	134	15,8	0,0031
Myh8	myosin, heavy polypeptide 8, skeletal muscle, perinatal	21	289	15,5	0,0077
Ank1	ankyrin 1, erythroid	88	1245	15,4	0,0035
Pdgfa	platelet derived growth factor, alpha polypeptide	125	1532	13,7	0,0095
Ppargc1b	peroxisome proliferative activated receptor, gamma, coactivator 1 beta	12	154	13,3	0,0025
Edn3	endothelin 3	7	109	13,0	0,0089
Tbx1	T-box 1	9	109	11,7	0,0008
Hdac11	histone deacetylase 11	53	520	9,8	0,0009
Des	desmin	52	442	8,6	0,0091
Gdf15	growth differentiation factor 15	45	390	8,6	0,0074
Drp2	Dystrophin related protein 2	73	563	8,3	0,0066
Tpm2	tropomyosin 2, beta	17	134	8,1	0,0019
Acvr2b	activin receptor IIB	21	159	8,2	0,0148

Supplementary table 2. Genes preferentially expressed by satellite cells. For each gene, the average expression intensity was calculated from Affymetrix analysis. Fold change and p-value are indicated.

CHAPTER 4. DEFINING THE ROLE OF PICS IN ADULT SKELETAL MUSCLE.

In this second part of the study, we sought to determine the role(s) of PICs in adult muscle. An examination of our micro-array analyses comparing PICs and satellite cells gene expression profiles revealed that myostatin is expressed by satellite cells whereas its antagonist follistatin is highly expressed by PICs, suggesting that PICs and satellite cells crosstalk through the TGFβ pathway. To test this hypothesis we investigated myostatin effects on PICs and satellite cells *in vitro*, as well as assessed the effects of co-culturing using a transwell membrane system to allow for paracrine signaling.

Our previous results suggest that PICs play a role in muscle hypertrophy either by directly fusing with fibers, since they display some myogenic potential *in vitro*; or by producing factors favoring myoblast differentiation (and see (Joe, Yi et al. 2010)). To investigate these 2 hypotheses, we induced muscle hypertrophy in male mice by delivering a soluble form of ActR2b, termed RAP-031, which has been shown to rapidly lead to an increase in muscle mass (Cadena, Tomkinson et al. 2010). In order to distinguish between PICs and satellite cells potential contribution to fiber growth, we used the $Pax7^{DTR/+}$ mice which express the diphtheria toxin (DT) receptor under the Pax7 promoter and allow the conditional ablation of satellite cells upon DT injection (Sambasivan, Yao et al. 2011). Using this mouse model or similar ones, recent studies have demonstrated that muscle regeneration is abrogated in the absence of satellite cells leading to the conclusion that non-satellite myogenic progenitors do not participate in fiber regeneration under normal physiological conditions (Lepper, Partridge et al. 2011; McCarthy, Mula et al. 2011; Sambasivan, Yao et al. 2011).

The results presented here suggest that the state of the muscle stem cell niche is not merely dependent upon the presence or absence of satellite cells. We demonstrate that myostatin inhibition leads to a significant activation and recruitment of PICs that is permissive for regeneration even when satellite cells are largely depleted. We propose that PICs allow myoblast recruitment and activation by releasing positive signals such as follistatin.

Myostatin inhibition promotes non-satellite stem cell recruitment in skeletal muscle regeneration.

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Myostatin is a potent inhibitor of skeletal muscle growth. Strategies aimed at increasing muscle mass have sought to inhibit myostatin signaling by the systemic delivery of antagonists such as a soluble form of the activin receptor 2b (ActR2b). It is unknown whether satellite cells, which are a potential source of additional myonuclei, nor other resident progenitors are important in muscle hypertrophy in response to myostatin inhibition. Here we show that PW1-positive interstitial cells referred to as PICs (PW1+ Interstitial Cells) produce follistatin that promotes satellite cell proliferation and further blocks the activity of myostatin, and that PICs are preferentially recruited following myostatin inhibition. In addition, whereas regeneration is normally absent in muscles depleted for satellite cells, we observed that exposure to soluble ActR2b prior to muscle damage rescues regenerative capacity. We propose that myostatin inhibition results in the activation of PICs which in turn promote a favorable environment for myoblast recruitment and commitment, and that the local muscle stem cell environment can be modified to promote regeneration in the absence or severe depletion of canonical stem cells.

Key words: myostatin, muscle hypertrophy, ActR2b, PICs.

INTRODUCTION

Preserving and/or promoting skeletal muscle mass represents a key therapeutic challenge for the treatment of chronic diseases such as muscular dystrophies and cancers, as well as in the case of age-related loss of muscle mass (sarcopenia). One attractive therapeutic target is myostatin, which is a member of the transforming growth factor β family (TGFβ) and is

expressed predominantly in skeletal muscle (McPherron, Lawler et al. 1997; Lee and McPherron 2001). Numerous species ranging from mouse to humans display a massive increase in muscle mass and size in response to a loss of function of the myostatin gene (Grobet, Martin et al. 1997; Kambadur, Sharma et al. 1997; McPherron, Lawler et al. 1997; McPherron and Lee 1997; Schuelke, Wagner et al. 2004; Clop, Marcq et al. 2006). Myostatin acts predominantly through binding to the activin transmembrane receptor type 2b (ActR2b) with its corresponding type 1 receptor Alk4 or Alk5 (Lee and McPherron 2001) leading to the phosphorylation of Smad proteins which translocate to the nucleus to control gene expression (Zhu, Topouzis et al. 2004). In particular, the cyclin-dependent kinase (Cdk) inhibitor p21 is up-regulated while Cdk2 activity is reduced, resulting in the suppression of retinoblastoma protein (Rb) phosphorylation and concurrent cell cycle arrest to the G_1 phase (Thomas, Langley et al. 2000; Joulia, Bernardi et al. 2003). Myostatin also inhibits MyoD, Myf5 and myogenin expression resulting in a block of myogenic progression (Thomas, Langley et al. 2000; Taylor, Bhasin et al. 2001; Langley, Thomas et al. 2002; Rios, Carneiro et al. 2002; Joulia, Bernardi et al. 2003).

Several approaches have been developed to block myostatin signaling including direct delivery of myostatin blocking antibodies or antagonists, or the delivery of their adeno-associated viruses form (Bogdanovich, Krag et al. 2002; Lee, Reed et al. 2005; Qiao, Li et al. 2008). These treatments result in a significant amelioration of muscle physiology in mouse models for muscular dystrophies by inducing fiber hypertrophy and improving muscle strength (Whittemore, Song et al. 2003; Haidet, Rizo et al. 2008). Pronounced muscle hypertrophy is observed following injection of a soluble form of the ActR2b (Lee, Reed et al. 2005; Cadena, Tomkinson et al. 2010) or with the natural myostatin antagonist follistatin (Haidet, Rizo et al. 2008; Nakatani, Takehara et al. 2008), however in these cases, it is not clear whether fiber hypertrophy is due to a net increase in myonuclei or an increase in myofiber protein content or a combination of both.

It is generally accepted that satellite cells constitute the major source of postnatal skeletal muscle stem cells during muscle regeneration (Mauro 1961; Montarras, Morgan et al. 2005; Relaix, Rocancourt et al. 2005; Zammit, Relaix et al. 2006). In addition, myostatin inhibits satellite cell proliferation *in vitro*, suggesting that blocking myostatin will lead to satellite

cell differentiation and fusion supporting myofiber growth. In accordance with this hypothesis, McCroskery and colleagues observed that *myostatin-null* ($Mst^{-/-}$) mice display a ~50% increase in the number of activated satellite cells as compared to wildtype (WT) animals (McCroskery, Thomas et al. 2003). Furthermore, a 5-fold increase in satellite cell numbers is observed following a single injection of the soluble ActR2b (Zhou, Wang et al. 2010). In contrast, other groups have provided evidence that muscle hypertrophy induced by myostatin inhibition does not involve nor require satellite cells but is simply the result of an increase in myofiber protein content (Amthor, Otto et al. 2009; Blaauw, Canato et al. 2009; Wang and McPherron 2012). One explanation for this discrepancy comes from a recent study demonstrating that myostatin inhibition by the soluble ActR2b leads to an initial increase in fiber size followed later by satellite cell activation and myonuclear addition (Wang and McPherron 2012). Taken together, these studies imply that a fiber size increase is a two-step process that may require nuclear addition to sustain the increase in fiber size.

Increasing evidence supports the notion that non-satellite cell populations possess high myogenic potential. These include the hematopoietic lineage (side population), vessel-associated cells (mesoangioblasts and pericytes), and interstitial resident cells (PICs, CD133+ cells, β4-integrin+ cells) (Gussoni, Soneoka et al. 1999; Sampaolesi, Torrente et al. 2003; Sampaolesi, Blot et al. 2006; Benchaouir, Meregalli et al. 2007; Dellavalle, Sampaolesi et al. 2007; Mitchell, Pannerec et al. 2010; Liadaki, Casar et al. 2012). While these progenitors readily participate in myogenesis following engraftment into damaged or diseased muscle, their direct contribution to postnatal muscle growth and regeneration *in vivo* is unknown or extremely limited (Dellavalle, Maroli et al. 2011). Indeed, the limited role of these atypical myogenic cells has been illustrated by two recent studies in which muscle regeneration is completely abrogated when the satellite cell population is depleted (Lepper, Partridge et al. 2011; Sambasivan, Yao et al. 2011). However, several of these non-satellite cell types are presently being used in clinical trials as a preferred stem cell donor for engraftment due to their ability to migrate, circulate an invade damaged muscle tissue in contrast to satellite cells that have thus far proved disappointing in pre-clinical and clinical settings (Pannérec, Marazzi et al., in press). One of the muscle resident progenitor populations with myogenic potential was identified by our group and referred to as PICs

(PW1+ Interstitial Cells), is characterized by PW1/Peg3 expression and its interstitial location. Lineage tracing studies demonstrated that PICs and satellite cells do not derive from the same embryonic progenitor (Mitchell, Pannerec et al. 2010). More recently, we reported that PICs display a gene expression profile consistent with a mesenchymal progenitor while satellite cells are committed to the skeletal muscle lineage, suggesting that PICs constitute an upstream population (Pannérec, Formicola et al., manuscript in preparation). *In vitro* studies confirmed these observations by revealing that PICs are multipotent progenitors capable to differentiate into smooth muscle, skeletal muscle and fat, while satellite cells only adopt a skeletal muscle phenotype (Pannérec, Formicola et al., manuscript in preparation). Lastly, we showed that the adult PIC population consists of at least 2 lineages, one that retains the capacity to undergo skeletal myogenesis and one that is committed to the adipogenic lineage (Pannérec, Formicola et al., manuscript in preparation). Given that adipogenic progenitors resident in skeletal muscle have been demonstrated to exert a pro-myogenic effect upon injured muscle (Joe, Yi et al. 2010), we proposed that PICs play a role in the regulation of muscle homeostasis.

In this study we demonstrate that PICs exert a beneficial effect on satellite cell proliferation in the presence of myostatin by secreting the myostatin antagonist follistatin. *In vivo*, pharmaceutical inhibition of myostatin leads to a massive increase in PICs number and promotes muscle regeneration in the absence of satellite cells. Taken together, we propose that PICs participate in myogenesis through the release of positive factors that favor myoblast recruitment. Our results imply that PICs and/or other non-satellite cell populations are recruited when satellite cells are either absent or too few to execute a regenerative response.

MATERIALS AND METHODS

Mice models. The animal models used were the *PW1*^{IRESnLacZ} transgenic mice (PW1$^{nlacZ/+}$), in which a nuclear operon lactose gene is expressed under the control of the *Pw1* locus in a bac construct (Besson, Smeriglio et al. 2011); the *myostatin-null* mice (Mst$^{-/-}$), in which the C-terminal region of myostatin was deleted and replaced by a neo cassette (McPherron, Lawler et al. 1997); the Pax7$^{DTR/+}$ mice, in which the diphteria toxin receptor in expressed under the control of the *Pax7*

promoter; and C57Bl6J mice (Elevage Janvier). All work with mice was carried out in adherence to French government guidelines.

RAP-031 treatment. Five-week old C57Bl6 or $Pax7^{DTR/+}$ males were treated with intra-peritoneal injections of RAP-031 or vehicle (TBS) at $10mg.kg^{-1}$ of body weight. Mice were weighed and dosed twice weekly for 2 weeks. At the end of the treatment period, mice were euthanized by cervical dislocation and weighted. *Tibialis anterior* (TA), *extensor digitorum longus* (EDL), *soleus*, *plantaris* and *gastrocnemius* muscles were removed, weighed, and snap frozen in liquid nitrogen-cooled isopentane.

Toxins injections. A total volume of 15-30µl was used for intramuscular injections of toxins or PBS into the TA muscle using 30G Hamilton syringes, under anaesthesia induced by intra-peritoneal injection of ketamine ($100mg.kg^{-1}$) and xylazine ($10mg.kg^{-1}$) in sterile saline solution. Diphtheria toxin (DT) from *Corynebacterium diphtheria* (Sigma Aldrich) was used at $1ng.g^{-1}$ of body weight. Muscle injury was induced by intramuscular injection of cardiotoxin from *Naja mossambica* (Sigma), at a concentration of 10µM.

Primary cell culture. Primary skeletal muscle cells from limb muscles of 1 week and 7 weeks old $PW1^{nLacZ/+}$ mice were FACS sorted as described previously (Pannérec, Formicola et al., manuscript in preparation). Immediately after sorting, satellite cells were plated on gelatin-coated dishes at a density of 100 cells per cm^2, in a growth medium (GM) composed of high-glucose Dulbecco's modified eagle medium (DMEM, Gibco) supplemented with $2.5ng.ml^{-1}$ bFGF (Invitrogen), 20% heat-inactivated FBS (Invitrogen), 10% heat-inactivated horse serum (Gibco), 1% (v/v) penicillin-streptomycin (Gibco), 1% (v/v) L-Glutamine (Gibco) and 1% (v/v) Na-pyruvate (Gibco). After 1 day in GM, cells were switched in GM containing 0-2µg recombinant myostatin (R&D Systems) and were allowed to grow for 2 more days. Proliferation was assessed by counting the number of cells par colony. For myogenic differentiation assay, cells grown 4 days in GM followed by 2 days in differentiation medium (DM) composed of DMEM supplemented with 5% (v/v) horse serum, 1% (v/v) penicillin-streptomycin and containing 0-2µg recombinant myostatin. Fusion index was quantified by counting the number of nuclei in MF20+ cells per total number of nuclei. For co-culture experiments, PICs were allowed to grow on top of satellite cells using insert wells (1µm, BD Biosciences), at a density of 3,000 cells per cm^2. After 1 day in GM, cells were switched in GM containing 0-2µg recombinant myostatin and human follistatin blocking antibody or its isotype control ($4µg.mL^{-1}$, R&D Systems). After 2-3 days, cells were stopped and the number of cells par colony was counted.

Histological analyses. For general muscle morphology, hematoxilin and eosin staining was performed on muscle cryosections (10µm). For immunofluorescence, TA cryosections and cultured cells were fixed in 4% (w/v) paraformaldehyde and processed for immunostaining as described previously (Mitchell, Pannerec et al. 2010). The following primary antibodies were used: PW1 ((Relaix, Weng et al. 1996) 1:3,000), Pax7 (Developmental Studies Hybridoma Bank, 1:15), M-Cadherin (Nanotools, 1:100), Ki67 (BD Biosciences, 1:100), laminin (Sigma, 1:100) and MF20 (Developmental Studies Hybridoma Bank, 1:100). Antibody binding was revealed using species-specific secondary antibodies coupled to Alexa Fluor 488 (Molecular Probes), Cy3 or Cy5 (Jackson Immunoresearch). Nuclei were counterstained with DAPI (Sigma). Fiber size distribution was measured from cryosections obtained from the mid-belly of TA stained with laminin, images were captured on a Zeiss AxioImagerZ1 microscope, and morphometric analysis was performed using MetaMorph7.5 (Molecular Devices).

Gene expression and western blot analyses. RNA extraction was performed on freshly FACS sorted cells or frozen TA powder using the RNeasy micro-kit or fibrous tissue kit, respectively (Qiagen). cDNA was generated by random-primed reverse transcription using the SuperScript First Strand kit (Invitrogen). The cDNAs were then analyzed by semi-real-time PCR using the Ready Mix PCR Master Mix (Thermo Scientific), or real-time PCR using the Light Cycler 480 SYBR Green I Master mix (Roche). See Supplementary Table 1 for primers used. For western blot, total proteins were prepared from FACS sorted cells or frozen TA powder using RIPA lysis buffer, and nuclear enriched extracts were prepared as previously described (Thornborrow and Manfredi 1999). Equal amounts of protein were separated by electrophoresis (10% SDS-polyacrylamid) and transferred to a PVDF membrane in 20% methanol transfer buffer. Membranes were probed with antibodies to myostatin (Millipore, 1:500), follistatin (Abcam 1:800), and tubulin (Calbiochem, 1:500). Antibody binding was visualized with HRP-conjugated secondary antibodies (Jackson ImmunoResearch) followed by enhanced chemiluminescence (Pierce).

Statistical analysis. All statistics were performed using an unpaired Student's t-test in the StatView software. Values represent the mean ± s.em. *$p<0.05$, **$p<0.01$ and ***$p<0.001$.

RESULTS

PICs and satellite cells differentially express genes of the TGFβ pathway

We have previously reported that PICs and satellite cells share myogenic capacity however they show significant differences in their gene expression profiles (Pannérec, Formicola et al., manuscript in preparation). Among these differences, we found that many genes from the TGFβ pathway are differentially expressed between PICs and satellite cells; in particular ActR2b is expressed by satellite cells while follistatin is expressed by PICs (**Fig.1a and supplementary table 2**). We looked at myostatin and follistatin expression in juvenile (9 day old) cell populations, sorted as previously described, using semi-quantitative PCR (Pannérec, Formicola et al., manuscript in preparation) (Mitchell, Pannerec et al. 2010): CD34-Sca1- cells (Neg.), CD34+Sca1- satellite cells (SAT), CD34+$Sca1_{MED}$ PICs ($Sca1_{MED}$) and CD34+$Sca1_{HIGH}$ cells ($Sca1_{HIGH}$ being a mixed population containing ~50% PICs) (**Fig.1b**). We confirmed follistatin expression in the $Sca1_{MED}$ and $Sca1_{HIGH}$ populations corresponding to PICs whereas myostatin expression was detected in satellite cells as observed by others (McCroskery, Thomas et al. 2003). In adult muscle, PICs are present only in the $Sca1_{HIGH}$ fraction and can be purified using a FACS sorting technique previously described (Pannérec, Formicola et al., manuscript in preparation). Similarly to early postnatal cells, we observed myostatin expression in satellite cells and follistatin expression in PICs (**Fig.1c**). As follistatin acts as an endogenous myostatin antagonist, these results suggest a reciprocal paracrine interaction between PICs and satellite cells.

PICs rescue satellite cell proliferation from myostatin inhibitory effect through follistatin release

To test whether PICs and satellite cells interact through a reciprocal paracrine regulatory pathway involving myostatin and follistatin secretion, we first compared how PICs and satellite cells respond to myostatin *in vitro*. We therefore cultured juvenile wildtype (WT) and $Mst^{-/-}$ cells in myogenic growth medium (GM) or differentiation medium (DM) in the presence of increasing doses of recombinant myostatin (**Fig.2**). As observed by others (McCroskery, Thomas et al. 2003), (Langley, Thomas et al. 2002), we found that satellite cell proliferation and differentiation are inhibited by myostatin in a dose-dependent manner

(**Fig.2a,b**). Moreover we noted that $Mst^{-/-}$ satellite cells possess a higher myogenic potential in standard conditions, suggesting that WT satellite cells respond to myostatin in an autocrine manner. In contrast, WT PIC proliferation was not affected by myostatin and $Mst^{-/-}$ PIC proliferation was inhibited only at high doses of myostatin, whereas both WT and $Mst^{-/-}$ PIC differentiation were inhibited by myostatin in a dose-dependent manner (**Fig.2c,d**). These results indicate that PICs are protected from myostatin effect in proliferative conditions, but lose this property upon acquiring a myogenic cell fate. To test whether PICs rescue satellite cells from myostatin inhibition, we cultured PICs and satellite cells in GM in transwell dishes at increasing levels of recombinant myostatin (**Fig.3a**). We observed that satellite cells are able to form significantly bigger colonies in the presence of PICs at all the doses of myostatin tested, demonstrating that PICs impart a compensatory effect into the media that overcomes the inhibitory effects of myostatin upon satellite cells (**Fig.3b**). We next evaluated the potential of adult PICs to rescue adult satellite cells. As observed with juvenile cells, adult satellite cell proliferation is inhibited by myostatin in a dose-dependent manner (**Fig.4a**). In contrast, co-culturing PICs under transwell conditions led to a significant increase in satellite cell proliferation comparable to baseline conditions. These results support the hypothesis that PICs produce protective factor(s) that overcome the inhibitory effects of myostatin upon satellite cells. Since we observed that PICs secrete follistatin, which is a potent myostatin inhibitor (Amthor, Nicholas et al. 2004), (Lee and McPherron 2001), we co-cultured PICs and satellite cells in a transwell assay in the presence of a follistatin blocking antibody (αFST) or a control IgG (αIgG) (**Fig.4a**). In the presence of αFST, the PIC-mediated rescue of satellite cell proliferation was significantly reduced but not eliminated, suggesting that PICs act in part through follistatin secretion but additional factors may also be secreted that promote satellite cell proliferation and differentiation (**Fig.4b**).

Myostatin inhibition leads to stem cell recruitment in adult mice

We injected the soluble activin receptor RAP-031 into C57Bl6 males beginning at 5 weeks postnatal for 2 weeks consisting of 2 injections per week as described previously (Cadena, Tomkinson et al. 2010) (**Fig.5a**). Western blot analyses confirmed that the active form of myostatin was down-regulated after 2 weeks of treatment while its precursor was still present (**supplementary Fig.1a**). After 2 weeks of treatment, we compared the body

weight of RAP-031-treated animals (RAP-031) with the age-matched constitutive *myostatin-null* ($Mst^{-/-}$) males and control C57Bl6 males (CTL). As reported by others, RAP-031 and $Mst^{-/-}$ animals displayed a marked increase in their body weight and visibly larger skeletal muscles (**Fig.5b,c**) (McPherron, Lawler et al. 1997; Lee, Reed et al. 2005; Cadena, Tomkinson et al. 2010). Among RAP-031 mice hindlimb muscles, the *tibialis anterior* (TA) was significantly bigger with larger muscle fibers after 2 weeks of treatment (**Fig.5d-f**). We next examined PIC and satellite cell numbers in CTL, $Mst^{-/-}$, and RAP-031 hindlimb muscles (**Fig.6 and supplementary Fig.2**). In $Mst^{-/-}$ TA we observed a 2-fold increase in satellite cells and a 3-fold increase in PICs number per 100 fibers, while in RAP-031 treated TA we observed a 2.5-fold increase in satellite cell number and 4-fold increase in PIC number per 100 fibers (**Fig.6a,b**). Similar results were obtained in *gastrocnenius*, *plantaris* and *soleus* muscles, suggesting that myostatin inhibition induces stem cell proliferation (**supplementary Fig.2**). To test this hypothesis, we immunostained TA cross-sections with the cell cycle marker Ki67 and observed a 5-fold increase in proliferating cells in RAP-031 muscles compared to CTL (**Fig.6c,d**). Specifically we observed a 2-fold increase in labeled PICs as compared to controls whereas the percentage of proliferative satellite cells was unchanged, indicating that PICs are preferentially recruited when myostatin is inhibited (**Fig.6c,d**). In contrast, we detected only very low levels of Ki67 labeling in $Mst^{-/-}$ muscles. Moreover, we observed a marked increase in the number of interstitial and sub-laminal nuclei per 100 fibers in RAP-031 muscles compared to CTL and $Mst^{-/-}$ muscles, while the nuclei distribution was unchanged (**Fig.7**). These results demonstrate that myostatin inhibition by the soluble ActR2b induces fiber hypertrophy concomitant with a rapid induction of PICs and an increase in myonuclear content, suggesting that RAP-031 treatment leads to stem cell recruitment, proliferation, and fusion with existing fibers.

PICs do not directly participate in fiber hypertrophy following myostatin inhibition

We have demonstrated that PICs promote satellite cell proliferation *in vitro* and are activated *in vivo* following myostatin inhibition. In addition, we find that fiber hypertrophy in response to RAP-031 involves new myonuclei addition. In a previous study, we established that PICs are multipotential progenitors capable of differentiating into skeletal muscle, smooth muscle, and adipocytes (Pannérec, Formicola et al., manuscript in

preparation). Therefore, we sought to determine whether PICs contribute directly to fiber hypertrophy by committing to the skeletal muscle lineage and fusing with existing fibers. In order to distinguish between satellite cell versus non-satellite cell contribution during hypertrophy, we used an inducible satellite cell-depleted mouse model, in which the diphteria toxin (DT) receptor is expressed under the control of Pax7 and, as such, DT injection induces the death of satellite cells (Sambasivan, Yao et al. 2011). Mice were injected with DT in one TA and PBS in the contralateral muscle, and were allowed to recover for 2 weeks prior to RAP-031 treatment (**Fig.8a**). Consistent with our previous results, animals treated with RAP-031 displayed a marked increase in body weight as compared to CTL animals and the control TAs were significantly bigger (**Fig.8b,c**). TAs injected with DT from RAP-031 mice did not show a significant increase in weight, however they did possess bigger myofibers (**Fig.8c,d**). Analyses of fiber size distribution in satellite cell-depleted TAs revealed that myostatin inhibition induces muscle hypertrophy in the absence of satellite cells (**Fig.8e**). However, in contrast to control TAs, we did not observe an increase in myonuclei numbers in TAs injected with DT suggesting that fiber hypertrophy does not involve myonuclear accretion (**Fig.8f**). We verified that satellite cells were depleted by staining for satellite cell using M-Cadherin. As reported previously (Sambasivan, Yao et al. 2011), we detected almost no satellite cells in DT treated TAs (**Fig.8g**). These data demonstrate that satellite cells are required for myonuclei addition during fiber hypertrophy, however fiber hypertrophy still occurs in the absence of satellite cells as previously reported (McCarthy, Mula et al. 2011). We note that the number of PICs markedly increased following myostatin inhibition, even in the absence of satellite cells, demonstrating that PICs recruitment by RAP-031 is not dependent upon satellite cells (**Fig.8h**). Taken together these results confirm that myostatin inhibition activates PICs, however they do not directly participate in fiber hypertrophy. Instead, our data suggest that satellite cells are the primary source of additional myonuclei in fiber hypertrophy.

Myostatin inhibition allows muscle regeneration in the absence of satellite cells

Our results show that PICs do not spontaneously fuse with existing myofibers *in vivo* during RAP-031-mediated fiber hypertrophy whereas our previous analyses demonstrated that PICs are capable of fusing with myoblasts and myotubes *in vitro* (Mitchell, Pannerec et al. 2010). Since PICs are activated rapidly in response to myostatin blockade, we wondered

whether we could mobilize PICs to participate in the myogenic program during muscle regeneration if they were activated by myostatin blockade prior to injury. We repeated the same experimental design used previously on the satellite cell-depleted mouse model, however we injured both TA muscles of each animal using cardiotoxin (**Fig.9a**). Two weeks after injury, histological analyses revealed that control muscles (non-DT injected) from mice pre-treated with RAP-031 do not display any overt differences as compared to CTL animals. Both groups have large newly formed fibers containing centrally located nuclei (**Fig.9b, left**). As reported by others (Lepper, Partridge et al. 2011; Sambasivan, Yao et al. 2011), muscles that were depleted for satellite cells displayed markedly impaired regeneration with high levels of infiltration and few regenerated fibers (**Fig.9b, right**). However, when mice were pre-treated with RAP-031 prior to injury, satellite cell-depleted TAs displayed a significantly improved regeneration with many newly formed fibers and reduced infiltration. Myonuclear content counts revealed that satellite cell-depleted muscles from CTL mice have ~15% of nuclei inside myofibers in the injured area versus 40% in mice pre-treated with RAP-031 (**Fig.9c**). We note that control muscles typically display ~50% of nuclei inside fibers in the injured area. We conclude that myostatin inhibition prior to injury almost completely rescues muscle regeneration in the absence of satellite cells. Immunostaining for PW1 and Pax7 on muscles cross-sections revealed that control muscles from both CTL and RAP-031 mice displayed many PICs and Pax7+ cells as well as PW1+ centrally located nuclei (**Fig.10**). As previously reported (Sambasivan, Yao et al. 2011), satellite cell-depleted TAs from CTL animals contained PICs but no Pax7+ cells. In contrast, satellite cell-depleted TAs pre-treated with RAP-031 contained both PICs and Pax7+ cells as well as many PW1+ centrally located myonuclei, resembling control muscles. This finding suggests that myostatin inhibition promotes muscle regeneration through the recruitment of non-satellite cell myogenic populations, or greatly increases the regenerative properties of a residual population of satellite cells that are normally unable to properly execute a regenerative response.

DISCUSSION

In this study, we show that PICs interact with satellite cells through paracrine signals. Micro-array analysis comparing PICs and satellite cells gene expression during early postnatal growth in limb muscles (Pannérec, Formicola et al., manuscript in preparation) revealed that satellite cells express myostatin and its receptor ActR2b whereas PICs express the myostatin antagonist follistatin. While satellite cell proliferation and differentiation is inhibited by myostatin in a dose-dependent manner, we show that PICs are insensitive to myostatin in proliferative conditions and can overcome the inhibitory effect of myostatin on satellite cells through follistatin secretion (**Fig.2-4**). We note that a blocking antibody to follistatin does not entirely overcome the inhibitory effects of myostatin, suggesting that PICs secrete additional myogenic promoting factors. Micro-array analysis revealed that PICs express high levels of IGF-1 (**see supplementary table 2**), a strong stimulator of myoblast proliferation, muscle growth and protein synthesis (Musaro, McCullagh et al. 2001; Fiorotto, Schwartz et al. 2003). IGF-1 acts through the Akt/mTOR pathway (Bodine, Stitt et al. 2001; Rommel, Bodine et al. 2001) whereas myostatin can override the Akt/mTOR pathway through Smad2/3 (McFarlane, Plummer et al. 2006). Interestingly, IGF-1 counteracts the inhibitory effects of myostatin, demonstrating that IGF-1/Akt signaling is dominant over the myostatin/Smad inhibition (Trendelenburg, Meyer et al. 2009). We have shown previously that adult PICs consist of 2 progenitor populations: the first being a multipotent cell with myogenic competence and the second being the PDGFRα expressing mesenchymal cells that constitute the resident interstitial adipogenic progenitors (Pannérec, Formicola et al., manuscript in preparation). As such, the promyogenic effect we report when PICs are co-cultured with satellite cells is entirely consistent with the promyogenic effects already demonstrated for the fibro/adipogenic progenitors (Joe, Yi et al. 2010).

We report here that the PICs number undergoes a large increase while the satellite cell number undergoes a more modest increase in response to myostatin blocking *in vivo*. Moreover we found that the percentage of proliferating PICs was doubled while the percentage of proliferating satellite cells was unchanged (**Fig.6**). In addition, the number of sub-laminal nuclei was markedly increased compared to CTL muscles suggesting that fiber

hypertrophy involves nuclear accretion. These data demonstrate that myostatin inhibition by RAP-031 leads to a recruitment of PICs as well as an increase in myonuclei content. In $Mst^{-/-}$ muscles, we also observe an increase in PICs and satellite cells numbers however we do not see an increase in proliferating cells, suggesting the increase in stem cell numbers occured during fetal or early postnatal life. Previous studies have demonstrated that increased muscle size in $Mst^{-/-}$ animals results from both fiber hypertrophy and hyperplasia (McPherron, Lawler et al. 1997), and the number of fibers is established by birth (Matsakas, Otto et al. 2010). We report here that $Mst^{-/-}$ muscles and WT muscles have the same myonuclear content. Taken together, these results demonstrate that constitutive loss-of-myostatin function and a pharmacological acute myostatin blockade lead to different cellular and molecular responses that nonetheless lead to an increase in muscle mass.

The generation of inducible satellite cell-depleted mouse models has allowed us to test the potential contribution of PICs to the myostatin-blockade-induced fiber hyperetrophy in the absence of satellite cells. We used the Pax7-driven DT model (Sambasivan, Yao et al. 2011) combined with RAP-031 treatment. DT injection has been shown previously to induce a slight loss of muscle mass (Sambasivan, Yao et al. 2011) thus while we do not see a significant increase in muscle weight following RAP-031 exposure to satellite cell depleted muscles, we do observe a clear increase in fiber size coupled with an increase in overall body weight confirming the hypertrophic effects of RAP-031 exposure (**Fig.8**). We note however that this increase in fiber size is not concomitant with an increase in myonuclei numbers. As reported previously (McCarthy, Mula et al. 2011), our results support the hypothesis that satellite cells constitute the only source of additional nuclei in models of muscle hypertrophy induced at the adult quiescent state, but that nuclear accretion is not an obligate step in fiber hypertrophy.

Several groups have recently reported that satellite cell ablation in adult muscle completely abrogates regeneration, leading to the conclusion that satellite cells are absolutely indispensable for muscle regeneration and that other muscle progenitors cannot actively participate in muscle repair in the absence of satellite cells (Lepper, Partridge et al. 2011; Murphy, Lawson et al. 2011; Sambasivan, Yao et al. 2011). The massive increase in PICs induced by myostatin inhibition coupled with our finding that PICs secrete pro-myogenic

signals led us to investigate whether RAP-031 treatment could promote muscle regeneration in the absence of satellite cells. Using the $Pax7^{DTR/+}$ mouse model, we show here that pre-treatment with RAP-031 rescues muscle regeneration in satellite cell-depleted muscles (**Fig.9**). Moreover, we find many Pax7+ cells in either interstitial or satellite cell positions, suggesting that muscle regeneration in this case involves the activation and/or commitment of myoblasts and replacement of the satellite cell compartment. Whether these Pax7+ cells arise from the small number of spared satellite cells that survive DT exposure (Sambasivan, Yao et al. 2011), or from atypical myogenic populations such as the PICs remains to be determined.

Satellite cells and mesoangioblasts can be recruited by trophic signals arising from endothelial and inflammatory cells (Arnold, Henry et al. 2007; Lolmede, Campana et al. 2009), and FAPs promote myoblast proliferation (Joe, Yi et al. 2010). It is therefore possible that myostatin inhibition favors the secretion of these promyogenic factors leading to the recruitment of multiple myogenic progenitors, whereas the level of secretion is not sufficient when myostatin remains active. We propose that PICs recruitment by RAP-031 provides a stem cell environment in which positive signals are sufficient to drive myogenic activation, either of the remaining satellite cells and/or non-satellite myogenic resident stem cells. While the PIC myogenic sub-fraction only accounts for <10% of total PICs in quiescent muscle (Pannérec, Formicola et al., manuscript in preparation), it might expand under myostatin inhibition and subsequently allow muscle regeneration by committing to the myogenic lineage. Taken together, we suggest that muscle repair rescue by myostatin inhibition is the result of the combined effects of satellite cell re-activation and atypical myogenic progenitors recruitment. This beneficial effect on the skeletal muscle stem cell niche might also account for the improvement of muscle physiology that is observed in diseased mouse models treated with myostatin inhibitors (Bogdanovich, Krag et al. 2002; Haidet, Rizo et al. 2008). More generally, these data suggest that myostatin inhibitors can improve muscle function even in late stage myopathies in which satellite cells are present in very low numbers and do not properly engage a regenerative response.

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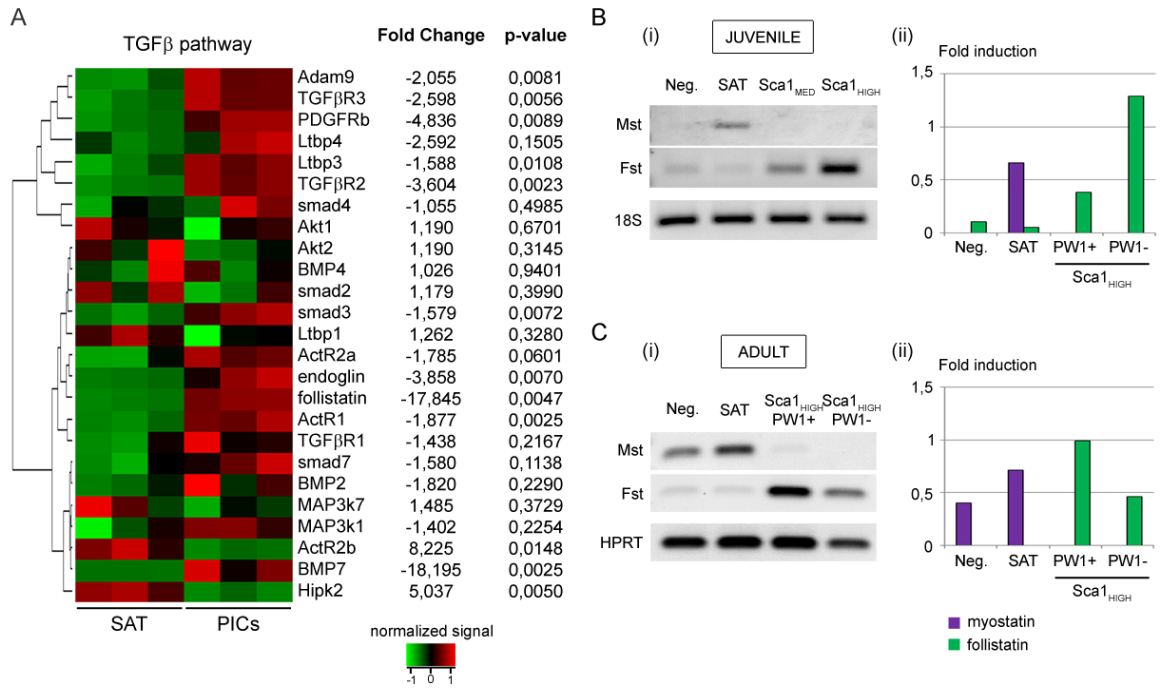


Figure 1. PICs and satellite cells show complementarities in TGFβ pathway genes (**a**) Gene Ontology clustering analysis of TGFβ pathway genes. Colouring was based on normalized signals as shown in the colour bar, gene tree is shown on the left, Fold Change and p-value are indicated for each gene. (**b**) Verification of myostatin (Mst) and follistatin (Fst) expression by semi-quantitative PCR on freshly isolated CD34-Sca1- cells (Neg.), CD34+Sca1- satellite cells (SAT), CD34+Sca1$_{MED}$ PICs (Sca1$_{MED}$) and CD34+Sca1$_{HIGH}$ cells (Sca1$_{HIGH}$) from 9-day old limb muscles. (i) Gel pictures. (ii) Quantification. (**c**) Verification of myostatin and follistatin expression by semi-quantitative PCR of freshly isolated CD34-Sca1- cells (Neg.), CD34+Sca1- satellite cells (SAT), CD34+Sca1$_{HIGH}$PW1+ PICs (Sca1$_{HIGH}$ PW1+) and CD34+Sca1$_{HIGH}$ PW1- cells (Sca1$_{HIGH}$ PW1-) from 7-week old limb muscles. (i) Gel pictures. (ii) Quantification.

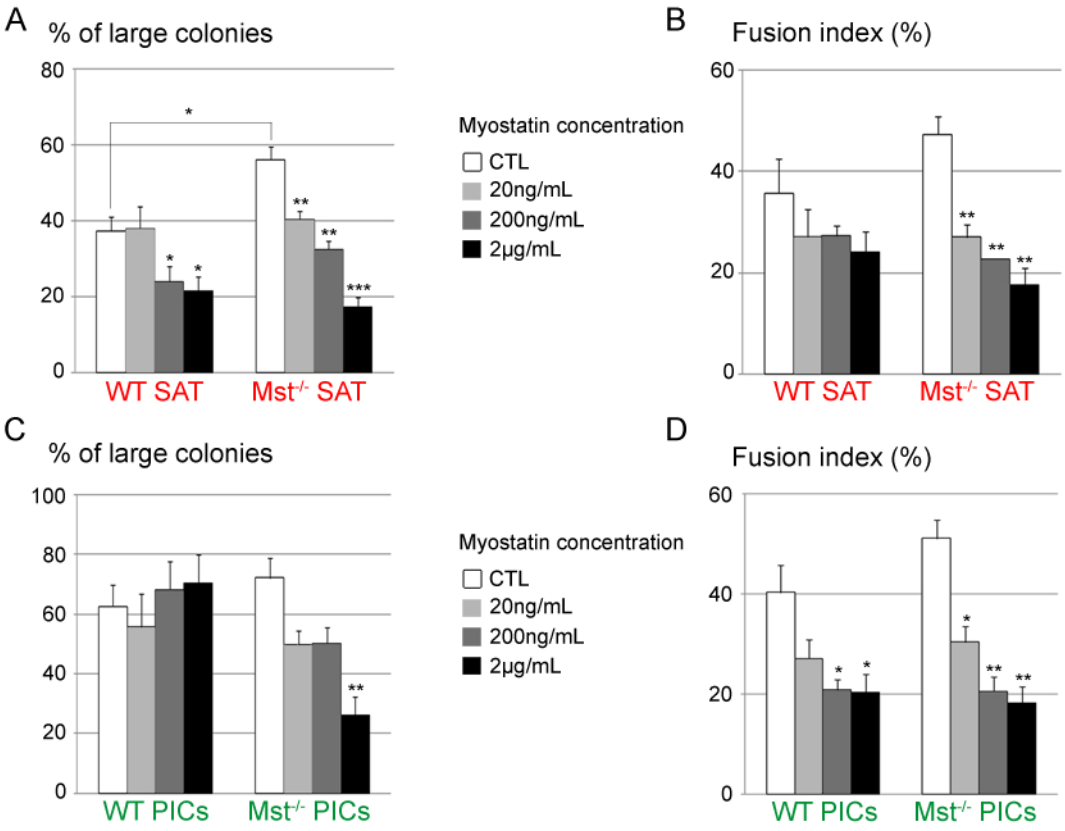


Figure 2. Myostatin effect on juvenile satellite cells and PICs *in vitro*. (**a,b**) Quantitative analysis of wild type (WT) or $Mst^{-/-}$ satellite cell proliferation (a) and differentiation (b) in the presence of 0, 20, 200 or 2 000ng.mL^{-1} recombinant myostatin. (**c,d**) Quantitative analysis of WT or $Mst^{-/-}$ PICs proliferation (c) and differentiation (d) in the same conditions. Proliferating values represent the mean percentage ± s.e.m. of colonies containing more than 30 cells after 3 days in GM. Fusion index represents the mean percentage ± s.e.m. of nuclei incorporated into MHC+ cells after 4 days in GM followed by 2 days in DM. For all graphs, statistical significance is indicated for values significantly different from their respective controls from at least 3 independent experiments. *p<0,05 **p<0,01 ***p<0,001.

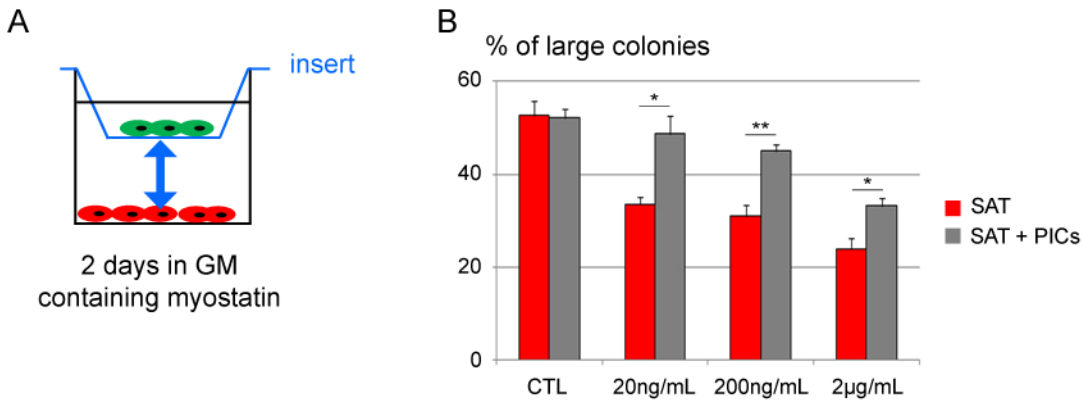


Figure 3. Juvenile PICs rescue satellite cell proliferation from myostatin inhibition. (**a**) Experimental design of insert well experiments, allowing exchanges of medium between the two compartments. In this experiment, PICs were allowed to grow in the top compartment while satellite cells were plated in the bottom compartment. (**b**) Quantitative analysis of satellite cell proliferation in GM containing 0, 20, 200 or 2 000ng.mL^{-1} recombinant myostatin. Satellite cells were either cultured alone (red bars), or in the presence of PICs (grey bars). Statistical significance represents the mean ±s.e.m of at least 3 different experiments. *p<0,05 **p<0,01.

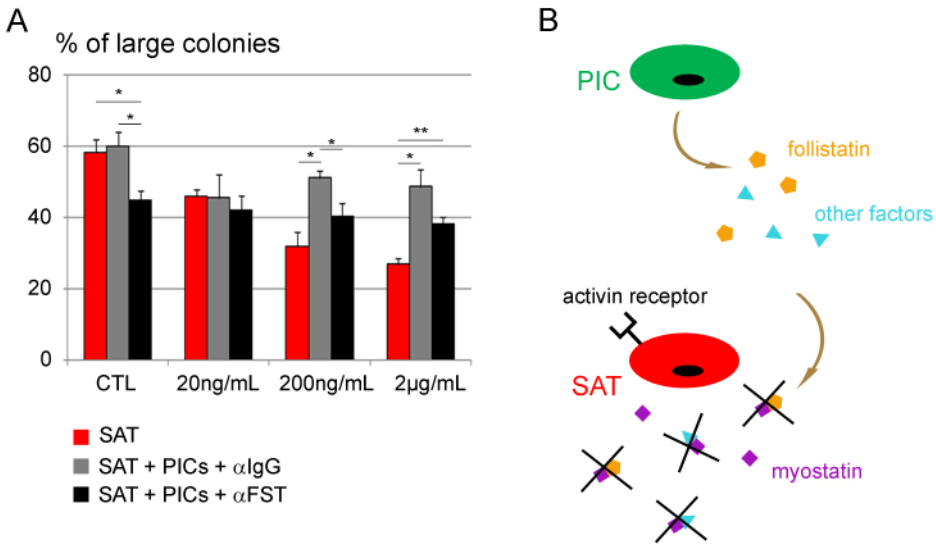


Figure 4. Adult PICs rescue satellite cell proliferation from myostatin inhibition through follistatin release. (**a**) Quantitative analysis of satellite cell proliferation in GM containing 0, 20, 200 or 2 000ng.mL^{-1} recombinant myostatin. Satellite cells were either cultured alone (red bars), or in the presence of PICs and a blocking antibody to follistatin (αFST, black bars) or its isotype control (αIgG, grey bars). Statistical significance represents the mean ±s.e.m of at least 3 different experiments. *p<0,05 **p<0,01. (**c**) Model for satellite cells and PICs interactions.

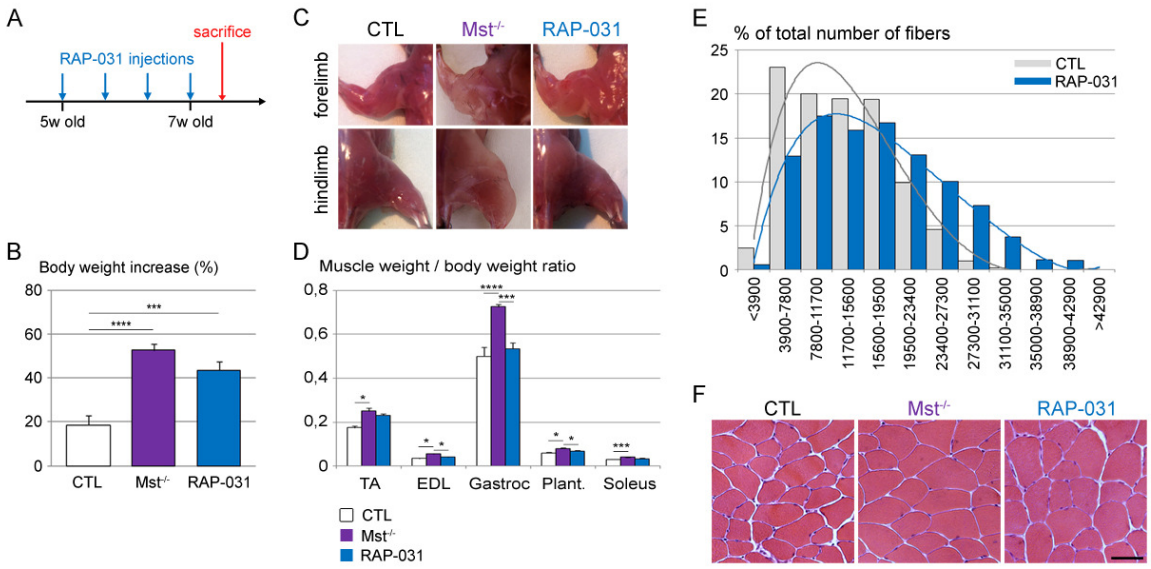


Figure 5. Myostatin inhibition in adult mice leads to a hyper-muscled phenotype. (**a**) Experimental design. Five weeks old C57Bl6 males were treated with intra-peritoneal injections of the myostatin inhibitor RAP-031 twice a week for 2 weeks. Animals were sacrificed 3 days after the last injection. (**b**) Graph showing percentage of body weight increase between 5-week and 7-week old C57Bl6 control (CTL), *myostatin-null* ($Mst^{-/-}$) or RAP-031-treated C57Bl6 (RAP-031) mice. (**c**) Images of forelimb (top panel) and hindlimb (bottom panel) from skinned CTL, $Mst^{-/-}$ or RAP-031 mice. (**d**) Hindlimb individual muscles weight comparison between CTL, $Mst^{-/-}$ or RAP-031 mice. TA=*tibialis anterior*; EDL=*extensor digitorum longus*; Gastroc=*gastrocnemius*; Plant.=*plantaris*. (**e**) Fiber size distribution in CTL (grey) and RAP-031 (blue) mice TA. (**f**) TA cross-sections from CTL, $Mst^{-/-}$ and RAP-031 mice stained with hematoxylin and eosin. Scale bar, 50µm. For all graphs, at least 6 animals of each genotype were used to calculate statistical significance. *p<0,05 **p<0,01 ***p<0,001 ****p<0,0001.

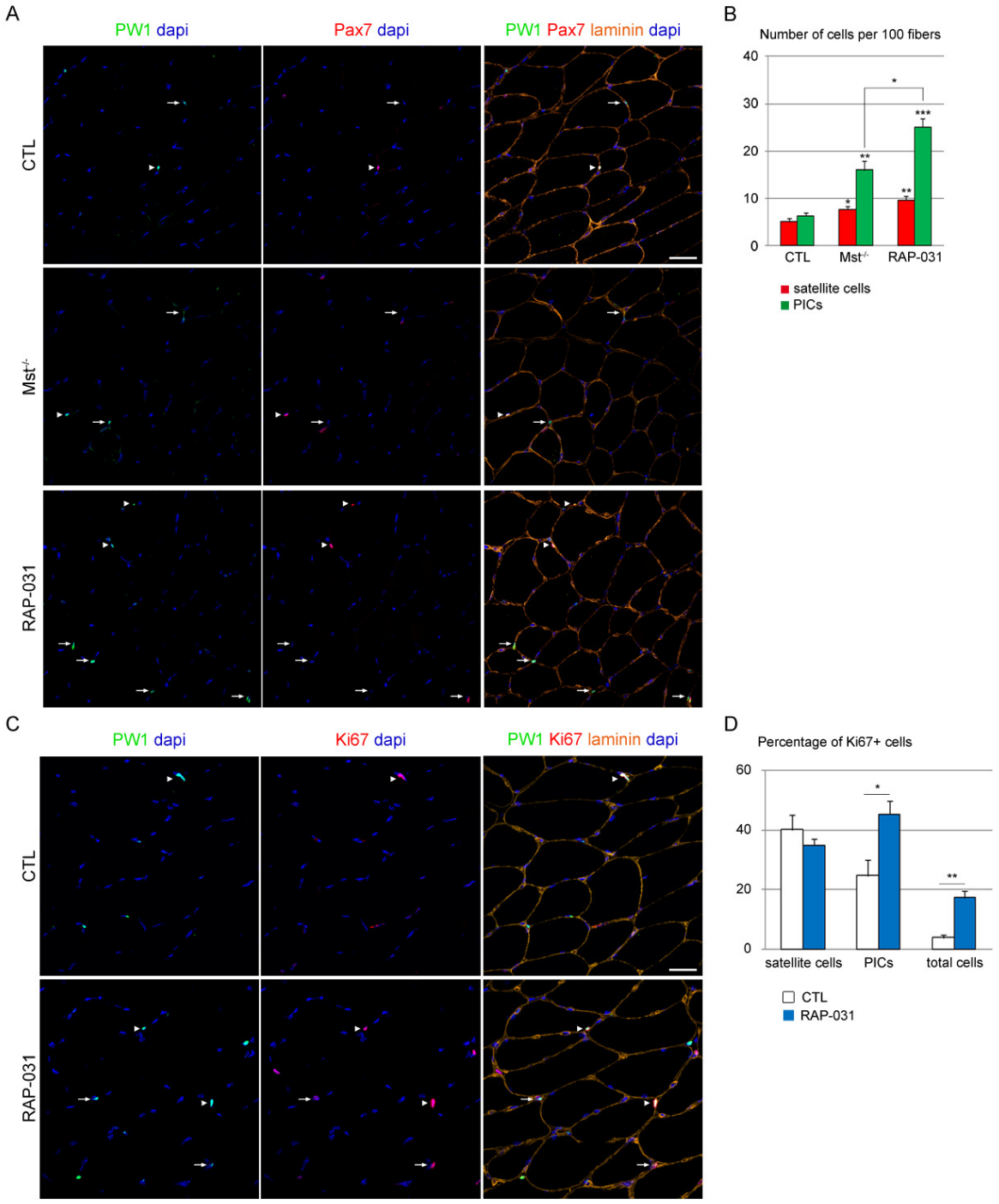


Figure 6. Myostatin inhibition leads to stem cell recruitment. (**a**) Representative cross-sections of CTL, Mst$^{-/-}$ and RAP-031 treated TA immunostained for PW1 (green), Pax7 (red), laminin (orange) and dapi (blue). Satellite cells (PW1+/Pax7+) are shown with arrowheads and PICs (PW1+/Pax7-) are shown with arrows. (**b**) Graph showing the number of PICs and satellite cells per 100 fibers counted on same TAs as in (a). (**c**) Representative cross-sections of CTL and RAP-031 treated TA immunostained for PW1 (green), Ki67 (red), laminin (orange) and dapi (blue). Proliferating satellite cells are shown with arrowheads and proliferating PICs are shown with arrows. (**d**) Percentage of proliferating (Ki67+) satellite cells, PICs and total cells counted on same

TAs as in (c). For all graphs, statistical significance was calculated from at least 3 different animals per condition. *p<0,05 **p<0,01 ***p<0,001 compared to respective WT controls. Scale bars, 30µm.

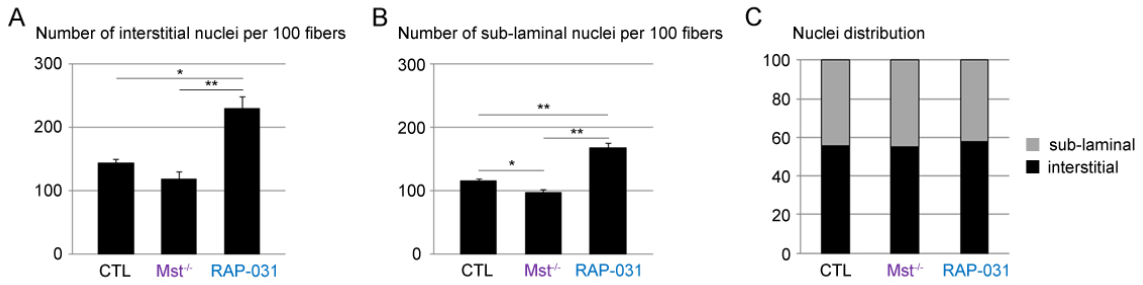


Figure 7. Myostatin inhibition leads to an increase in nuclei number. (**a**) Number of interstitial nuclei per 100 fibers in TA from CTL, Mst$^{-/-}$ and RAP-031 treated mice. (**b**) Number of sub-laminal nuclei per 100 fibers in TA from CTL, Mst$^{-/-}$ and RAP-031 treated mice. (**c**) Nuclei distribution in same muscles. Statistical significance was calculated from at least 3 different animals per condition. *p<0,05 **p<0,01 ***p<0,001.

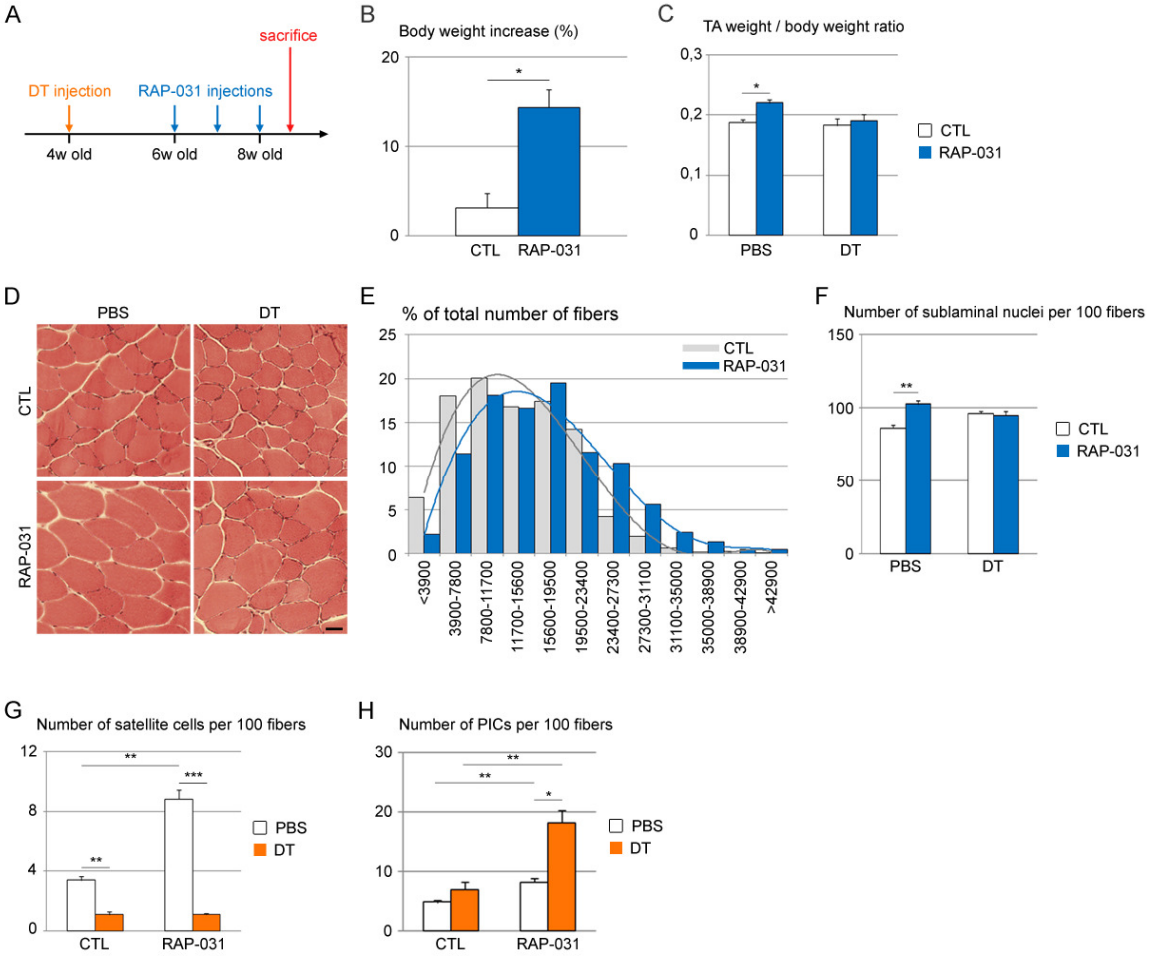


Figure 8. PICs do not directly participate in fiber hypertrophy following myostatin inhibition. (**a**) Experimental design. Diphteria toxin (DT) was injected in the right TA of Pax7$^{DTR/+}$ males while the contralateral muscle was injected with PBS, 2 weeks before RAP-031 treatment. (**b**) Graph showing percentage of body weight increase between 6-week and 8-week old CTL and RAP-031 treated Pax7$^{DTR/+}$ mice. (**c**) Graph showing the weight of TAs from CTL and RAP-031 treated Pax7$^{DTR/+}$ mice. (**d**) TA cross-sections from CTL or RAP-031 treated mice were stained with hematoxylin and eosin. Scale bar, 60µm. (**e**) Fiber size distribution in CTL (grey) and RAP-031 (blue) TAs injected with DT. (**f**) Number of sublaminal nuclei per 100 fibers in muscles described in (c). (**g**) Number of satellite cells per 100 fibers in muscles described in (c). Satellite cells were determined by M-Cadherin and laminin staining. (**h**) Number of PICs per 100 fibers in muscles described in (c). PICs were determined by PW1 and laminin staining. For all graphs, statistical significance was calculated from at least 3 animals of each condition. *p<0,05 **p<0,01 ***p<0,001.

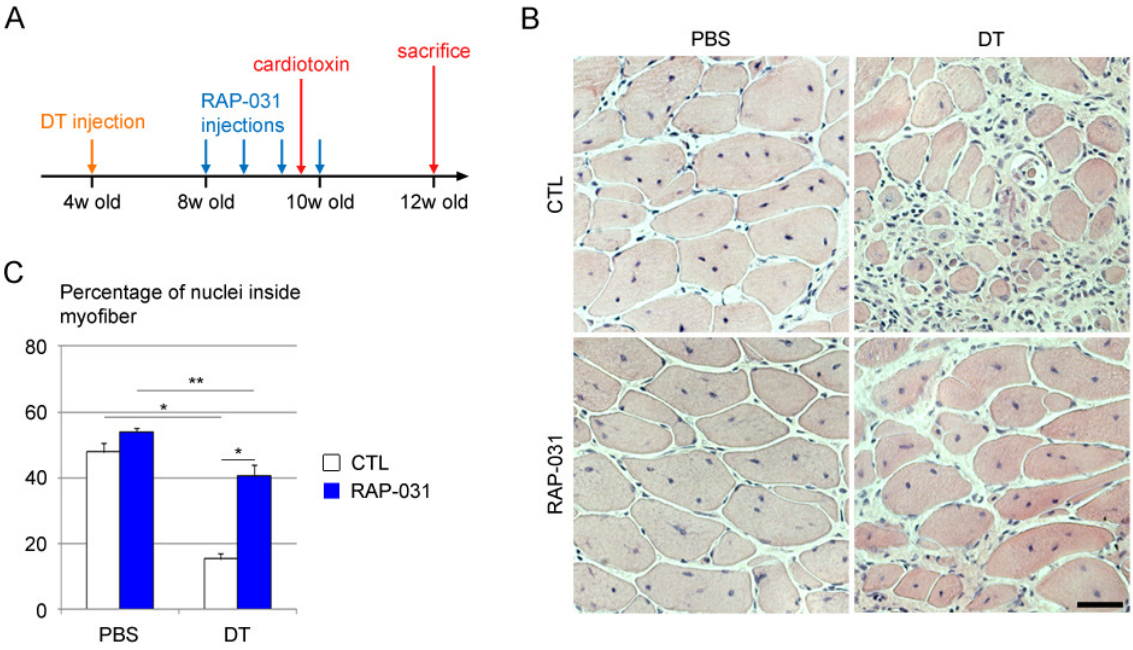


Figure 9. Myostatin inhibition favors muscle regeneration. (**a**) Experimental design. Animals were treated with RAP-031 4 weeks after the DT injection, and TAs were damaged using cardiotoxin. Animals were sacrificed 2 weeks after injury. (**b**) Hematoxilin and eosin staining of TA cross-sections from CTL or RAP-031 treated mice. For each animal one TA was injected with DT, and the other one with PBS. Scale bar, 50µm. (**c**) Percentage of centrolocated nuclei in the damaged area of the same muscles as in (b). Statistical significance was calculated from at least 3 different animals. *p<0,05 **p<0,01.

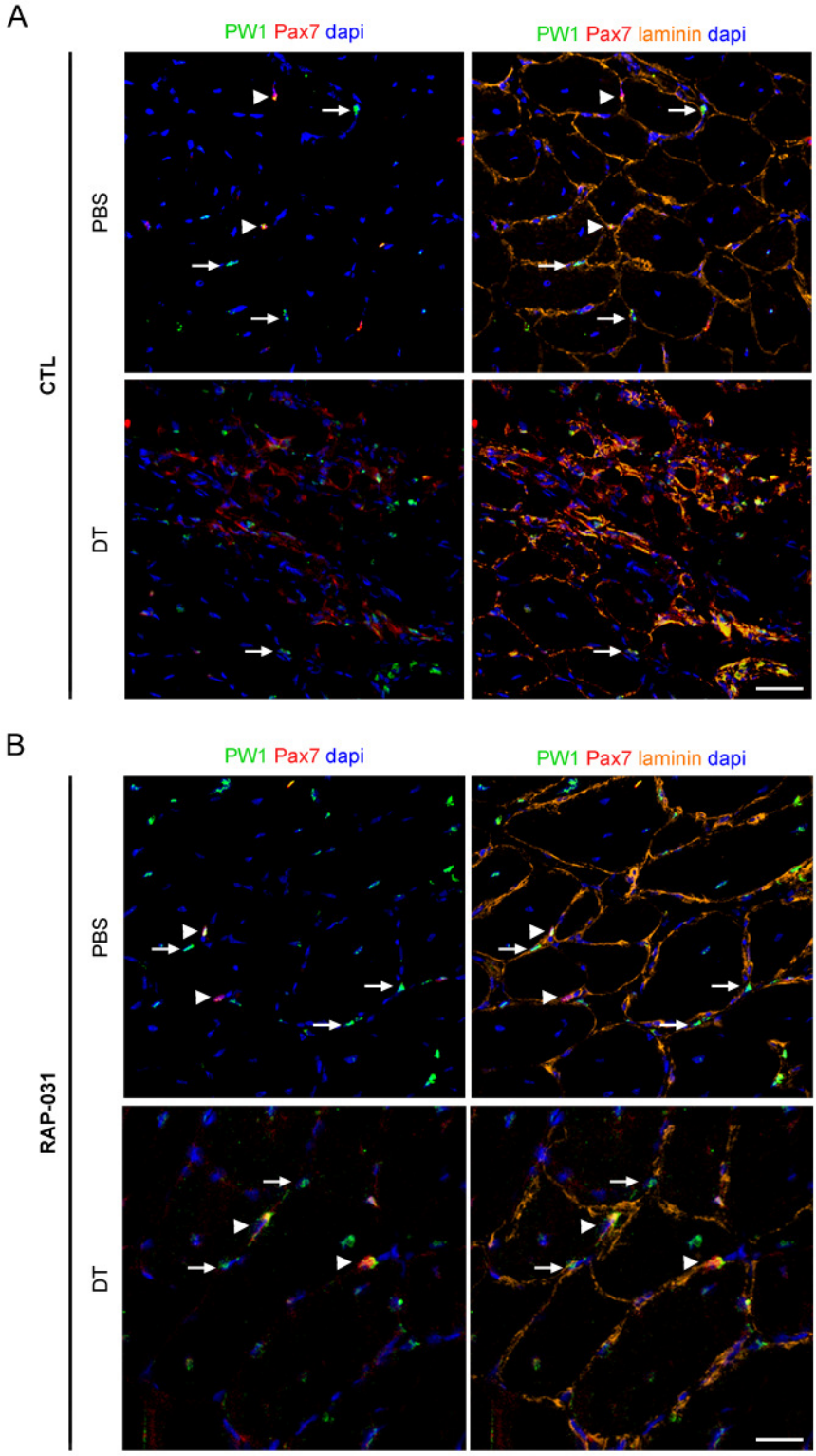
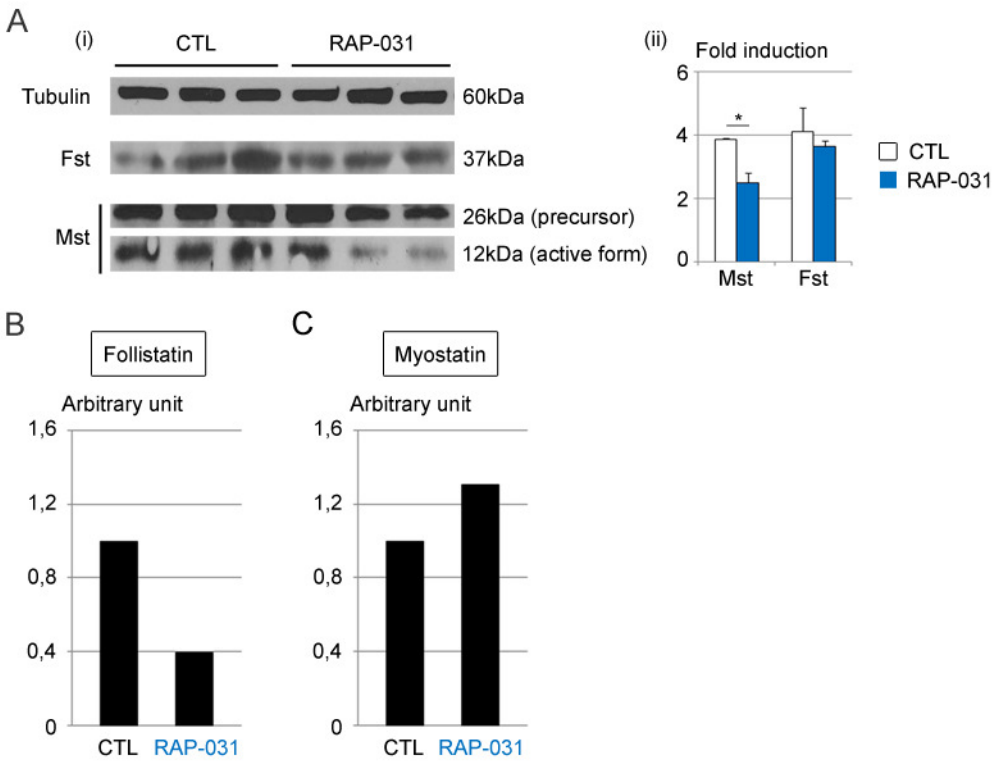
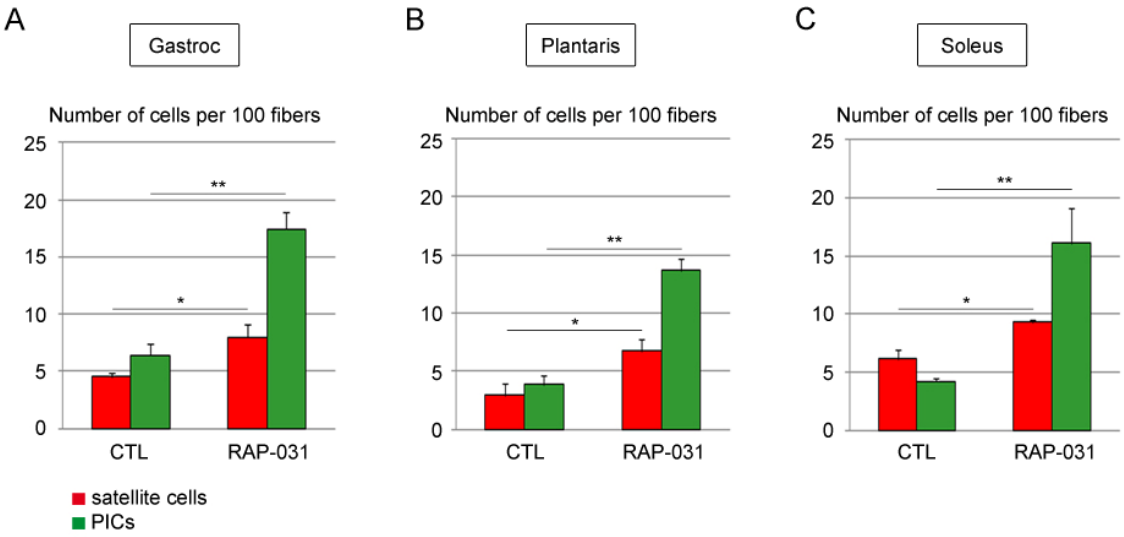


Figure 10. Myostatin inhibition induces the recruitment of Pax7+ progenitors. Representative cross-sections of TA muscles from CTL (**a**) or RAP-031 treated animals (**b**) injected with PBS or DT, immunostained for PW1 (green), Pax7 (red), laminin (orange) and dapi (blue). PICs are designated by arrows and Pax7+ cells are designated by arrowheads. Scale bars, 25µm.



Supplementary Figure 1. Follistatin and myostatin profiles in muscles treated with RAP-031. (**a**) Western blot analyses for tubulin, follistatin (Fst) and myostatin (Mst) on control (CTL) or RAP-031 treated TA. (i) Images, (ii) Quantification. (**b,c**) Real time quantitative PCR for follistatin (b) and myostatin (c) on CTL or RAP-031 treated TA. For each gene, at least 3 different TAs were used to calculate the average arbitrary unit and expression intensity was normalized to that of CTL.



Supplementary Figure 2. Number of PICs and satellite cells in hindlimb muscles. Number of PICs and satellite cells per 100 fibers in gastrocnemius (**a**), plantaris (**b**), and soleus (**c**) muscles from CTL and RAP-031 treated mice. PICs were determined with PW1 and laminin, and satellite cells were determined with M-Cadherin. Statistical significance was calculated from at least 3 different animals of each genotype. *p<0,05 **p<0,01.

Gene	Forward primer	Reverse primer
follistatin	CCCCAACTGCATCCCTTGTAAA	TCCAGGTGATGTTGGAACAGTC
myostatin	GGCTCAAACAGCCTGAATCCAA	CCAGTCCCATCCAAAGGCTTCAAA
18S	CGGCTACCACATCCAAGGAA	TATACGCTATTGGAGCTGGAA
HPRT	CTGGTGAAAAGGACCTCTCG	TGAAGTACTCATTATAGTCAAGGGCA

Supplementary Table 1. Primers used for semi quantitative and quantitative PCR.

Gene symbol	Gene name	Average intensity		Fold Change	p-value
		PICs	SAT		
Fst	follistatin	381	11	-32,8	0,0073
Pdgfra	platelet derived growth factor receptor, alpha polypeptide	897	124	-28,3	0,0016
Bmp7	bone morphogenetic protein 7	123	6	-18,2	0,0025
Fgf1	fibroblast growth factor 1	462	31	-16,6	0,0060
Igf1	insulin-like growth factor 1	3034	347	-8,8	0,0007
Tgfbr2	transforming growth factor, beta receptor II	9019	2521	-3,6	0,0023
Acvr2a	activin receptor IIA	1035	593	-1,8	0,0601
Bmp2	bone morphogenic protein 2	348	185	-1,8	0,2290
Smad3	MAD homolog 3	1580	983	-1,6	0,0072
Smad4	MAD homolog 4	3878	4012	1,0	0,8623
Bmp4	bone morphogenic protein 4	1386	1439	1,0	0,9401
Smad2	MAD homolog 2	967	1139	1,2	0,3990
Akt1	thymoma viral proto-oncogene 1	4719	5529	1,2	0,6701
Akt2	thymoma viral proto-oncogene 2	1305	1562	1,2	0,3145
Acvr2b	activin receptor IIB	21	159	8,2	0,0148
Myod1	myogenic differentiation 1	47	2275	57,1	0,0036
Fgfr4	fibroblast growth factor receptor 4	13	1032	86,5	0,0048

Supplementary Table 2. Genes highly expressed by PICs and satellite cells. For each gene, the average expression intensity was calculated from Affymetrix analysis. Fold change and p-value are indicated.

CHAPTER 5. DISCUSSION

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1. **PICs: multipotent progenitors or mixed population?**
    - Adult PICs versus juvenile PICs
    - Adult PICs versus other stem cells of the skeletal muscle niche

2. **PICs as a therapeutic target**
    - PICs are a source of positive signals for muscle growth
    - PICs constitute an easily recruitable population

1. PICs: multipotent progenitors or mixed population?

1.1. Adult PICs versus juvenile PICs

Recent studies have led to the identification of non-myogenic cell populations resident in skeletal muscle that participate in the regulation of muscle homeostasis and regeneration (Arnold, Henry et al. 2007; Joe, Yi et al. 2010; Uezumi, Fukada et al. 2010; Murphy, Lawson et al. 2011). The discovery of PICs by our group some years ago (Mitchell, Pannerec et al. 2010) adds to a growing list of non-satellite cell populations with myogenic capacities resident in skeletal muscle (Gussoni, Soneoka et al. 1999; Sampaolesi, Torrente et al. 2003; Benchaouir, Meregalli et al. 2007; Dellavalle, Sampaolesi et al. 2007; Liadaki, Casar et al. 2012). The relationship and potential overlaps between these populations and the mechanisms regulating their interactions are a central focus of the studies presented here.

In the first part of the work, we established a FACS sorting strategy to purify PICs and satellite cells from both young and, most importantly, adult mice using the $PW1^{nLacZ/+}$ reporter mouse. This strategy allowed us to examine the $Sca1_{HIGH}$/PW1+ fraction of PICs, present in both juvenile and adult muscles, which could not be studied previously due to the inability to sufficiently purify the PW1+ cells. Briefly, we found that the population of $Sca1_{HIGH}$/PW1+ cells increased in adult muscles while the population of $Sca1_{MED}$ cells that we initially reported (Mitchell, Pannerec et al. 2010) declined rapidly after birth until it was no longer detectable after 3 weeks of postnatal development. While all fractions were multipotent and capable of differentiating into 3 distinct mesenchymal lineages *in vitro* (smooth muscle, skeletal muscle and fat), we noticed that the $Sca1_{MED}$ myogenic potential was superior to that of $Sca1_{HIGH}$/PW1+ cells. A recent study reported that adult satellite cells do not require Pax7 expression after 3 weeks of age whereas before this stage, Pax7 is required for both regeneration and normal postnatal growth (Lepper, Conway et al. 2009). Our initial examination of PICs showed that the constitutive Pax7 mutant mouse display a massive increase in PICs prior to any overt sign of muscle phenotypes (Mitchell, Pannerec et al. 2010) giving the first indications that satellite cells and PICs are under a shared regulation and thus constitute a larger stem cell niche. Furthermore, we find that the PIC

population undergoes a clear inflection at 3 weeks of postnatal life such that the juvenile $Sca1_{MED}$ population disappears. Taken together, these data suggest that there is a specific postnatal role for the $Sca1_{MED}$ population and that this population requires Pax7 function for postnatal myogenesis. We note that upon conversion to the myogenic lineage, PICs express Pax3 and Pax7, thus if this process were occurring, it would not have been detected using lineage analyses.

While our studies clearly address the lineage potentials of PICs during postnatal growth and adult stages in muscle, the results gained thus far support a model in which PICs play a role that is Pax7 dependent to drive early postnatal muscle growth. This stage involves marked satellite cell fusion leading to myofiber nuclear accretion and occurs between birth and 3 weeks old (Mantilla, Sill et al. 2008; White, Bierinx et al. 2010). As the number of PICs decreases between birth and 7 days old (Mitchell, Pannerec et al. 2010), we postulate that $Sca1_{MED}$ PICs constitute a transient highly myogenic population that either fuse with fibers or up-regulate Sca1 between 2 and 3 weeks of age such that only the $Sca1_{HIGH}$ population remains in the adult.

1.2. Adult PICs versus other stem cells of the skeletal muscle niche

We have previously reported that juvenile PICs can participate in fiber formation as well as give rise to satellite cells upon engraftment into damaged muscle (Mitchell, Pannerec et al. 2010). Here, we demonstrate that both young and adult PICs can differentiate into 3 different mesenchymal lineages while satellite cells can only adopt a skeletal muscle phenotype, suggesting that PICs constitute a more plastic and upstream population compared to satellite cells. Our micro-array analyses confirmed that these 2 populations are very different such that satellite cells express genes consistent with commitment to the skeletal muscle lineage while PICs display a more stem-like profile with the expression of a wide range of genes related to mesenchymal lineages. In particular we find that PICs express many genes implicated in vascular development such as VEGF or angiogenin, and most interestingly the pericyte marker NG2. Pericytes are vessel-

associated cells characterized by the expression of NG2 and alkaline phosphatase (AP) that possess high myogenic potential, although distinct from satellite cells, and are considered the adult equivalent of mesoangioblasts (Dellavalle, Sampaolesi et al. 2007). A recent study using an inducible Cre-AP mouse model demonstrated that pericytes directly contribute to fiber growth and can enter the satellite cell compartment during unperturbed postnatal development (Dellavalle, Maroli et al. 2011). Moreover pericytes are often found adjacent to a satellite cell, which is also the case for PICs (Christov, Chretien et al. 2007; Mitchell, Pannerec et al. 2010; Dellavalle, Maroli et al. 2011). These similarities between PICs and pericytes raised the possibility of a common vessel-associated embryonic progenitor. At the time we obtained our micro-array results, we established a collaboration with Dr. Cossu to test this hypothesis by investigating PW1 expression in the Cre-AP mouse model. Unfortunately, we could not obtain reliable reporter gene expression in this model hampering further progress. Nonetheless, PW1 is expressed by mesoangioblasts and PW1 expression appears to be required for mesoangioblast myogenicity and capability to cross the vessel walls (Messina et al, manuscript in preparation).

The observation that adult PICs possess a high adipogenic potential raised the question of a possible overlap with 2 recently described populations of adipogenic progenitors that are located in the interstitial space of the muscle, FAPs (Joe, Yi et al. 2010) and mesenchymal PDGFRα+ progenitors (Uezumi, Fukada et al. 2010). These two complementary studies suggest that interactions between myogenic and adipogenic cells regulate muscle homeostasis. Here we demonstrate that PDGFRα+ mesenchymal progenitors constitute a sub-population of PICs and represent the only fraction capable to differentiate into adipocytes. These results suggest that FAPs, representing the globality of Sca1 expressing cells, are a mixed population that can be divided into the adipogenic PDGFRα+ cells, i.e mesenchymal progenitors, and the other cells. Therefore, PDGFRα+ mesenchymal progenitors not only constitute a sub-population of PICs but also a sub-population of FAPs. Whether PDGFRα- FAPs and PDGFRα- PICs are overlapped populations remains to be determined. Joe and colleagues did not observe any myogenic potential in FAPs (Joe, Yi et al. 2010), and we accordingly observed very low myogenicity in

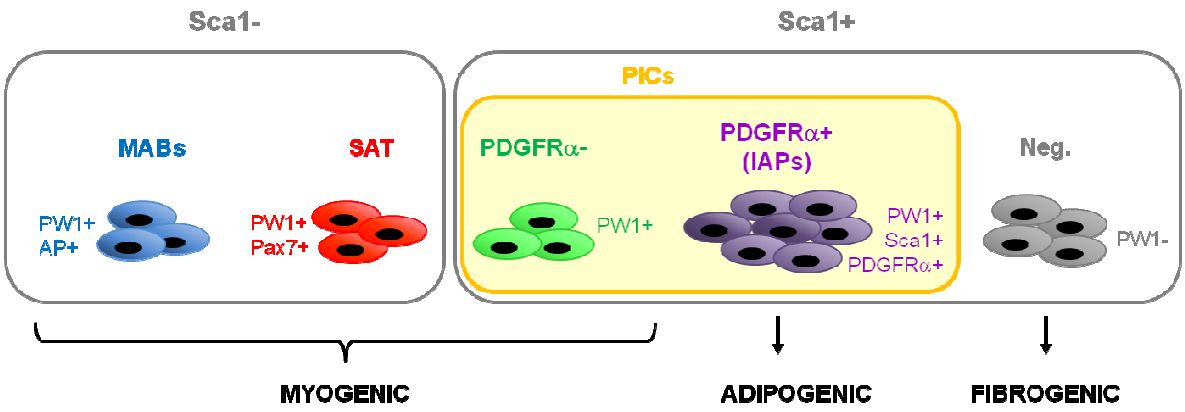

Figure 19. Redefinition of the skeletal muscle stem cell hierarchy. Model presenting stem cell hierarchy upon PW1 expression, in which each progenitor population expresses PW1 and a specific marker. Myogenic potential resides in satellite cells, mesoangioblasts (MABs) and a sub-population of PICs, while adipogenic potential arises from the IAP fraction of PICs. Cells which do not express PW1 give rise to fibroblasts. *IAPs=interstitial adipogenic progenitors; Neg.=PW1-/PDGFRα- cells.*

the $Sca1_{HIGH}$/PDGFRα- cells (~15%). However the myogenic potential of the $Sca1_{HIGH}$/PDGFRα- cells increased to about 50% upon enrichment for PW1 expression, suggesting that the $Sca1_{HIGH}$ fraction does retain some myogenic potential but in a too small sub-population to be detected when sorted in its globality. Taken together, our results reveal that PICs can be sub-divided into a PDGFRα expressing fraction that retain adipogenic capacity and a smaller myogenic fraction that do not express PDGFRα. These analyses were performed on 7 weeks old mice, corresponding to a stage where regeneration potential is high. While it is tempting to speculate that the PW1+ cells all arise from a common precursor, we are only developing now the appropriate tools to determine this *in vivo*. We can state that engraftment of PICs into injured muscle gives rise to myofibers, satellite cells and PICs (Mitchell, Pannerec et al. 2010), however there is some debate as to whether engraftment can lead to a more 'permissive' outcome as compared to what really occurs *in situ*.

Taken together, we propose a stem cell identification scheme in which all cells possessing stem or progenitor properties can be identified by PW1 expression and further sub-classified by the expression of one or more specific markers (i.e. Pax7 for the satellite cells and PDGFRα for the adipocytes) (**Fig.19**). Since myogenic and non-myogenic stem cells interact with each other to regulate muscle homeostasis and regeneration, we propose that the "satellite cell niche" is really part of a larger "skeletal muscle stem cells niche" in which the various stem cells reside and for which there is compelling evidence that they crosstalk to maintain tissue integrity and mount regenerative responses (Pannérec, Marazzi et al., in press – **Appendix 2**). This scheme is based upon the assumption that all adult stem cells do express PW1, which may not be the case. PW1+PDGFRα+ cells are adipocytes whereas the PW1+Pax7+ cells are satellite cells. However, the PW1+PDGFRα- fraction of the PICs are myogenic but do not express Pax7 nor any other skeletal muscle lineage gene until they overtly adopt a myogenic phenotype. One potential PIC myogenic marker is β4-integrin, which has recently been reported to mark a population of interstitial myogenic progenitors (Liadaki, Casar et al. 2012). β4-integrin+ cells do not express any myogenic marker upon isolation but readily differentiate

into myotubes both *in vitro* and following engraftment into damaged muscle (Liadaki, Casar et al. 2012). However while the vast majority of β4-integrin+ cells do not express CD45, they display some heterogeneity in the expression of CD34 and Sca1 which will probably complicate subsequent comparison with PICs. Second, the fate of the diverse PW1+ muscle-resident populations must be investigated *in vivo*. Presumably PDGFRα- PICs would directly participate in muscle regeneration in a model of muscle injury while PDGFRα+ PICs would give rise to adipocytes in models of fatty infiltration, but interactions between these cell types most probably exist that contribute to the regulation of muscle homeostasis. Finally, PICs identity must be reconsidered. PICs were initially defined by their anatomical location outside the muscle basal lamina and by the expression of PW1 (Mitchell, Pannerec et al. 2010). While no specific marker for PICs was established that distinguished them from satellite cells, we provide evidence here that the lineage potentials of adult PICs can be determined based on the expression of PDGFRα. While this property has not been investigated in juvenile muscle, "PW1+ Interstitial Cells" designates two distinct adult stem cell populations.

2. PICs as a therapeutic target

2.1. PICs are a source of positive signals for muscle growth

The second part of our studies stemmed from the observation that follistatin was one of the "top" genes to be highly expressed in PICs but not in satellite cells from our micro-array analyses. We therefore focused on the TGFβ family and more particularly myostatin, which is the major target and binding partner of follistatin and for which there is a compelling literature that myostatin and follistatin play major roles in muscle biology.

Myostatin has been demonstrated to inhibit myoblast proliferation and differentiation *in vitro* (Thomas, Langley et al. 2000; Taylor, Bhasin et al. 2001; Langley, Thomas et al. 2002). Here we demonstrate that PIC differentiation into skeletal muscle is inhibited by myostatin, but that PICs can rescue satellite cells from myostatin-inhibition in proliferative conditions through follistatin release. A recent report corroborates our results

by demonstrating the capacity of follistatin to partially rescue C2C12 proliferation from myostatin inhibition (Zhu, Li et al. 2011). Our results suggest that PICs constitute an internal positive regulator of muscle mass by producing positive signals that counteract the action of negative factors present in the environment and thus favor muscle growth. We, in this work, and others have demonstrated that satellite cells are endogenously express myostatin (McCroskery, Thomas et al. 2003). We note that it has also been reported that myostatin exerts its effect on myoblasts through an autocrine rather than endocrine manner, leading to the proposal that some mechanism differentially regulates the bioavailability of endogenous and exogenous myostatin to muscle cells (Rios, Fernandez-Nocelos et al. 2004). Our data leads us to propose that follistatin is secreted by PICs and then binds preferentially to exogenous myostatin such that satellite cells auto-regulate their proliferation through an autocrine manner. This hypothesis lends itself to further investigation by determining which follistatin isoform is produced by PICs, as the short Fst288 isoform tends to concentrate near the plasma membrane while the long Fst315 isoform is widespread in tissues and circulation (Xia and Schneyer 2009).

A recent study demonstrated that muscle hypertrophy induced by follistatin is mostly dependent upon the activation of the IGF-1/Akt/mTOR pathway (Kalista, Schakman et al. 2012). IGF-1 is a potent stimulator of myogenesis (Coleman, DeMayo et al. 1995; Musaro, McCullagh et al. 2001) which up-regulation is mandatory for myoblast differentiation *in vitro* (Yoshiko, Hirao et al. 2002). Coupled with the observation that $Mst^{-/-}$ mice show increased levels of circulating IGF-1 (Williams, Interlichia et al. 2011), these data suggest that IGF-1 contributes to muscle hypertrophy induced by follistatin. Our micro-array analysis revealed that PICs highly express IGF-1 in addition to follistatin, suggesting they can directly promote cell proliferation and differentiation. This hypothesis will be further investigated by performing additional co-culture experiments using IGF-1 blocking antibodies.

Taken together our results suggest that PICs positively mediate muscle growth by producing factors that either protect surrounding cells from negative regulators such as

myostatin, or directly induce cell proliferation by activating the potent IGF-1/Akt/mTOR pathway.

2.2. PICs constitute an easily recruitable population

The observation that PICs participate in the follistatin/myostatin axis led us to investigate the potential role for PICs in myostatin blockade-induced muscle hypertrophy using the inhibitor RAP-031 (Cadena, Tomkinson et al. 2010). Two weeks of RAP-031 treatment led to an increase in muscle mass and myofiber size accompanied by a marked increase in PICs number suggesting that PICs are recruited rapidly following myostatin inhibition. Treatment of satellite cell-depleted muscles with RAP-031 led to fiber hypertrophy in the absence of myonuclei accretion, as reported by others (McCarthy, Mula et al. 2011). These data demonstrate that neither PICs nor other non-satellite myogenic population participate in fiber hypertrophy following myostatin inhibition, but that nuclear accretion is not an obligate step in fiber hypertrophy. This finding reconciles previous studies in which the role of satellite cells in fiber hypertrophy appeared in conflict (McCroskery, Thomas et al. 2003; Amthor, Otto et al. 2009; Blaauw, Canato et al. 2009; Zhou, Wang et al. 2010).

Since myostatin is expressed in resting muscle and represses satellite cells, we propose that muscle homeostasis requires a basal level of myostatin that maintains satellite cells in quiescence. When myostatin is inhibited, satellite cells re-enter the cell cycle and progress through the myogenic program until fusion with nearby fibers. Our results suggest that PICs participate in satellite cell activation, not only by secreting follistatin but also by secreting IGF-1 which promote myoblast proliferation and differentiation (Coleman, DeMayo et al. 1995; Yoshiko, Hirao et al. 2002). However we could not establish the mechanisms leading to PICs recruitment and proliferation in the absence of myostatin. Micro-array analyses and *in vitro* experiments suggest that PICs are not sensitive to myostatin since they poorly express the cognate receptors, ActR2b and ActR2a in basal conditions. PICs recruitment is probably an indirect effect of myostatin

inhibition, activating other regulatory factors and driving the re-organization of the skeletal muscle niche. Another hypothesis is that PW1 may be a myostatin target gene. Previous data from our group and others demonstrated that PW1 acts as a growth arrest gene in the p53 pathway (Deng and Wu 2000; Relaix, Wei et al. 2000; Coletti, Yang et al. 2002; Schwarzkopf, Coletti et al. 2006) and ongoing work in our lab reveals that transient down-regulation of PW1 expression can trigger a variety of adult stem cells to re-enter the cell cycle (Besson et al, manuscript in preparation, Bonafanti et al, personal communication).

Lastly, we demonstrated that myostatin inhibition rescue muscle regeneration in the absence of satellite cells. Satellite cell-depleted muscles pre-treated with RAP-031 display many regenerating fibers, reduced fibrosis, and the abundant presence of interstitial and sub-laminal Pax7+ cells. This finding suggests that either atypical myogenic progenitors are recruited to the myogenic lineage or that small proportions of satellite cells remaining after DT treatment are more efficient when myostatin is blocked. We are presently testing whether the regeneration is due to satellite cells or a non-satellite cell lineage using additional mouse models developed in collaboration with Dr. Tajbakhsh. In any case, RAP-031 treatment can overcome the inability to regenerate when there are no or very reduced numbers of satellite cells.

Based on the recent proposal that muscle repair is dependent upon FAPs interactions with myogenic cells (Natarajan, Lemos et al. 2010) and our finding that FAPs and PICs are overlapping populations, we propose a model in which interstitial adipogenic progenitors (IAPs) allow to create a beneficial environment for muscle regeneration under myostatin blockade (**Fig.20**). When satellite cells are ablated, the level of trophic signals secreted by IAPs is not sufficient and/or not able to recruit myogenic progenitors. Reciprocal signals limiting IAPs expansion are missing, leading to severe fat deposition (Sambasivan, Yao et al. 2011). In contrast, pre-activation of the muscle environment by a myostatin inhibitor activates IAPs and promotes signal secretion, shifting the balance from a adipogenic/fibrogenic response to a myogenic response.

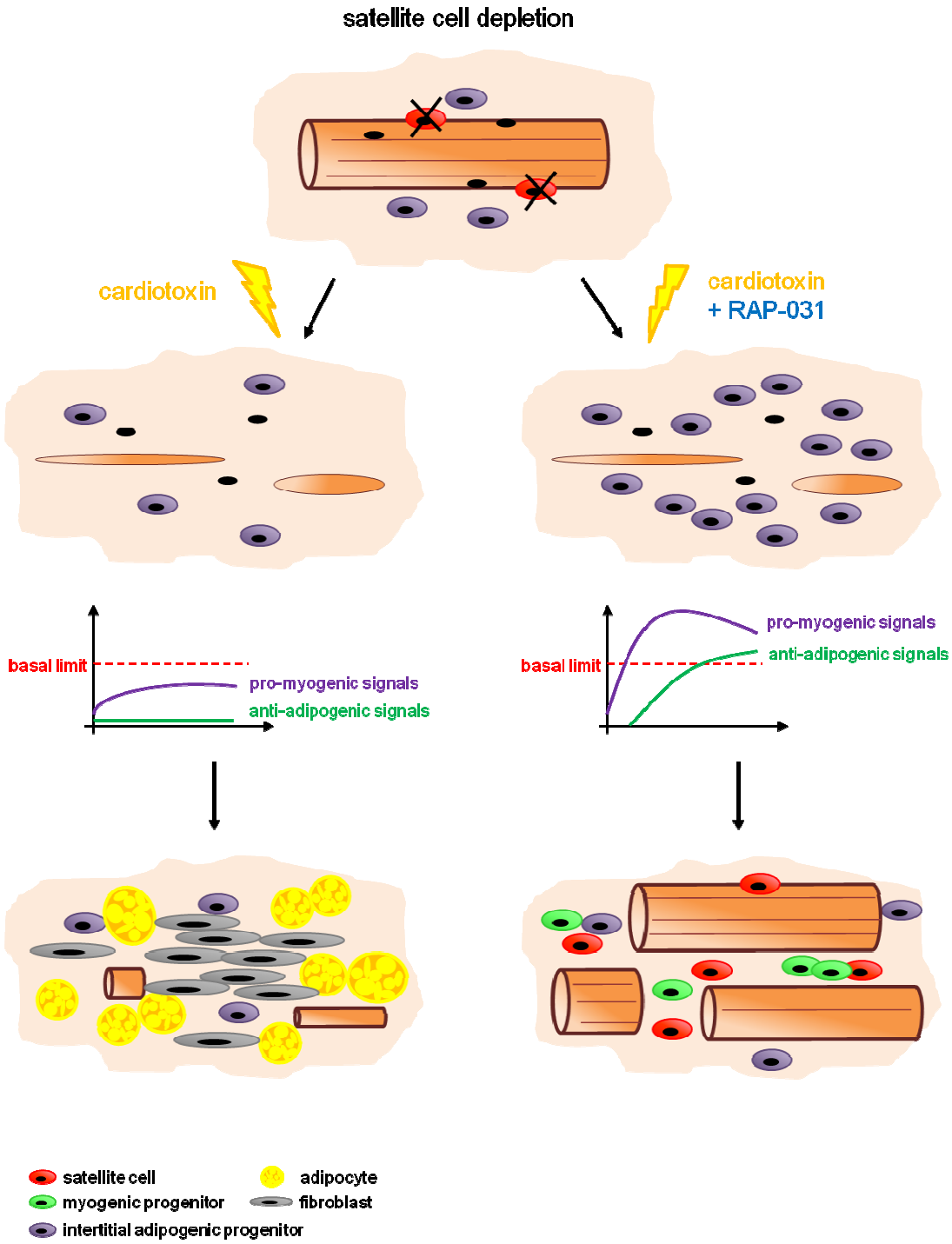

Figure 20. Model for muscle regeneration rescue by RAP-031. When satellite cells are absent, cardiotoxin injury induces the expansion of interstitial adipogenic progenitors (IAPs) which readily differentiate into fibroblasts and adipocytes, impairing muscle regeneration (left). When muscles are treated with RAP-031 prior to injury, IAPs proliferate and create an environment favoring the recruitment of myogenic progenitors by releasing positive signals (right). In turn, differentiating myoblasts produce signals inhibiting adipogenic differentiation thus restraining IAPs expansion and allowing muscle regeneration.

To conclude, further studies focusing on the larger muscle stem cell environment promise to be invaluable to the eventual understanding of stem cell function as well as for designing new therapeutic approaches in degenerative muscle diseases.

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APPENDIX 1

nature cell biology

Identification and characterization of a non-satellite cell muscle resident progenitor during postnatal development

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Satellite cells are resident myogenic progenitors in postnatal skeletal muscle involved in muscle postnatal growth and adult regenerative capacity. Here, we identify and describe a population of muscle-resident stem cells, which are located in the interstitium, that express the cell stress mediator PW1 but do not express other markers of muscle stem cells such as Pax7. PW1$^+$/Pax7$^-$ interstitial cells (PICs) are myogenic *in vitro* and efficiently contribute to skeletal muscle regeneration *in vivo* as well as generating satellite cells and PICs. Whereas Pax7 mutant satellite cells show robust myogenic potential, Pax7 mutant PICs are unable to participate in myogenesis and accumulate during postnatal growth. Furthermore, we found that PICs are not derived from a satellite cell lineage. Taken together, our findings uncover a new and anatomically identifiable population of muscle progenitors and define a key role for Pax7 in a non-satellite cell population during postnatal muscle growth.

Postnatal skeletal muscle growth and regeneration is dependent on muscle-resident progenitor cells[1–9]. Considerable work has demonstrated that satellite cells are a major source of progenitor cells in postnatal skeletal muscle[2,5,6,8,10–13]. Satellite cells were first defined by their anatomical location between the basal lamina and muscle fibre plasma membrane[14]. The identification of genes specifically expressed in satellite cells, such as the paired box transcription factor, *Pax7* (ref. 8) and MCadherin[15], allows for their identification using standard microscopy. Pax7 is required for postnatal muscle growth and maintenance of the satellite cell population, and both Pax3 and Pax7 participate in the establishment of the myogenic lineage during early development[12,16]. Constitutive Pax7 mutant muscle shows a significant reduction in the satellite cell population during the first weeks of postnatal life, which is attributed to decreased proliferative capacity[17–19] and increased apoptosis[19]. Studies using primary muscle culture and isolated single myofibres support a role for Pax7 in satellite cell self-renewal[13,20,21]. A recent report demonstrated that Pax7 and Pax3 are not required during adult life[22], suggesting that key cellular events during postnatal muscle development remain to be elucidated.

Non-satellite cell progenitors, such as bone marrow-derived circulating stem cells and muscle resident cell populations, can also participate in myogenesis[23–26]. However, as a consequence of the methods used to isolate these myogenic progenitors, such as cell culture selection techniques and flow cytometry-based sorting using cell surface markers or Hoechst dye exclusion, critical information is missing regarding the precise anatomical localization of these non-satellite cell progenitors, as well as their physiological contribution to muscle growth and repair[23,24,26–29], with the exception of mesoangioblasts, which can be localized as alkaline phosphatise-positive cells associated with the vessels[30].

We used a differential screening approach to identify factors involved in skeletal muscle stem cell commitment leading to the isolation of PW1/Peg3 (ref. 31). PW1 is expressed in primary myoblasts and myogenic cell lines[31–34]. PW1 expression initiates in early embryonic mesoderm and is downregulated in tissues as they differentiate[31]. In postnatal skeletal muscle, PW1 expression is detected in satellite cells and a subset of interstitial cells and is markedly upregulated during muscle regeneration[32,33]. PW1 participates in TNF–NFκB signalling and p53-mediated cell stress pathways[32–37]. Transgenic mice expressing a truncated form of PW1 (ΔPW1) that inhibits TNF and p53 signalling pathways[5] show a profound failure in postnatal muscle growth reminiscent of the Pax7 constitutive mutant phenotype[8,33]. ΔPW1-expressing mice are severely atrophic despite the presence of active Pax7$^+$ cell clusters underneath the basal lamina, implicating a role for PW1 in stem cell behaviour and postnatal muscle growth[33]. Together with p53, PW1 participates in regulating intrinsic and extrinsic stress pathways in myoblasts, placing PW1 as a key regulator of muscle atrophy[32,35].

In this study, we show that PICs can be isolated and their cell fate determined. PICs show bipotential behaviour *in vitro,* generating both smooth and skeletal muscle. Using single cell analyses, we observed that both lineages can be derived from a single PIC. Whereas freshly isolated

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Received 17 November 2009; accepted 14 January 2010; published online 31 January 2010; DOI: 10.1038/ncb2025

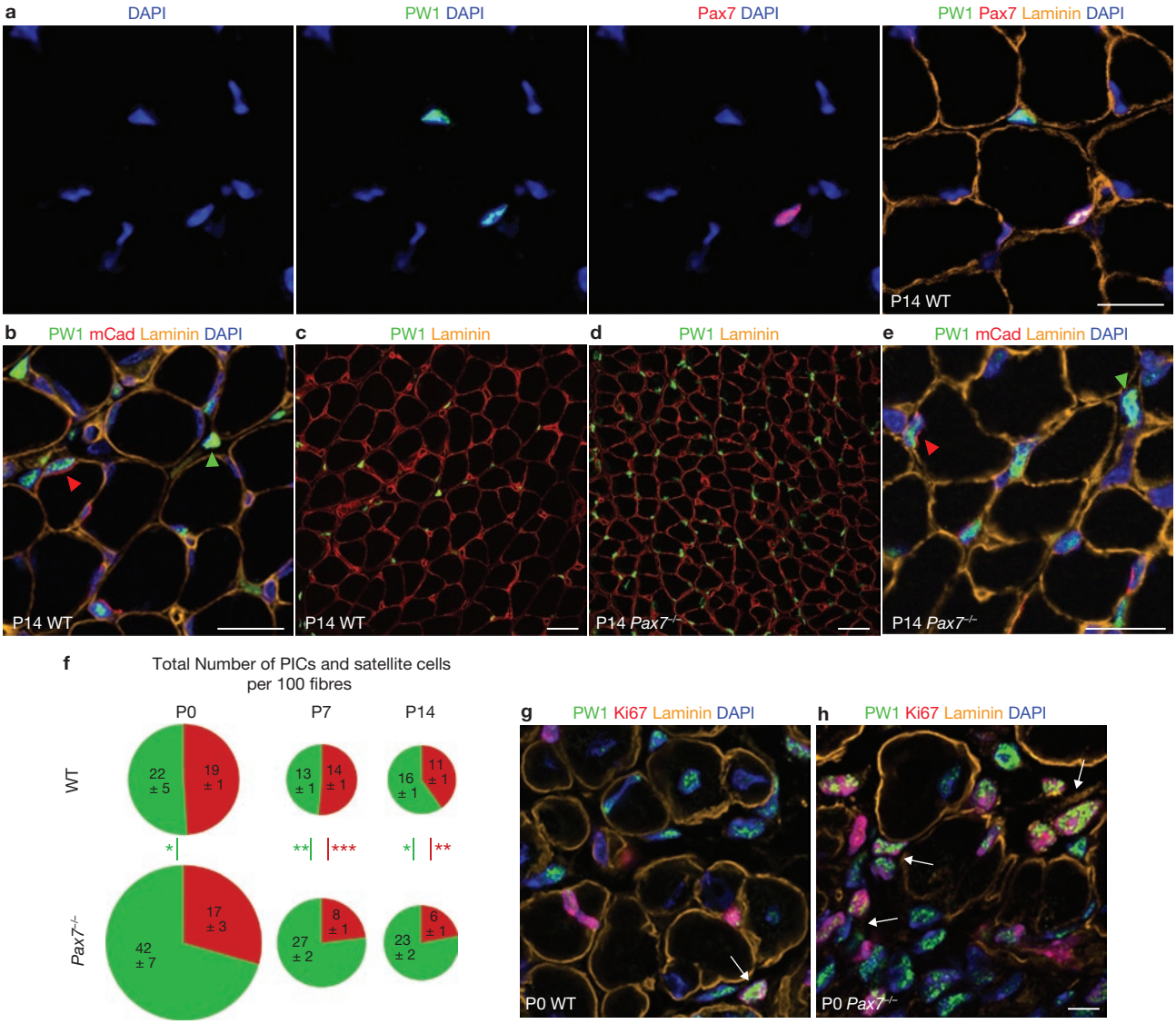

Figure 1 PW1 identifies two distinct populations in postnatal muscle under the regulation of Pax7. (**a**) Cross-section of a P14 hindlimb muscle immunostained for PW1 (green) and Pax7 (red) to identify satellite cells. Laminin staining (orange) shows the basal lamina. Nuclei were counterstained by DAPI (blue). We note that PW1 is expressed in satellite cells (colocalized with Pax7) in addition to a subset of interstitial cells (Pax7-negative). Scale bar, 50 μm. (**b**) Cross-section of a P14 hindlimb muscle from wild-type (P14 WT) mice immunostained for PW1 (green), mCadherin (red) to identify satellite cells and laminin (orange). DAPI staining identifies nuclei (blue). We note PW1 is expressed in satellite cells (mCadherin-positive, red arrowhead) and in a subset of interstitial cells that do not express mCadherin (green arrowhead). Scale bar, 50 μm. (**c**, **d**) Cross-sections of P14 hindlimb muscles from wild-type (P14 WT; **c**) and Pax7$^{-/-}$ (P14 *Pax7*$^{-/-}$; **d**) mice immunostained for PW1 (green) and laminin (red). The number of PW1^{+} cells is markedly increased in Pax7$^{-/-}$ muscle. Scale bars, 100 μm. (**e**) Cross-section of Pax7$^{-/-}$ P14 hindlimb muscle (P14 *Pax7*$^{-/-}$) immunostained for mCadherin (red), PW1 (green) and laminin (orange). DAPI staining (blue) identifies nuclei. Satellite cells (red arrowhead) and PICs (green arrowhead) are readily identified. Scale bar, 50 μm. (**f**) Quantification of PICs (green) and satellite cells (red) per 100 fibres for wild-type and *Pax7*$^{-/-}$ mice at P0, P7 and P14 from cross-sections immunostained as shown in **b** and **e**. Values represent the mean number of positive cells ± s.e.m. per 100 fibres and statistical analyses were performed using Student's *t*-test, $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$ ($n = 4$ per genotype and age). Circle area is relative to the total cell number. In *Pax7*$^{-/-}$ muscle, the number of PICs is significantly increased at all stages examined. (**g**, **h**) Cross-sections of P0 hindlimb muscles from wild-type (P0 WT) and *Pax7*$^{-/-}$ (P0 *Pax7*$^{-/-}$) mice immunostained for Ki67 (red), to identify proliferating cells, PW1 (green) and laminin (orange). Wild-type and *Pax7*$^{-/-}$ PICs express Ki67 (proliferation marker, arrows). We note clusters of proliferating PICs in *Pax7*$^{-/-}$ muscle only. Scale bar, 50 μm.

PICs do not express Pax7 or MyoD, we observed that they convert to a Pax7^{+}/MyoD^{+} state before forming skeletal muscle *in vitro*. Furthermore, we detected pronounced myogenic capacity of PICs following injection into damaged muscle tissue *in vivo*, comparable to that observed with freshly isolated satellite cells. Pax7 mutant mice (*Pax7*$^{-/-}$) show a marked increase in PICs during postnatal development, which is inversely proportional to the decrease in satellite cells. Satellite cells isolated from *Pax7*$^{-/-}$ mice proliferate well and are highly myogenic *in vitro*, however, *Pax7*$^{-/-}$ PICs show almost no skeletal myogenic capacity but retain the ability to become smooth muscle. Using lineage analyses, we show that PICs are not derived from a Pax3-expressing parental cell and thus do not share a satellite cell lineage; however, PICs do express Pax3 upon conversion to skeletal muscle. These data identify a new bipotent resident stem cell in skeletal muscle and are consistent with a model whereby Pax7

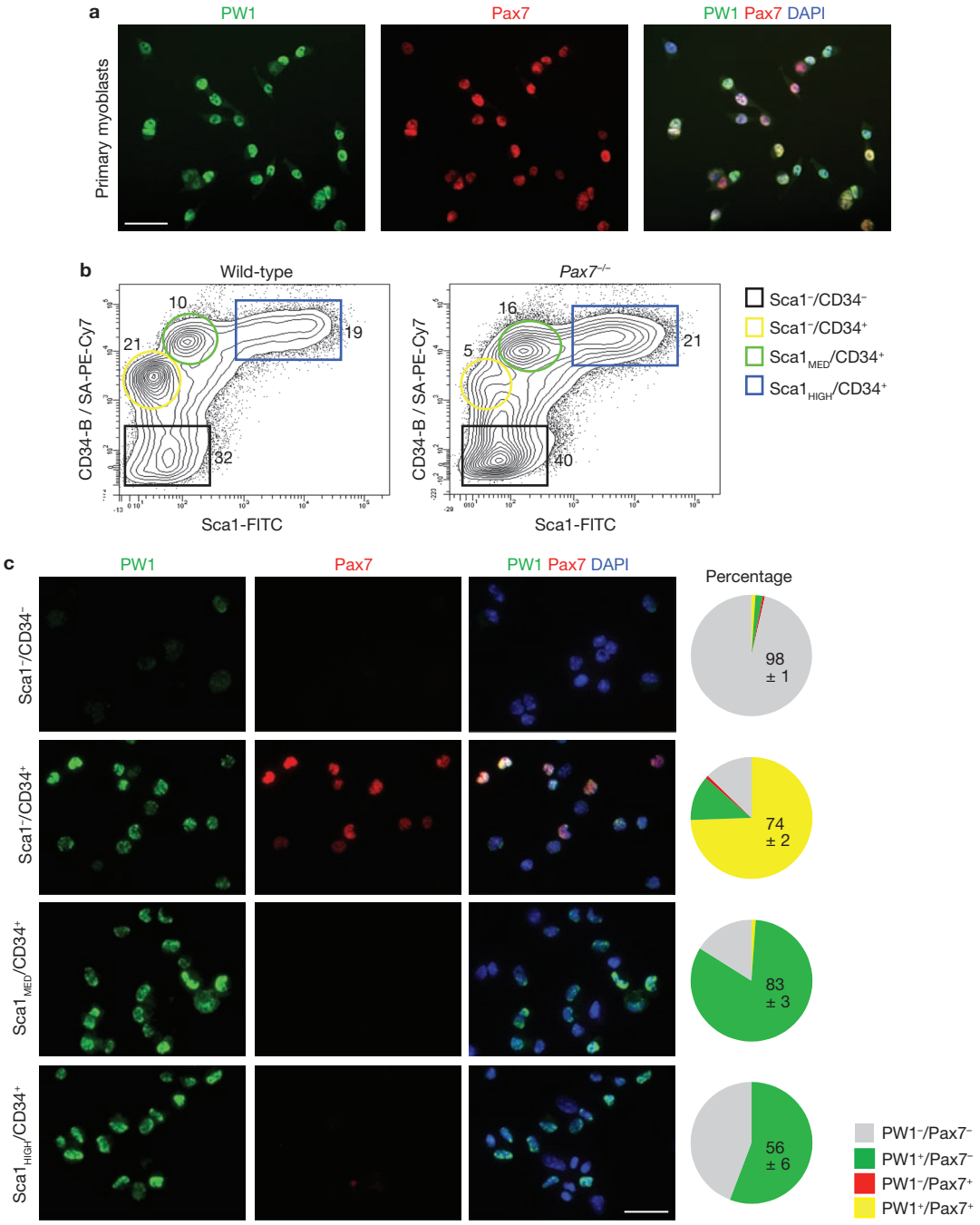

Figure 2 PICs are enriched in the muscle-resident Sca1$^+$/CD34$^+$ population. (**a**) Proliferating primary bulk muscle cultures immunostained for PW1 (green) and Pax7 (red) show co-expression of both genes. Nuclei were counterstained with DAPI (blue). PW1-expressing cells that do not express Pax7 cannot be identified *in vitro*. Scale bar, 30 μm. (**b**) Flow cytometric analyses of single cells from P10 hindlimb muscles from wild-type or *Pax7*$^{-/-}$ mice stained with antibodies against Sca1 and CD34. CD45$^+$ and Ter119$_{HIGH}$ cells were excluded as described in Supplementary Information, Fig. S1. The gates used to isolate Sca1$^-$/CD34$^-$, Sca1$^-$/CD34$^+$, Sca1$_{MED}$/CD34$^+$ and Sca1$_{HIGH}$/CD34$^+$ cells are shown. The four fractions are indicated directly on the scatterplot. Values represent the mean percentage of the parent population from four independent experiments. (**c**) Immunolocalization of PW1 (green) and Pax7 (red) in freshly sorted cytospun cell fractions, as shown in **b**. Nuclei are shown by DAPI staining (blue). PW1$^+$/Pax7$^+$ cells are in the Sca1$^-$/CD34$^+$ fraction and PW1$^+$/Pax7$^-$ cells are in the Sca1$^+$/CD34$^+$ fraction. Quantification of the staining (far right) shows that the Sca1$_{MED}$/CD34$^+$ population is highly enriched in PW1$^+$/Pax7$^-$ cells (PICs). Values are presented as the mean percentage ± s.e.m., $n = 5$. Scale bar, 30 μm.

expression is required for the postnatal recruitment of PICs to skeletal muscle during postnatal growth. Furthermore, these data implicate PICs as a key cell population that cannot be recruited into the skeletal muscle lineage in the absence of Pax7 function and is likely to contribute to the Pax7 muscle phenotype during postnatal growth.

RESULTS

PW1 identifies two distinct populations in postnatal muscle

To define the cell types in which PW1 is expressed in postnatal muscle, we stained muscle tissue sections for PW1, laminin to delineate the basal lamina and Pax7 (Fig. 1a) or MCadherin[15] (Fig. 1b) to identify satellite

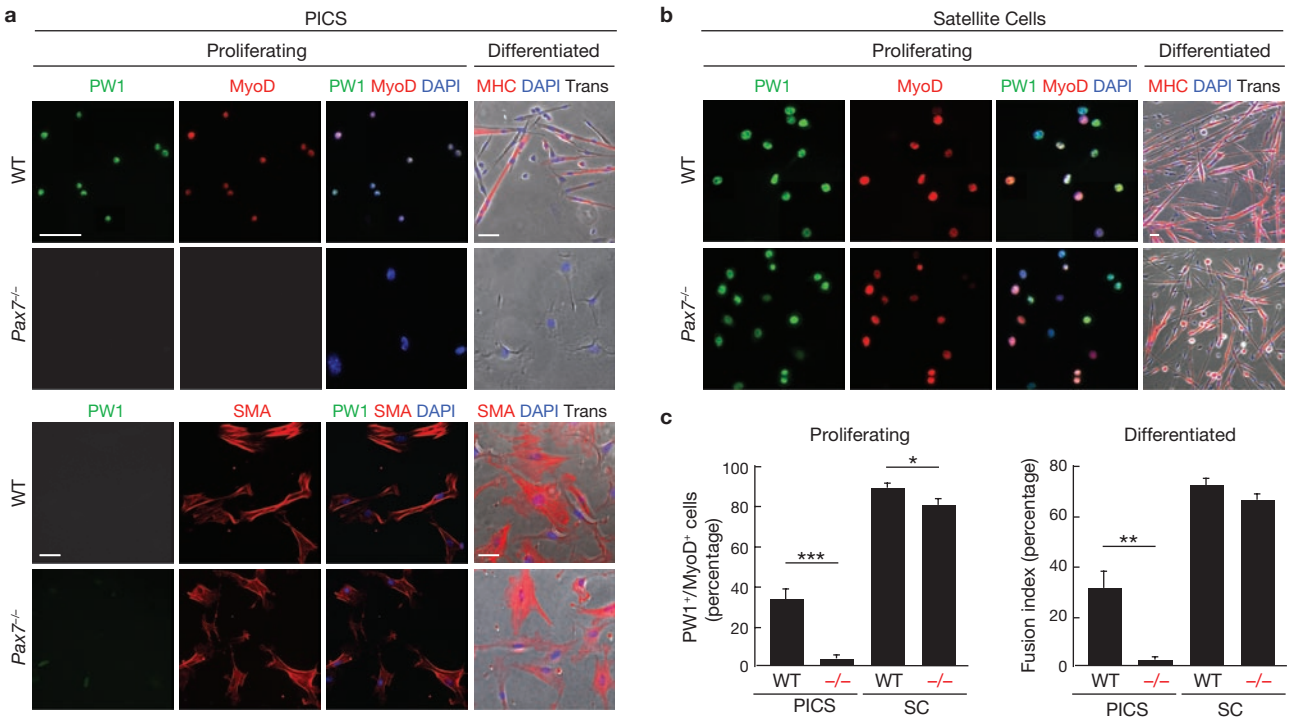

Figure 3 PICs spontaneously convert to skeletal myogenic cells via a Pax7-dependent pathway. (**a**) Top panels: myogenic cells were identified in cultures of proliferating wild-type and *Pax7*$^{-/-}$ PICs by immunostaining for PW1 (green) and MyoD (red). After 32 h in differentiation medium (Differentiated, far right), wild-type and *Pax7*$^{-/-}$ PICs were immunostained for myosin heavy chain (MHC; red) to determine biochemical differentiation. Bottom panels: immunolocalization of PW1 (green) and smooth muscle actin (SMA; red) in proliferating and differentiated wild-type and *Pax7*$^{-/-}$ PICs. Nuclei were counterstained with DAPI. Cultured PICs that express smooth muscle actin lose their PW1 expression. We note that whereas wild-type PICs acquire both myogenic and smooth muscle phenotypes, *Pax7*$^{-/-}$ PICs almost exclusively become smooth muscle. Under these conditions, only MyoD-negative cells express smooth muscle actin (Supplementary Information, Fig. S1). Scale bars, 50 μm. (**b**) Representative photomicrographs of proliferating wild-type and *Pax7*$^{-/-}$ satellite cells immunostained for PW1 (green) and MyoD (red). After 32 h in differentiating medium (Differentiated, far right), wild-type and *Pax7*$^{-/-}$ satellite cells were immunostained for myosin heavy chain (MHC; red). Nuclei are shown by DAPI. Note that *Pax7*$^{-/-}$ satellite cells are myogenically competent. Scale bar, 50 μm. (**c**) Quantitative analysis of myogenic competence and differentiation of cells treated as described in **a** and **b**. Proliferating values represent the mean percentage ± s.e.m. of proliferating cells co-expressing MyoD and PW1. Differentiated values show the mean percemtage ± s.e.m. of nuclei incorporated into MHC^{+} cells. *Pax7*$^{-/-}$ satellite cells are as myogenic as wild-type satellite cells. *Pax7*$^{-/-}$ PICS in culture lose PW1 expression, do not express MyoD and do not differentiate into skeletal muscle. $n = 5$ for each assay and genotype.

cells. Interstitial PW1^{+}/Pax7^{-} cells are abundant at birth, remain identifiable into adult life and then decline in older muscle, but increase following muscle injury (data not shown)[32,33,35]. As *Pax7*$^{-/-}$ mice have a significantly reduced lifespan[8,17,38], we restricted our analyses to the first 2 weeks of postnatal development. We observed that PW1 was expressed in two distinct compartments: satellite cells and a subset of interstitial cells (Fig. 1a, b)[32,33]. Most satellite cells expressed PW1 at postnatal day (P) 14 (93 ± 4%; $n = 3$ mice, > 350 fibres per mouse). PW1 expression was also detected in a subpopulation of interstitial cells (23 ± 3%; $n = 3$ mice, > 350 fibres per mouse). PICs did not express skeletal muscle, smooth muscle, endothelial or macrophage-specific gene markers at detectable levels, however, PICs were positive for vimentin (Supplementary Information, Fig. 1a–e), characteristic of muscle progenitors[39,40].

The PIC population is markedly increased in Pax7 mutant muscle

As the satellite cell compartment is reduced in the *Pax7*$^{-/-}$ mouse[8], we predicted that overall levels of PW1^{+} cells would decrease, reflecting the decrease in satellite cells. Surprisingly, we observed a marked increase in PW1^{+} cells in the Pax7 mutant by P14 (Fig. 1c, d). We quantified the number of satellite cells and PICs in wild-type, *Pax7*$^{+/-}$ and *Pax7*$^{-/-}$ muscle during postnatal development (Fig. 1f). We found that *Pax7*$^{+/-}$ muscle showed no significant phenotype, therefore only data from wild-type and *Pax7*$^{-/-}$ mice are presented. We considered MCadherin-positive cells present under the basal lamina to be satellite cells[41–43]. As in wild-type muscle (Fig. 1b), satellite cells and PICs were detected in *Pax7*$^{-/-}$ muscle (Fig. 1e). The percentage of satellite cells expressing PW1 did not vary significantly (93 ± 4% versus 98 ± 2% for wild-type and *Pax7*$^{-/-}$, respectively, at P14; $n = 3$ mice per genotype, > 350 fibres from randomly chosen fields per mouse; see Methods), however, we observed clear differences in the total number and ratio of satellite cells and PICs (Fig. 1f). At birth (P0), wild-type and *Pax7*$^{-/-}$ mice showed similar numbers of satellite cells, however, during postnatal growth (P7 and P14) this population declined significantly in Pax7 mutant mice. In contrast, the number of PICs was significantly elevated in Pax7 mutant mice at all stages examined. The most notable difference was observed at birth where twofold more PICs were present in Pax7 mutant mice (Fig. 1f), resulting in an expansion of the interstitial compartment (39 ± 2 versus 52 ± 6 interstitial cells per 100 fibres for wild-type and *Pax7*$^{-/-}$mice, respectively, at $P = 0$; $n = 3$ mice per genotype, $P = 0.05$, > 350 fibres per mouse). Overall, we observed a shift in the ratio of PICs to satellite cells from ~ 1:1 in wild-type to ~ 3:1 in Pax7 mutant mice (Fig. 1f). The increase in PICs at birth represents the earliest phenotype detectable in *Pax7*$^{-/-}$ muscle, preceding the decline in satellite cell number.

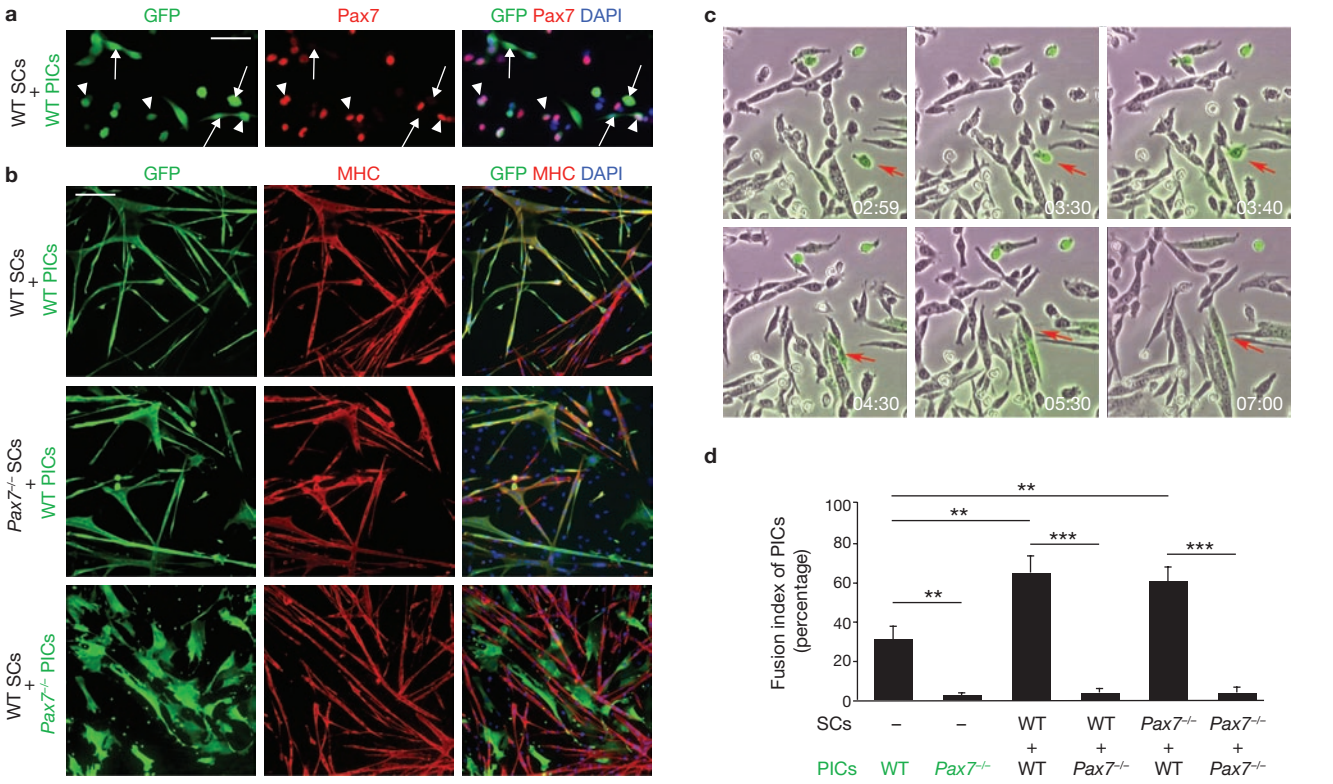

Figure 4 Pax7 is required for PICs to participate in myogenesis. (**a**) GFP-labelled wild-type PICs (WT PICs) grown with wild-type unlabelled satellite cells (WT SCs) for 96 h in growth medium. Immunostaining for GFP and Pax7 shows Pax7-positive GFP-negative satellite cells (red) in close proximity to Pax7-negative GFP-positive PICs (green; arrows) and GFP-positive PICs that have switched on Pax7 (arrowheads). Nuclei were counterstained with DAPI. Scale bar, 50 μm. (**b**) Top Panels, wild-type unlabelled satellite cells (WT SCs) grown in co-culture with WT GFP-labelled PICs (WT PICs) and allowed to differentiate in differentiation medium for 32 h. Immunolocalization of MHC (red) and GFP (green) shows GFP$^+$MHC$^+$ myotubes. Middle Panels, Pax7 mutant unlabelled satellite cells (Pax7$^{-/-}$ SCs) grown with GFP-labelled wild-type PICs (WT PICs) and allowed to differentiate in differentiation medium for 32 h. Immunolocalization of MHC (red) and GFP (green) shows GFP$^+$MHC$^+$ myotubes. Bottom panel, wild-type unlabelled satellite cells (WT SCs) grown in co-culture with wild-type GFP-labelled Pax7 mutant PICs (*Pax7*$^{-/-}$ PICs) and allowed to differentiate in DM for 32 h. No GFP$^+$MHC$^+$ myotubes were observed in co-cultures containing *Pax7*$^{-/-}$ PICs. Nuclei were counterstained with DAPI. Scale bar, 100 μm. (**c**) Frames from a time-lapse recording of co-cultures of wild-type GFP-labelled PICs (green) and wild-type unlabelled satellite cells (see also, Supplementary Information, Movie 1). Cells were transferred to low-serum conditions at time, 0. Time elapsed is shown in h:min. GFP$^+$ PICs readily fuse with satellite cell-derived myoblasts (arrows). (**d**) Quantitative analysis of myogenic differentiation of GFP-labelled PICs grown with unlabelled satellite cells (SCs), as shown in **b**. *Pax7*$^{-/-}$ PICs do not differentiate even when co-cultured with wild-type satellite cells. Values represent the percentage of nuclei in MHC$^+$ cells for GFP$^+$ cells only (mean ± s.e.m., $n = 4$ for each condition).

To determine the proliferative status of PICs, we stained wild-type and *Pax7*$^{-/-}$ muscles at various stages of postnatal development for Ki67. We noted clusters of Ki67$^+$ PICs in *Pax7*$^{-/-}$ at P0, whereas only scattered Ki67$^+$ PICs were observed in the wild-type muscle (Fig. 1g, h), revealing that loss of Pax7 is concomitant with an increase in PIC proliferation. By 1 week after birth, PICs and satellite cells were mitotically quiescent in wild-type muscle with few proliferative cells at birth.

PICs are present in the skeletal muscle Sca1$^+$/CD34$^+$ population

Primary bulk cultures (cultures of all single cells) derived from postnatal muscle gave rise to numerous myogenic cells, all of which co-expressed PW1 and Pax7 following several days in culture (Fig. 2a). In contrast, we never observed PW1$^+$ cells that expressed Pax7 or MyoD in culture, even though PICs were as abundant as satellite cells in muscle tissue (Fig. 1f). As a prerequisite step to explore PIC cell fate, we developed a fluorescence-activated cell sorting (FACS) approach using cell-surface markers previously used to isolate muscle stem cell populations[26,29,44]. Freshly sorted cells from P7–10 pooled muscle were immediately cytospun onto glass slides and analysed for PW1 and Pax7 expression. The Ter119$_{HIGH}$ and CD45$^+$ populations were completely PW1- and Pax7-negative whereas the remaining fraction contained a mixture of PW1$^+$ and PW1$^-$ cells (Supplementary Information, Fig. S1f–h). This fraction was then sorted for Sca1 and CD34, revealing four distinct populations (Fig. 2b; Supplementary Information, Fig. S1f). Satellite cells (PW1$^+$/Pax7$^+$) were present in the Sca1$^-$/CD34$^+$ fraction (Fig. 2c), in agreement with previous studies[2,44]. Upon isolation, PW1$^+$/Pax7$^-$ cells were considered to be PICs, corresponding to the only population of PW1$^+$/Pax7$^-$ cells *in vivo*. These cells were enriched in the two Sca1$^+$/CD34$^+$ fractions (Fig. 2c). As the Sca1$_{MED}$ fraction contained > 80% PICs, this fraction was further investigated *in vitro* and *in vivo*. The same approach was used to isolate satellite cells and PICs from *Pax7*$^{-/-}$ mice. In agreement with our *in vivo* quantification (Fig. 1f), loss of Pax7 resulted in a diminished satellite cell fraction (21 ± 4% for wild-type, 5 ± 1% for Pax7$^{-/-}$; $n = 4$; $P < 0.01$) and a larger Sca1$_{MED}$/CD34$^+$ fraction (10 ± 2% for wild-type and 16 ± 2% for Pax7$^{-/-}$; $n = 4$; $P < 0.05$; Fig. 2b), of which 72 ± 2% were PICs ($n = 3$). Significant changes in other fractions were not observed (Supplementary Information, Fig. S1h).

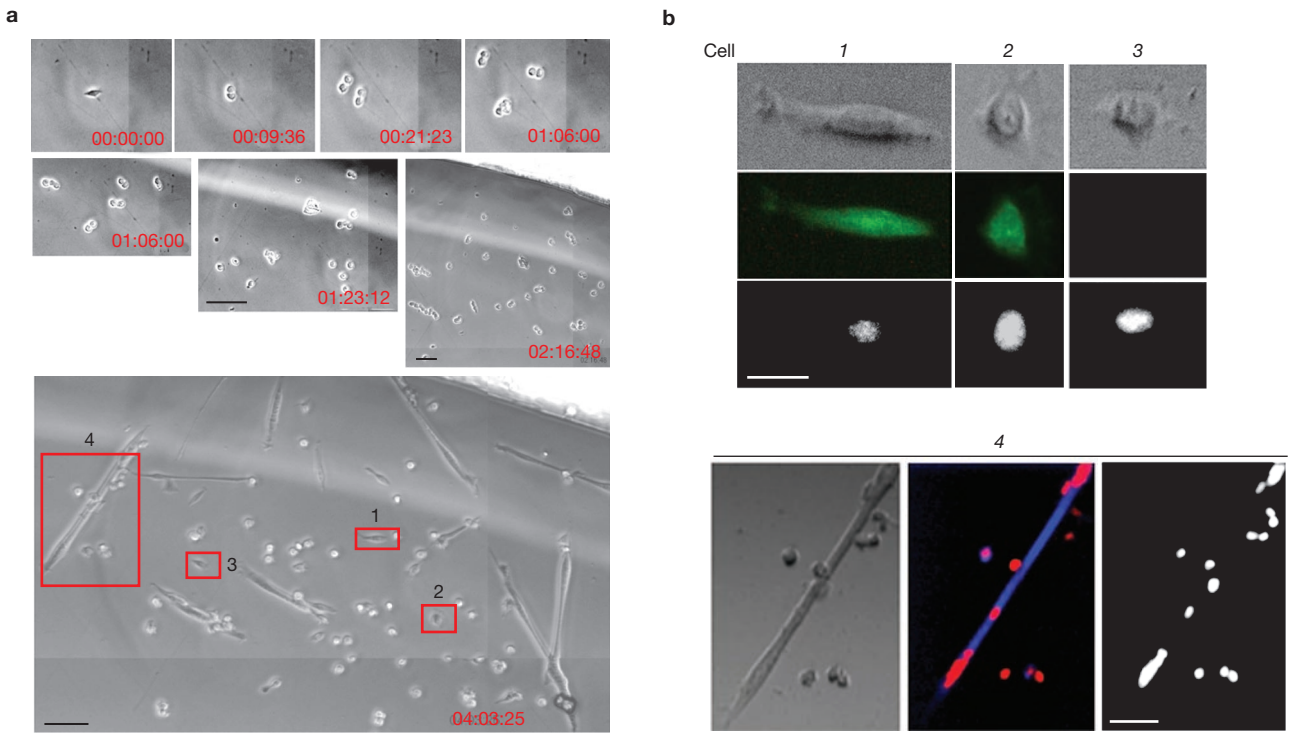

Figure 5 PICs are bipotent and give rise to smooth and skeletal muscle. (**a**) Representative frames from a time-lapse recording of freshly isolated PICs. Cells were observed for 3 days in proliferating medium and 24 h in differentiating medium (time in day:h:min). During this period, cells from different clones did not mix (Supplementary Information, Movie 2). A single cell divides several times during proliferation (from 0:00:00 until 3:00:00). After 24 h in differentiation medium, some of the daughter cells fused to form myotubes (bottom panel). Scale bars, 50 μm for middle panels and 100 μm for bottom panel. (**b**) Immunostaining for SM22 (green) MyoD (red) and MHC (blue) at the end of acquisition (4:03:25) was performed on selected daughter cells shown in the boxed, numbered cells in **a** (bottom panel), and shows lineage differences. The high magnification images show representative outcomes. Two examples of smooth muscle cells (numbers 1 and 2), which are positive for SM22 and negative for MyoD, are shown. We also observed a few cells that were negative for all markers used (number 3) and myotubes as well as single cells positive for MHC and MyoD (number 4). In this experiment, one PIC generated 149 nuclei, of which 117 corresponded to skeletal muscle ($MyoD^+$), 35 corresponded to smooth muscle ($SM22^+/MyoD^-$) and 15 showed no detectable staining. Scale bars, 30μm for top panel and 100 μm for bottom panel.

PICs show pronounced myogenic capacity *in vitro*

Freshly purified PICs were placed in culture to test for myogenic differentiation. After 4 days in culture, ~ 30% of cells were positive for MyoD and PW1 (Fig. 3a), and Pax7 (data not shown) and gave rise to myosin heavy chain (MHC)-expressing multinucleate myotubes after 24 h in differentiation conditions (Fig. 3a). Cells that did not form myotubes did not express skeletal muscle markers nor PW1, however, most expressed smooth muscle actin (Fig. 3a), suggesting that PICs also give rise to smooth muscle. Further analyses revealed that only smooth muscle actin-negative cells were positive for MyoD and PW1 (supplementary Information, Fig. S1i).

Pax7 is required for myogenic specification of PICs

As loss of Pax7 results in a decrease in satellite cells and an increase in PICs (Fig. 1f), we investigated whether loss of Pax7 altered PIC behaviour in culture. PICs and satellite cells from wild-type and *Pax7*$^{-/-}$ mice were purified and directly compared *in vitro*. We observed that wild-type and *Pax7*$^{-/-}$ satellite cells proliferated equally well in culture and that ~ 80% of cells expressed both MyoD and PW1. Furthermore, loss of Pax7 had no impact on the differentiation potential of satellite cells *in vitro* (Fig. 3b, c). The behaviours of wild-type and Pax7 mutant PICs were markedly different. In proliferating cultures, only ~3% of *Pax7*$^{-/-}$ PICs were $MyoD^+/PW1^+$, resulting in a fusion index of ~ 2%, in contrast to > 30% myogenicity of wild-type PICs (Fig. 3a, c). The smooth muscle fate of PICs was unaffected (Fig. 3a).

To investigate whether skeletal myoblasts influence the myogenic potential of PICs, we co-cultured wild-type GFP-labelled PICs with wild-type unlabelled satellite cells, and tracked PICs using GFP as a marker. Under proliferating conditions, we observed PICs that did not express Pax7 in close proximity to PICs that expressed Pax7, together with $Pax7^+$ satellite cells (GFP^-; Fig. 4a). We noted that $GFP^+/Pax7^-$ cells adopted a small, rounded phenotype, suggesting satellite cells alter PIC behaviour *in vitro* (Fig. 4a; Supplementary Information, Fig. S2). In differentiated cultures, > 60% of all nuclei in GFP^+ cells were in myotubes (verified by staining for MHC) compared with ~ 30% in cultures of PICs alone (Fig. 4b, top panels and Fig. 4d). We performed live-cell imaging of differentiating GFP-labelled PICs with unlabelled satellite cells to follow cell interactions over time. We observed that GFP^+ cells (PICs) readily fused with unlabelled myoblasts and myofibres to form fibres composed of both satellite cells and PICs (Fig. 4c; Supplementary Information, Movie 1).

Both wild-type and *Pax7*$^{-/-}$ satellite cells had comparable effects on the myogenic potential of wild-type PICs, revealing that Pax7 is not required by satellite cells to fuse with PICs (Fig. 4b, d). In contrast, *Pax7*$^{-/-}$ PICs were myogenically deficient when co-cultured with satellite cells from either wild-type or *Pax7*$^{-/-}$ mice, giving rise to ~ 3% of nuclei in GFP^+ cells being incorporated into myotubes (Fig. 4b, d). These data demonstrate that Pax7 is required for the myogenic capacity of PICs, whereas loss of Pax7 has little effect on purified satellite cells.

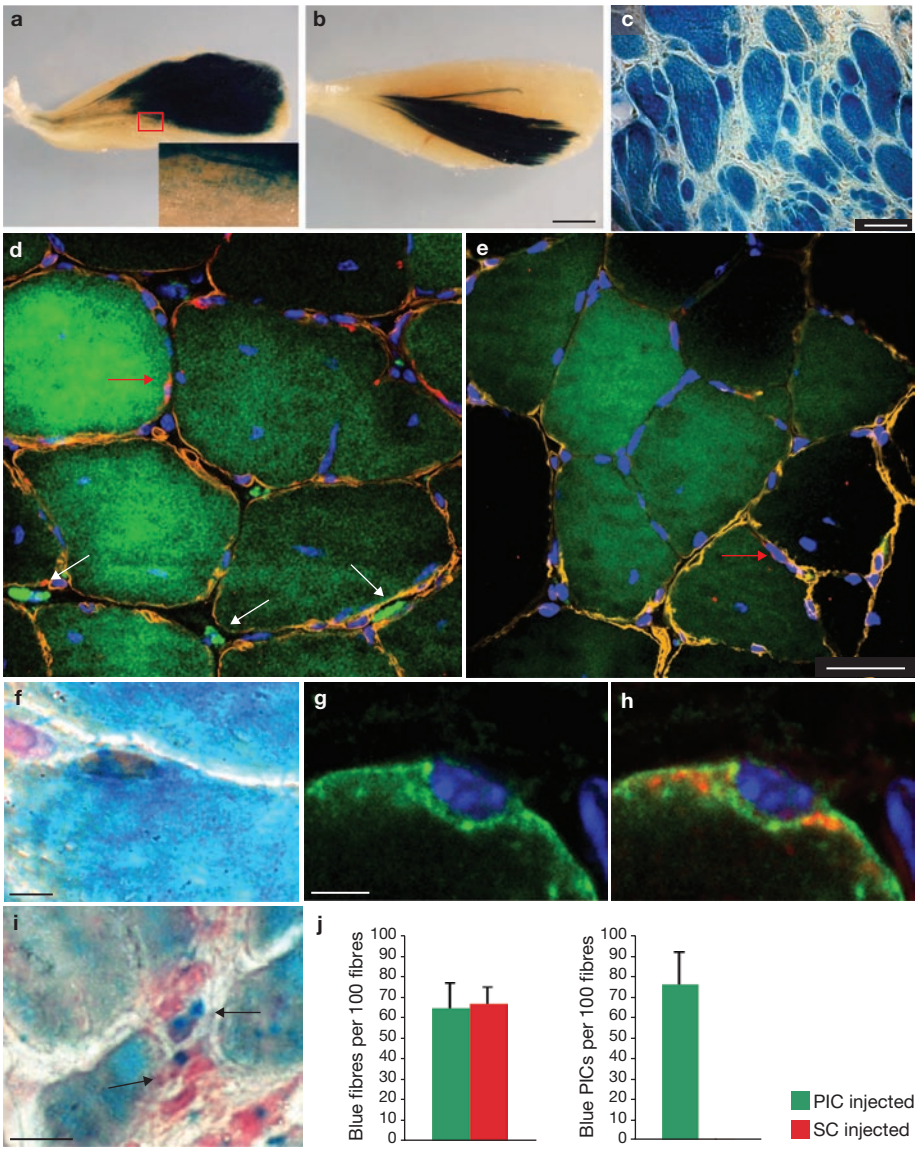

Figure 6 PICs are myogenic and recolonize the PIC niche *in vivo*. (**a**, **b**) Tibialis anterior muscle 2 weeks after injection with either 15,000 PICs (**a**) or satellite cells (**b**) freshly isolated from 10-day-old *R26LacZ* hind- and forelimbs. Muscles were tested for β-galactosidase activity. In PIC-transplanted muscle, LacZ staining is diffuse (see inset). (**c**) Cross-section of the tibialis anterior of nude mice injected with *R26LacZ* PICs as shown in **a**. β-Galactosidase staining (blue) revealed PIC contribution to myofibres and interstitial cells. (**d**, **e**) Representative cross-section of the tibialis anterior of nude mice 2 weeks after injection with 15,000 PICs (**d**) or satellite cells (**e**) freshly isolated from 9-day-old β-actin–EGFP limb muscles. Sections were immunostained for mCadherin (red) and laminin (orange). GFP fluorescence is shown in green. Nuclei were counterstained with DAPI (blue). Interstitial GFP-positive cells are only observed in PIC-transplanted muscles (white arrows). mCadherin labelling is present in GFP-positive fibres corresponding to cells in the satellite cell compartment (red arrows). Scale bar, 50 μm. (**f**) High resolution cross-section of the tibialis anterior injected with *R26LacZ* PICs and immunostained for Pax7 (brown). Scale bar, 10 μm. (**g**, **h**) High resolution cross-section of the tibialis anterior injected with *β-actin–EGFP* PICs and immunostained for GFP (green) and mCadherin (red), showing PIC-derived satellite cells adjacent to a weakly positive GFP fibre. Scale bar, 10 μm. (**i**) High resolution cross-section of tibialis anterior injected with *R26LacZ* PICs and immunostained for PW1 (brown), showing PW1-expressing blue interstitial cells (arrows). Scale bar, 50 μm. (**j**) Left panel, histogram showing the number of blue fibres normalized to fibre number in regenerating areas. We noted that PICs and satellite cells show comparable contributions to muscle fibres following injection. Values represent the mean percentage ± s.e.m. from four independent experiments. Right panel, histogram showing the number of blue interstitial cells per 100 fibres following *R26LacZ* PIC (green bar) and satellite cell (red bar) injection normalized to 100 fibres in regenerating areas. We observed that satellite cells do not generate PICs whereas ~75 PICs per 100 fibres are present following PIC injection. Values represent the mean percentage ± s.e.m. from four independent experiments. At least 250 fibres were counted for each experiment.

PICs are bipotent

The observation that PICs give rise to smooth and skeletal muscle suggests that PICs have two cell fates. We used live-cell imaging of single cell cultured PICs to determine the complete cell fates of resultant daughter cells over the course of 3–4 days under growth conditions followed by 24 h of differentiation. We followed the division and movements of single and daughter cells (Fig. 5a; Supplementary Information, Fig. S2 and Movie 2). As shown in Fig. 5, single PICs gave rise to daughter cells that acquired both skeletal and smooth muscle fates, as determined by staining for MyoD, MHC and SM22 (Fig. 5b). We did not see mixed cell

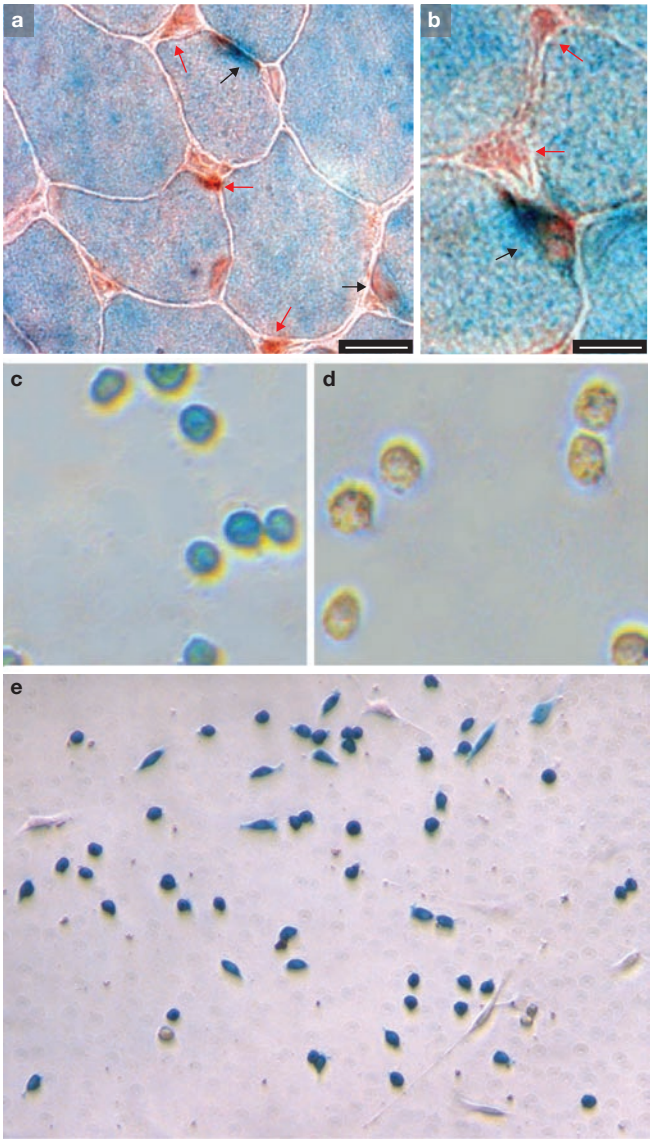

Figure 7 PICs are not derived from satellite cells. (**a**) Photomicrograph of a 2-week-old muscle from a *Pax3*$^{Cre/+}$ *X Rosa26*$^{LacZ/+}$ mouse. Sections were reacted with X-gal (blue) and stained for PW1 (brown). We note that all the muscle fibres and satellite cells (black arrows) are LacZ positive whereas no interstitial cells (red arrows) are positive for LacZ. Scale bar, 30 μm. (**b**) High resolution photomicrogaph of muscle as in **a**, showing a satellite cells marked by Pax3-Cre and stained for PW1 (black arrow). Interstitial cells positive for PW1 (PICs) are not marked by Pax3-Cre (red arrows). Scale bar, 10 μm. (**c**, **d**) Freshly isolated cytospun satellite cells (**c**) and PICs (**d**) were obtained from *Pax3*$^{Cre/+}$ X *Rosa26*$^{LacZ/+}$ crossed mice. Satellite cells are positive for β-galactosidase staining (**c**) whereas freshly isolated (cytospun) PICs are negative for β-galactosidase staining (**d**). (**e**) Colonies derived from freshly isolated PICs, as shown in **d**, and grown for several days in culture become positive for β-galactosidase staining.

fate outcomes with satellite cells (data not shown), suggesting that, unlike satellite cells, PICs are bipotent.

PICs are myogenically competent *in vivo* and participate in skeletal muscle regeneration

Evidence that satellite cells are muscle stem cells derives from studies demonstrating their potential to generate myofibres and satellite cells following muscle engraftment[2,13,45]. Therefore, we purified PICs from *Rosa26*$^{LacZ/+}$ mice or *β-actin–EGFP* mice to follow cell fate after injection into focally injured tibialis anterior muscles of nude mice. Freshly isolated satellite cells were injected into the contralateral limb tibialis anterior for direct comparison. We found that PICs participated as efficiently as satellite cells in myofibre formation, upon overall muscle inspection (Fig. 6a–c, i). However, PICs recolonized muscle tissue with less sharply defined boundaries compared with muscle recolonized by satellite cells (Fig. 6a, see inset, versus b). Histological examination of cross-sections of regenerated muscle following injection of labelled PICs revealed numerous labelled cells in the interstitium in all sections examined, whereas we did not observe labelled interstitial cells in muscle injected with satellite cells (Fig. 6c–e, j), suggesting PIC self-renewal. No labelled fibres or interstitial cells were observed in injured controlateral muscles that received an injection of PBS (data not shown). Close inspection of sectioned muscle injected with *R26*$^{LacZ/+}$ PICs revealed that PICs gave rise to Pax7^{+} satellite cells (Fig. 6f) as well as PW1^{+} interstitial cells (PICs; Fig. 6i). Satellite cell identity was further confirmed in regenerated muscle following injection with GFP-labelled PICs using antibodies against MCadherin and GFP (Fig. 6g). We found a significant contribution of PICs to their own compartment whereas no labelled PICs were generated following satellite cell injection (Fig. 6j). While PICs form smooth muscle *in vitro*, we primarily observed a contribution of injected PICs to myofibres *in vivo,* suggesting the tissue environment exerts a role in PIC specification.

PICs are not derived from satellite cells

PICs show myogenic regenerative capacity at levels comparable to satellite cells, suggesting a common cell lineage. Lineage analyses using *Pax3*^{Cre} mice crossed with *Rosa*^{lacZ} mice confirmed that all cells derived from Pax3-expressing cells are myofibres and satellite cells (Fig. 7a, b)[2,12,19]. In contrast, we did not observe Pax3-derived cells in the interstitium (Fig. 7a, b). PICs isolated from the *Pax3*^{Cre} × *Rosa*^{lacZ} mice contained almost no β-galactosidase-stained (blue) cells whereas satellite cells were blue (Fig. 7c, d). When PICs were cultured, we observed that all myogenic colonies were blue, demonstrating Pax3 expression upon entry of PICs into the skeletal muscle lineage (Fig. 7e). These data demonstrate that PICs are not derived from satellite cells.

DISCUSSION

Satellite cells are considered to be the main source of myonuclei in postnatal muscle[9,13,21,46]. However, the role of the satellite cell as a unique muscle progenitor has been challenged by reports of vascular or bone marrow cells with myogenic potential, as well as of resident multipotent stem cells[9,24,30,44,46–48]. As these cells do not express satellite cell markers (Pax7, MCadherin and NCAM) upon isolation, it is assumed that they lie outside the satellite cell niche. Defining a precise anatomical location for these progenitor cells has been challenging due to the lack of cellular markers, with the exception of Alp in the mesoangioblast/pericyte[30]. In this study, we provide evidence for muscle progenitors that are defined by their location in the interstitium coupled with PW1 expression. Similarly to satellite cells[17,49], this subpopulation of interstitial cells (PICs) is more abundant at birth and declines until about 2–3 weeks after birth, maintaining a 1:1 ratio with satellite cells. This decline in satellite cells may reflect recruitment to muscle during postnatal growth.

We demonstrated previously that PW1-expressing cells increase during regeneration, greatly exceeding the number of Pax7-positive cells[32];

however, their precise role during myogenesis was not defined. A key role for Pax7 was demonstrated by the observation that *Pax7*$^{-/-}$ mice show a dramatic decrease in satellite cells during postnatal growth[8,12,17,19]. We note that there is a pronounced increase in PICs before the decline in satellite cells, representing the earliest developmental muscle phenotype in the Pax7 constitutive mutant. *Pax7*$^{-/-}$ bulk muscle cultures are reported to have limited myogenic potential and to participate in non-muscle lineages such as fat and blood, suggesting a primary role for Pax7 in lineage specification[8,50]. Increased cell death has also been reported in Pax7 mutant satellite cells, suggesting a role for Pax7 in satellite cell maintenance[8,17]. Here, we report that purified Pax7 mutant satellite cells are myogenically competent, form normal-sized colonies and are able to form myotubes in culture. Although our studies do not address Pax7 mutant satellite cell capacity for self-renewal or other critical stem cell properties, we show a clear defect in the behaviour of Pax7-deficient PICs, which are not myogenic under any conditions tested.

The capacity of PICs to participate in myogenesis *in vivo* at levels comparable to those obtained with freshly isolated satellite cells, demonstrates that PICs are a significant myogenic progenitor population. However, only PICs give rise to more PICs in addition to satellite cells and myofibres. These data show that PICs re-populate their niche at high levels, fulfilling one criterion for stem cell self-renewal. In this regard, skeletal muscle may be comparable to other tissues, such as the skin, in which multipotent bulge stem cells give rise to specified populations of progenitors, including keratinocytes, which either remain quiescent or progress to form epidermis[51,52].

We show that PICs require *Pax7* for myogenic specification, a role originally proposed for this gene[8,20,38], which may have a critical role in postnatal muscle. The capacity for satellite cell self-renewal may not be sufficient until after postnatal growth is complete. This is consistent with a recent report confirming that Pax7 is required for postnatal muscle growth but not for adult muscle regeneration[22]. Whereas our lineage analyses confirm that satellite cells are derived from a Pax3 lineage[2,12,19], PICs express Pax3 during myogenic commitment. It remains to be determined whether PICs are a source of satellite cells during normal postnatal development.

PW1 was isolated from a screen designed to identify early stem cell compartments that give rise to the myogenic lineage[31]. PW1 protein associates with TRAF2, which acts as an adaptor in TNF signalling, leading to NFkB activation[34,37]. PW1 is also a p53-effector protein that associates with Bax and Siah1 in mediating cell death in non-muscle cells and can directly regulate myogenic differentiation through the caspase pathway[32,36,54–56]. It is striking that two muscle stem cell populations (satellite cells and PICs) constitutively express PW1, which may reflect a role in rapid and efficient recruitment after trauma and stress. □

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturecellbiology/.

Note: Supplementary Information is available on the Nature Cell Biology website.

ACKNOWLEDGEMENTS

We gratefully thank F. Relaix for fruitful discussions and critical reading of the manuscript. We thank A. Galy, E. Negroni and L. Arandel for technical advice and C. Blanc (Cytométrie en Flux platform, Institut Fédératif de Recherche 113) for FACs assistance. This work was supported by a grant from the NIH (NCI PO1 CA80058-06, subproject 3), a French Ministry of Research 'Chaire d'Excellence' to D.S. and the Muscular Dystrophy Association of America to D.S. and E.R.G. K.M. is a recipient of an INSERM 'contrat de jeunes chercheurs'. B.C. is a recipient of a grant from Fondation pour la Recherche Médicale (FRM). E.R.G. is supported in part by the Inserm Avenir Program. This work benefited from funding from the European Community's Seventh Framework Programme project OPTISTEM (Optimization of stem cell therapy for degenerative epithelial and muscle diseases contract number Health-F5-2009-223098). The Myology Group is the beneficiary of a Strategic Plan Support from the Association Française contre les Myopathies (AFM) and is affiliated with the Institute of Myology/ Association Institut Myologie.

AUTHOR CONTRIBUTIONS

All authors designed research; K.J.M., A.P., B.C., A.P. and V.B. performed research; B.C., V.B. and E.R.G. contributed new reagents/analytic tools; all authors analysed data and K.J.M., G.M. and D.A.S. wrote the paper.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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METHODS

Mice. The animal models used were *Pax7* $^{+/+}$, *Pax7* $^{LacZ/+}$ and *Pax7* $^{LacZ/LacZ}$ C57Bl/6J mice[56], C57Bl6-*β-actin–EGFP* transgenic mice, in which cytoplasmic GFP is expressed ubiquitously under the control of the chicken β-actin promoter[57], *ROSA26 flox–LacZ*; *Pax3* $^{Cre/+}$, *PGK-Cre* mice (*R26LacZ*) and nu/nu mice (Elevage Janvier). All work with mice was carried out in adherence to French government guidelines.

Primary cell culture. Primary skeletal muscle cell cultures from the limb muscle of P10 mice were prepared by enzymatic digestion, as described previously[58]. Cells were plated on gelatin-coated dishes at a density of 1000 cells per cm^2 in BIOAMF-2 complete medium (ATGC). For myogenic differentiation, cells were transferred to differentiation medium (DM) for 24–36 h: DMEM (Gibco) containing 2% (v/v) horse serum (Gibco) and 1% (v/v) penicillin-streptomycin (Gibco).

FACS analysis. For fluorescence-activated cell sorting, limb muscles from 7–14-day-old *Pax7* $^{+/+}$, *Pax7* $^{LacZ/LacZ}$, i or *R26LacZ* mice were minced and digested in HBSS (GIBCO) containing 2 μg ml^{-1} collagenase A (Roche), 2.4 U ml^{-1} dispase I (Roche), 10 ng ml^{-1} DNAse I (Roche), 0.4 mM $CaCl_2$ and 5 mM $MgCl_2$ for 90–120 min at 37 °C with agitation every 15 min. Three successive cycles of washing and filtration were performed before re-suspension of the cell pellet in HBSS containing 0.2% (w/v) BSA (Sigma), 1% (v/v) penicillin-streptomycin, 10 ng ml^{-1} DNAse I and 10% (v/v) mouse serum (Jackson Immunoresearch). Cells were incubated for 5 min on ice before adding the following primary antibodies at a concentration of 10 ng ml^{-1}: rat anti-mouse CD34-biotin (Ram34; eBiosciences), rat anti-mouse CD45-APC (BD Biosciences), rat anti-mouse Ter119-APC or Ter119-PE (BD Biosciences) and rat anti-mouse Sca1-FITC or Sca1-PE (BD Biosciences). Cells were incubated for 30 min on ice. Cell pellets were washed before incubation with streptavidin-PE-Cy7 (1/500; BD Biosciences) for 30 min on ice. Cells were finally washed, filtered and re-suspended in HBSS containing 0.2% (w/v) BSA and 1% (v/v) penicillin-streptomycin. Flow cytometry analysis and cell sorting were performed on a FACSAria (Becton Dickinson), with appropriate isotype matching controls. $Ter119_{HIGH}$ and $CD45^+$ cells were gated and excluded by negative selection. The remaining cells were then gated and sorted based on their CD34 and Sca1 expression. Purified cell populations were cultured as described above. For immuncytochemical analyses, freshly sorted cells (10,000 cells per slide) were immediately centrifuged onto glass slides using a Cytospin (ThermoFisher Scientific) for 5 min at 1000 rpm and immunostained for PW1 and Pax7, as described below. Quantitative analyses were performed by counting the number of positive cells out of a minimum of 300 cells in randomly chosen fields for each of three independent experiments.

Tissue injury and transplantation. Skeletal muscle regeneration was induced by a focal freeze-crush injury of the tibialis anterior of 2 month-old female nu/nu mice, as described previously[59]. At 24 h post-injury, freshly isolated PICs ($CD34^+$ $Sca1_{MED}$ $CD45^-$ $Ter119_{HIGH}$) and satellite cells ($CD34^+$ $Sca1^-$ $CD45^-$ $Ter119_{HIGH}$) from hind- and forelimb muscles of 7–10-day-old *β-actin–EGFP* or *R26LacZ* mice were washed, re-suspended in PBS and directly injected into the damaged muscle (10,000–15,000 cells per tibialis anterior). As a control, injured tibialis anterior muscles were injected with PBS. Muscles were collected 14 days post-injection.

Histological analyses. For immunofluorescence and immunohistochemistry experiments, entire hindlimbs from 0–14-day-old *Pax7* $^{+/+}$, *Pax7* $^{LacZ/+}$, *Pax7* $^{LacZ/LacZ}$ and *Pax3* $^{Cre/+}$ *X Rosa26* $^{LacZ/+}$ mice and tibialis anterior muscles from transplanted nu/nu mice were snap frozen in liquid nitrogen-cooled isopentane or directly in liquid nitrogen. β-Galactosidase activity in *R26LacZ*-transplanted tibialis anterior muscles and *Pax3* $^{Cre/+}$ *X Rosa26* $^{LacZ/+}$ mice was revealed either before freezing or on transverse cryosections using X-gal, as described previously[10]. Cryosections (10 μ), cultured cells and cytospin preparations were fixed in 4% (w/v) paraformaldehye and processed for immunostaining as described previously[32–34]. Primary antibodies used were against: PW1 (ref. 31; 1:4,000), Pax7 (Developmental Studies Hybridoma Bank; 1:20), laminin (Sigma; 1:100), MCadherin (NanoTools; 1:100), Ki67 (BD Biosciences; 1:100), myoD (BD Biosciences; 1:100), smooth muscle actin (Sigma; 1:300), MF20 (Developmental Studies Hybridoma Bank; 1:2), GFP (Biovalley and Abcam; 1:400), Pecam-1 (BD Biosciences; 1:500), vimentin (Progene; 1:500) and F4/80 (AbCam; 1:500). For immunofluorescence, antibody binding was revealed using species-specific secondary antibodies coupled to Alexa Fluor 488 (Molecular Probes), Cy3 or Cy5 (Jackson Immunoresearch). As described previously[6], nuclei were counterstained with DAPI (Sigma). For immunohistochemistry, antibody binding was revealed using species-specific secondary antibodies coupled with horseraddish peroxidase (Jackson Immunoresearch) and the enzymatic reaction was performed according to the manufacturer's instructions (VECTOR NovaRED substrate kit, AbCys). For quantitative analyses of immunostained tissue, positive cells in at least 350 fibres from randomly chosen fields were counted from three animals for each age per genotype. Cells in culture were quantified by counting at least 400 cells from randomly chosen fields for each of three independent experiments. Fusion indexes were quantified by counting the number of nuclei in $MF20^+$ cells per total number of nuclei. For quantitative analysis of *R26LacZ* PICs and satellite cells, injected tibialis anterior muscles, blue fibres and interstitial cells from fields of the area of regeneration were counted from four animals for each experimental condition and were normalized to the fibre number in areas counted. At least 200 fibres (positive and negative) for each independent experiment were counted. Values represent the mean ± s.em. and statistical analyses were performed using Student's *t*-test, $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$.

Microscopy and live-cell imaging. Images were acquired using a Leica DM-IL inverted fluorescence microscope, Leica DM fluorescence microscope or Leica SPE confocal microscope. For detection of GFP^+ myofibres, a narrow range of emission wavelength (511–532 nm) was used to avoid detection of autofluorescence[61].

For live-cell imaging, cell cultures were monitored using a Nikon Ti microscope equipped with an incubator to maintain cultures at 37 °C and 5% CO_2 (Okolab), a CoolSNAP HQ 2 camera (Roper Scientific) and an XY motorized stage (Nikon), driven by metamorph software (Molecular Devices). For myogenicity of the PICs, fluorescence (GFP) and phase contrast images were acquired every 10 min. For cell lineage, isolated PICs were plated on gelatin-coated dishes with custom-made 5-mm diameter silicon wells (sylgard 184, Dow Corning), to allow the observation of all the area of the well, using the XY stage. Cells were transferred just after plating to the microscope and images of the whole well were acquired every 12 or 30 min during 4 or 5 days. Cells were plated at a density of 1000 cells per cm2 in BIOAMF-2 complete medium (ATGC) for 3 or 4 days and then transferred to DMEM (Gibco) containing 2% (v/v) horse serum (Gibco) and 1% (v/v) penicillin-streptomycin (Gibco) for myogenic differentiation (1 day). Cells were fixed in 4% (w/v) paraformaldehyde immediately after the acquisition of the last image, permeabilized with cold methanol and blocked with 4% (w/v) BSA in PBS. Cells were immunostained using primary antibodies against smooth muscle actin (Sigma), MHCfast (AbCys), SM22a (AbCam) and MyoD (BD Biosciences) followed by secondary antibodies coupled to Cy3 or Cy5 (Jackson Immunoresearch). Nuclei were counterstained with DAPI (Sigma). Only wells containing one single cell at day 0 were analysed.

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DOI: 10.1038/ncb2025

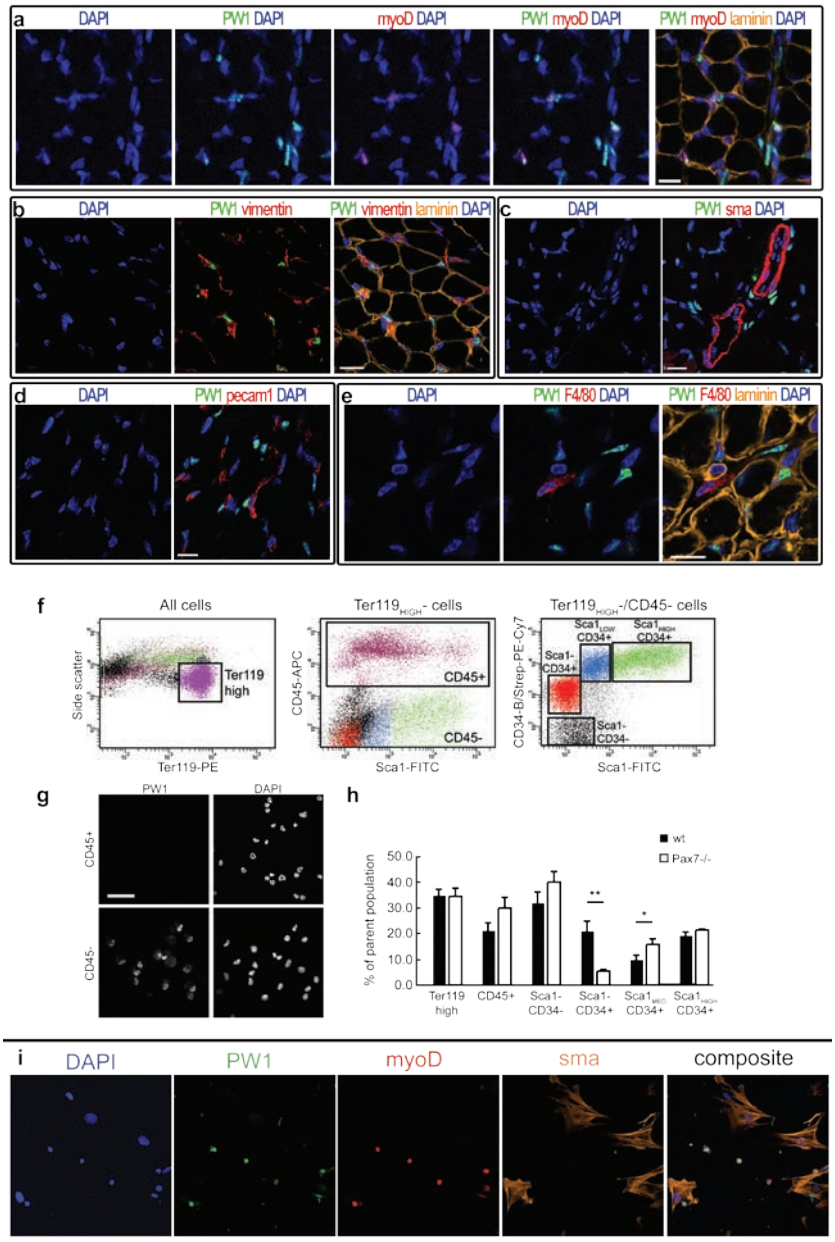

Figure S1 a-e. PICs are negative for markers of skeletal muscle, smooth muscle, endothelial cells and macrophages. Cross-sections of wildtype hindlimb muscle at P14 (**a**-**d**) or P2 (**e**), immunostained as indicated in the figure. Nuclei are visualized with DAPI. Scale bars = 50 μm. (**a**) MyoD+/PW1+ cells are observed underneath the basal lamina. PICs are negative for MyoD. (**b**) PICs express the intermediate filament vimentin. (**c**, **d**) PICs are often closely associated with vessels but PW1 (green) does not colocalise with either smooth muscle actin (red) (**c**) or the endothelial marker CD31/pecam1 (red) (**d**). (**e**) PICs are negative for the macrophage marker F4/80 (red). **f-h.** Isolation of muscle-resident populations by FACS using stem cell markers. (**f**) FACS profiles of single cells from P10 wildtype hindlimb muscles stained with antibodies against Ter119, CD45, Sca1 and CD34. $Ter119_{HIGH}$ cells were negatively selected to exclude erythrocytes (left panel). All CD45+ cells were negatively selected (middle panel). Ter119-/CD45- cells were then separated on the basis of Sca1 and CD34 expression (right panel). The gates used to isolate Sca1-/CD34-, Sca1-/CD34+, $Sca1_{MED}$/CD34+ and $Sca1_{HIGH}$/CD34+ cells are shown. (**g**) Representative photomicrographs of freshly sorted CD45+ and CD45- cells immunostained with PW1. Nuclei were counterstained with DAPI. PW1+ cells are only present in the CD45- population. Scale bar = 30 μm. (**h**) Quantification of % of cells in each population for P10 wildtype (black columns) and Pax7 -/- (open columns) hindlimb muscles. Pax7 -/- muscle has fewer Sca1-/CD34+ cells and more $Sca1_{MED}$/CD34+ cells. No significant differences were observed in the sizes of all other fractions. Values represent the mean % ± SEM (from 4 independent experiments) of positive cells from the following parent populations: all cells for $Ter119_{HIGH}$; Ter119- cells for CD45; and Ter119-/CD45- for the Sca1/CD34 fractions. **i.** Smooth muscle actin positive cells (sma) do not express skeletal muscle myoblast markers. Immunolocalisation of PW1 (green), MyoD (red), and smooth muscle actin (sma; orange) in PICs cultured for 4 days in growth media (differentiating). Nuclei were counterstained with DAPI. Only the smooth muscle actin negative cells are positive for MyoD and PW1.

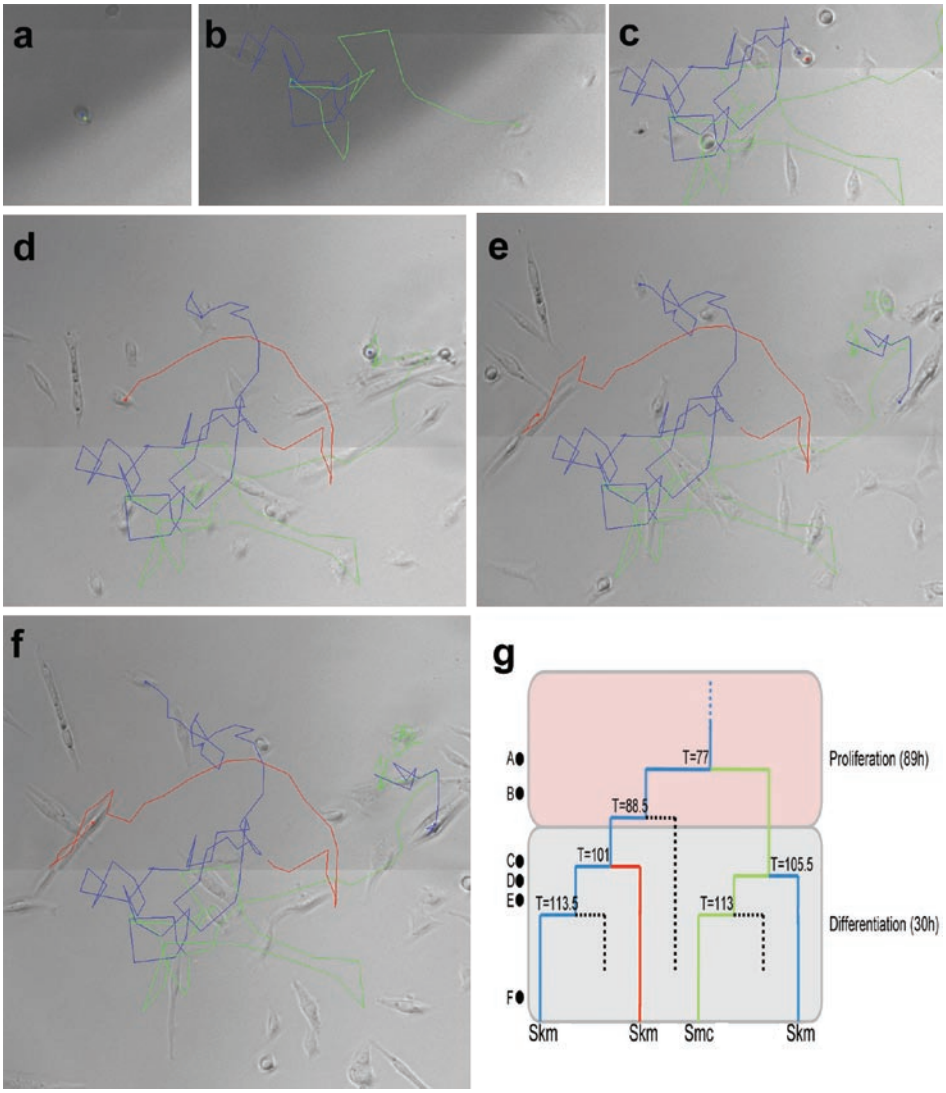

Figure S2 A geneology tree of a single PIC giving rise to smooth and skeletal muscle. (**a**-**f**) Representative frames from a time-lapse recording of freshly isolated PICs. Images were acquired over a 4 day period in growth medium (**a**-**b**) and 30 hours in differentiating medium (**c**-**f**). The parental cell shown in panel (**a**) divides several times (**a**-**d**) and coloured lines represent the divisions and movements of 4 cells derived from the initial cell. (**g**) Genealogy tree of the cell in the first frame (**a**) showing division time, colour-coded as shown in panels (**a**-**f**). The letters at the left correspond to the frames depicted in panels a-f and the time of cell divisions are indicated on the tree. The cell fates were determined by immunofluorescence staining as shown in Figure 5 (Skm-skeletal muscle, Smc-smooth muscle).

Supplementary Movie Legends

Movie 1 Real-time visualization of PICs contributing to myogenesis *in vitro*. Time-lapse recording of co-cultures of wildtype GFP-labelled PICs (green) and wildtype unlabeled satellite cells. Arrows indicate events during which a GFP-labelled PIC fuses with wildtype unlabeled satellite cells or myotubes. Frames from this movie are shown in Figure 4.

Movie 2 Real time visualization of cells shown in Figure 5. Real time lapse recording of entire cell history shown in Figure 5.

APPENDIX 2

Stem cells in the 'hood' : the skeletal muscle stem cell niche

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Summary

It is generally accepted that the principal resident progenitor underlying regenerative capacity in skeletal muscle is the satellite cell. Satellite cells are present throughout life even though regenerative capacity declines with age and disease. Recently, other stem cell populations have been identified with that can participate in muscle growth and regeneration. These cells may provide therapeutically useful sources of muscle stem cells as compared to satellite cells; however, the roles of these non-satellite cell populations during muscle homeostasis, regeneration and aging are unclear. Here, we discuss how the stem cell neighborhood influences satellite cell behavior and bring together recent discoveries pertaining to a wide variety of adult stem cells that may apply specifically to muscle stem cells and their niche.

Keywords

Satellite cell, regeneration, stem cell, aging, skeletal muscle, non-satellite stem cell, adult stem cells.

Diversity of muscle progenitors

Adult skeletal muscle is composed primarily of multinucleate myofibers, each surrounded by a basal lamina. A population of single cells, referred to as satellite cells, are found underneath the muscle fiber basal lamina and closely juxtaposed to the muscle fiber plasma membrane [1] (Box 1 and Fig.1). Under normal physiological conditions, satellite cells are quiescent in adult muscle and can be identified by the expression of a number of genes including Pax7 and α7-integrin [2] [3]. Upon injury, satellite cells activate the expression of the myogenic regulatory factors Myf5 and MyoD, re-enter the cell cycle, differentiate and ultimately fuse to form new fibers ([1, 4, 5] and for review see [6]). While the majority of satellite cells participate in muscle repair, a small proportion of cells exit the cell cycle and replace the satellite cell population [7] [8] providing a mechanism to support multiple rounds of injury. While substantial evidence points to satellite cells as the major skeletal muscle progenitor/stem cell, there are numerous reports of non-satellite cell populations with myogenic capacity following engraftment into muscle tissue (Table1).

One of the first non-satellite cell populations with myogenic capacity came from experiments in which bone marrow-derived cells were transplantated into lethally irradiated mice. Following muscle injury, these bone marrow-derived cells where shown to participate directly in skeletal muscle regeneration [9] [10]. Further analyses of the hematopoietic population revealed a novel population of cells, referred to as side population cells (SP) based on their ability to exclude Hoechst dye [11]. Following engraftment, SP cells reconstitute the hematopoietic lineage as well as give rise to new satellite cells and myofibers, albeit at extremely low levels [12, 13]. Transplantation of SP cells into *mdx* mice (a model for Duchenne Muscular Dystrophy) restored dystrophin expression and improved muscle function [9]. In humans, a sub-population of hematopoietic cells expressing the cell surface antigen, CD133, also displays myogenic capacity following co-culture with myoblasts [14]. Intramuscular and intra-arterial injection of these cells into *scid/mdx* mice resulted in significant recovery of muscle morphology, function, and dystrophin expression [15] and these cells are presently being tested in the clinic [16]. More recently, our group reported the identification of a muscle-resident population with myogenic potential located within the interstitial space and characterized by PW1/Peg3 expression referred to as PICs (PW1+ Interstitial Cells) [17]. PICs isolated from early postnatal muscle are able to generate new fibers, contribute to the satellite cell pool following engraftment into damaged muscle, and

to give rise to more PICs (the latter property not shared by satellite cells). Lineage tracing experiments demonstrated that PICs and satellites cells do not share the same (Pax3 dermamyotome somite) embryonic origin. Coupled with the observation that PICs also give rise to non-skeletal muscle lineages, we suggested that PICs constitute an upstream stem population and play a role during postnatal growth [17], which corresponds to a period of rapid muscle mass and nuclear accumulation [18]. In this context, it will be important to determine the potential of PICs in the adult. A recent study defined another interstitial population of adult muscle stem cells characterized by β4-integrin expression, which can participate in muscle repair following engraftment into *mdx* mice [19]. Whether these ß4-integrin+ cells are the same population as PICs remains to be determined. An additional progenitor population with high therapeutic potential are the mesoangioblasts (Mabs), which can be isolated from either embryonic [20, 21] or postnatal muscle vasculature [22]. Mabs display robust participation in muscle repair following engraftment or arterial delivery in both mice [23] and dogs [24] and are presently in clinical trial. Although the anatomical location of adult Mabs is unclear, compelling data suggest that they are derived from pericytes that also show robust myogenic capacity *in vitro* and *in vivo* [25]. Pericytes can be distinguished from endothelial cells by Alkaline Phosphatase (AP) expression, and do not express myogenic nor endothelial markers. Using lineage tracing, it was shown that pericytes contribute to post-natal muscle growth and regeneration *in situ* demonstrating *bona fide* contribution to normal muscle growth by a non-satellite cell population during post-natal development [26]. Studies outlined above establish clearly that non-satellite cell progenitors are competent to contribute to muscle repair, and raise the possibility that multiple cell types support adult skeletal muscle regeneration and postnatal muscle growth. However, with the exception of pericytes, direct contribution of these progenitors to postnatal myogenesis and muscle repair has not been firmly demonstrated, and in the case of pericytes, this contribution is still relatively minor.

Recently, two laboratories have created murine models in which satellite cells can be conditionally depleted in order to test whether muscle regeneration is possible in the absence of satellite cells [27] [28]. In one case, the human diphteria toxin receptor was expressed under the control of the murine *Pax7* locus so that injection of diphteria toxin kills cells expressing Pax7 [28]; and in the other study mice expressing the tamoxifen-inducible CRE under the control of Pax7 were crossed with mice expressing an inducible diphteria

toxin, leading to the induction of diphteria toxin expression in Pax7+ cells and subsequent loss of satellite cells [27]. Both studies demonstrated that elimination of the satellite cells in adult muscle leads to a severely impaired regenerative response following injury. These studies lead to the conclusion that satellite cells are indispensable for muscle repair. It was further demonstrated that vasculature and innervation were not affected by diphteria toxin, and that other progenitors such as PICs and presumably pericytes were still present following satellite cell depletion based on PW1 expression in interstitial cells [28]. Based on these results, it would appear that non-satellite myogenic populations cannot give rise to new muscle fibers under normal physiological conditions. However, several *caveats* must be considered in the interpretation of these experiments. First, it is not clear whether the depletion of satellite cells is a neutral event. Specifically, depletion of satellite cells leads to a decrease in muscle mass in the absence of any apparent injury, thus it is possible that some stress or inflammatory processes disturb the muscle environment prior to injury. Secondly, it is possible that the presence of satellite cells is necessary to recruit non-satellite cell progenitors into the myogenic lineage through physical and/or molecular paracrine interactions. The impact of the removal of any single population on the stem cell niche may be paramount for the understanding of these results.

The cellular neighborhood

Satellite cells grown *in vitro* are capable of proliferating and forming multinucleate myotubes and it is this inherent and stable myogenic property first observed in the 1960's [29, 30] [31] that is primarily responsible for the significant advances made in the field of muscle stem cell biology. While primary satellite cells display pronounced myogenic capacity, even in defined media, their behavior can be modulated dramatically by growth factors, neighboring cells, and the surrounding cellular matrix. It has been shown that even brief periods of satellite cell growth *in vitro* leads to a marked reduction in their myogenic and self-renewal capacity when re-engrafted into damaged muscle as compared to freshly isolated satellite cells [4]. Blau and colleagues demonstrated that a more elastic cell substrate (in contrast to 'hard' plastic) increased primary myoblast self-renewal and regenerative capacity when grafted into injured muscle [32]. In addition to the physical properties of the satellite cell environment, cytokines and other diffusible molecules produced by surrounding cells are important. Christov *et al* [33] observed that satellite cells

are invariably located near small vessels and that endothelial cells promote satellite cell proliferation through IGF-1, FGF, HGF and VEGF secretion. In turn, differentiating myoblasts promote angiogenesis. They proposed that a positive feedback between these two cell types promotes faster and properly patterned tissue repair. Following muscle damage, pro-inflammatory monocytes accumulate at the injury site and clear away cellular debris. This step is followed by a conversion of monocytes into anti-inflammatory macrophages that stimulate myogenesis and fiber growth [34]. Depletion of inflammatory cells completely blocks muscle regeneration, demonstrating their crucial role in muscle repair [34]. In addition, macrophages exert an anti-apoptotic effect on myoblasts through cell adhesion molecules and recruit mesoangioblasts through HMGB1 and MMP9 secretion [35, 36]. Taken together, these data demonstrate vascular-mediated paracrine effects on the stem cell niche that play a key role in coordinating stem cell responses [37, 38]. In addition to the presence of vascular cells, other interstitial cells support muscle regeneration. First, a population of connective tissue fibroblasts that are identified by Tcf4 expression proliferate in close proximity to satellite cells following injury [39]. Conditional ablation of Tcf4+ cells prior to muscle damage leads to smaller regenerated fibers and a decreased population of satellite cells due to premature differentiation, suggesting that fibroblasts provide critical signals during regeneration [39]. This was confirmed by *in vitro* experiments in which myoblasts cultured in the presence of Tcf4+ fibroblasts form larger myotubes containing more nuclei ([40] and see [41]). In turn, the authors showed that satellite cell ablation not only leads to a complete loss of muscle regeneration, as observed by others, but also to a defect in fibroblast recruitment at the onset of regeneration followed by connective tissue expansion [39]. Taken together, these results demonstrate that satellite cells and fibroblasts reciprocally regulate the expansion of each other to ensure efficient muscle repair. It was further proposed that fibroblasts and atypical myogenic stem cells are recruited to the site of injury by factors secreted by satellite cells [42].

In addition to the myogenic and fibroblast populations, two resident adipogenic populations have been recently identified in skeletal muscle. Mesenchymal progenitors are characterized by PDGFRα expression [43], and fibro/adipogenic progenitors (FAPs) have been isolated upon Sca1 expression [44]. These cells display a strong adipogenic potential *in vitro* and differentiate into fat when engrafted into pathological muscle, which is not the case when engrafted into healthy muscle. In addition, FAPs are activated upon injury and

promote myoblasts differentiation through cell-cell signaling [44] whereas adipogenesis of PDGFRα+ cells is strongly inhibited by myotubes [43]. It is tempting to speculate that mesenchymal PDGFRα+ cells and FAPs are overlapping populations, nonetheless, these adipogenic progenitors share the property of adopting different fates depending on the surrounding environment as well as promote differentiation of neighboring myogenic progenitors [44]. The authors propose that a balance between satellite cell-dependent myogenesis and PDGFRα+ cells/FAPs dependent adipogenesis regulates muscle homeostasis and regeneration. Taken together, these data provide a clear demonstration of interactions between multiple resident cell populations that promote activation of muscle progenitors. The de-regulation of any single cell population in muscle tissue is therefore likely to have a strong impact on all the resident populations and therefore must be taken into account when proposing a 'central' role for any single cell type during the regenerative process (Fig.1). Similarly, changes in this local environment may contribute to muscle pathologies and age-related loss of muscle stem cell competence.

Age-related loss of regenerative capacity and the stem cell neighborhood

A progressive loss of stem cell competence occurs with age and is associated with chronic diseases in mammals (Box 2). In diseased and aged muscle, myofibers are replaced by fat and fibrous tissue and the remaining fibers decrease in mass. The number of satellite cells in muscle declines soon after birth from 30% of myonuclei in the neonate to 4% in the adult followed by a small decrease to 2% in the old mouse [45]. It has been demonstrated that satellite cells undergo a decrease in levels of Pax7 expression leading to a loss of myogenicity accompanied by increased levels of apoptosis [46]. However, not all satellite cells appear to undergo this decrease and retain myogenic and self-renewing capacities comparable to that of young satellite cells, suggesting that other factors contribute to age-related changes in muscle regenerative capacity [46] [45, 47]. An aged-related delay in the early inflammatory response following muscle injury was reported in mice, although this did not impair the regeneration process [48]. *In vivo*, satellite cell proliferative and regenerative capacities are restored in old mice when exposed to circulating factors from young mice [49]. In contrast, serum obtained from old mice provokes an 'old age' muscle regenerative phenotype when injected into young mice. This old age-inducing effect has been attributed

to elevated circulating Wnt molecules, activating downstream targets such as Axin2 and β-catenin in aged satellite cells and leading to a conversion to a fibrogenic lineage [50]. Conversely, canonical Wnt signaling inhibition by Frizzled-related protein 3 or DKK1 in old mice restores regenerative potential [50]. Wnt molecules may be secreted by tissue-resident endothelial precursors in old muscle. Specifically, endothelial precursors are activated during neo-angiogenesis following muscle injury and inhibit myoblast proliferation through Wnt3a production [51, 52]. In addition, impaired muscle regeneration in aged animals has also been linked to a decline in Notch signaling [53]. Insufficient up-regulation of the Notch ligand, Delta-1, in satellite cells following injury in old animals leads to a decrease in their myogenic capacities, which can be restored by forced activation of Notch. In turn, *in vivo* inhibition of Notch signaling inhibits muscle regeneration in young mice and results in a phenotype similar to that of old muscle [53]. In addition, Notch signaling has been recently shown to be necessary for satellite cell maintenance, such that satellite cells undergo accelerated terminal differentiation without self-renewal in the absence of Notch, resulting in satellite cell depletion [54]. Loss of Notch activation in aged muscle has been associated with high levels of TGFβ-1 and cyclin-dependent kinase (cdk) inhibitors in satellite cells [55]. Abnormally high levels of pSmad-3 in aged satellite cells impair regenerative capacity, whereas regenerative competence can be restored *in vivo* by pSmad-3 shRNA blockade. Activation of Notch blocks the pSmad-3 mediated up-regulation of the cdks p15, p16, p21 and p27, whereas inhibition of Notch induces these cdks [55, 56]. Therefore, endogenous Notch and pSmad-3 antagonize each other in the control of satellite cell proliferation and deregulation of this balance in old animals is proposed to lead to regenerative potential decrease.

Taken together, these data reveal that the progressive decrease in regenerative capacity during muscle aging is due to a progressive switch from Notch to Wnt/TGF-b molecules secretion in the stem cell environment rather than intrinsic loss of myogenic cells competence.

Can we gain insight from lower vertebrate regeneration?

Fish and amphibians retain the capacity to fully regenerate tissues throughout life, whereas progressive loss of regenerative capacity is common in mammals. Specifically, the adult zebrafish heart can regenerate following ventricular resection [57] while mammals lose

this capacity soon after birth [58]. A striking demonstration of stem cell capacity in lower vertebrates was recently reported in newt lens subjected to 15 rounds of injury over 16 years without any decline in regenerative capacity [59]. Lastly, in addition to cell and organ replacement, amphibians can form a blastema following limb amputation consisting of dedifferentiated mesenchymal cells that will re-pattern and re-differentiate to generate the entire appendage. While limb amputation in amphibians activates resident Pax7+ satellite cells [60], a concomitant dedifferentiation of mature fibers is also observed, which give rise to mononucleated cells accounting for about 30% of the blastema [61]. While dedifferentiation is an extremely rare event in mammals, one recent report suggested that a similar process is occurring in skeletal muscle upon injury through the use of a Cre-Lox-βgal system based on the expression of muscle creatine kinase to specifically tag differentiated multinuclear myofibers [62]. In this study, mononuclear βgal+ cells were detected among which some were also Pax7+ and capable of re-differentiate into myotubes *in vitro* after injury [62]. While the contribution of dedifferentiated myofibers during muscle regeneration remains a subject of scrutiny, this study suggests that myofibers are an additional source of myogenic progenitors for muscle repair.

Understanding mechanisms underlying dedifferentiation promises tremendous therapeutic applications. Blau and colleagues recently demonstrated that committed myoblasts are reprogrammed by inactivating the tumor suppressors Rb and ARF [63]. Upon Rb and p16/19 inactivation, myonuclei re-entered the cell cycle and myotubes dedifferentiated in mononucleated cells. These cells expand under growth conditions and re-differentiate into mature myotubes. Following engraftment, these cells are incorporated into pre-existing fibers [63]. Similarly, Conboy and colleagues showed that inhibitors of tyrosine phosphatases and apoptosis provoke the dedifferentiation of myotubes into myogenic competent mononucleated cells. They obtained 'reprogrammed' muscle progenitors expressing Pax7 and MyoD capable of differentiating into skeletal muscle *in vitro* and *in vivo* [64]. Even if dedifferentiation does not normally occur during mammalian tissue regeneration, the underlying cellular machinery has not been lost during mammalian radiation and can be artificially re-activated. Presumably this loss of cellular plasticity and stem cell competence arose during evolution, implying that the loss of this regenerative capacity carries some unique advantage. Moreover the observation that genes involved in tumor suppression need to be de-activated, at least transiently, in order to provoke this

process in mammals suggests that there may have been an evolutionary trade-off between mechanisms preventing cancer and mechanisms supporting robust regenerative capacities.

Adolescence - a critical period for stem cells?

A pre-programmed adult body size, and by consequence, organ size, is a hallmark of mammals whereas many lower vertebrates display large variations in adult body size and in some cases, postnatal growth is maintained throughout life (reviewed in [65] and [66]). The continuous growth of lower vertebrates is presumably linked to their enhanced regenerative capacities, and recent studies demonstrate that mammals possess an enhanced regenerative capacity during the initial days or weeks after birth. In the case of the heart, which can regenerate in fish but not in mice, it had been recently shown that a partial ventricle resection of the mouse heart performed within the first postnatal day provoked complete regeneration through induction of heart cardiomyocyte proliferation whereas this capacity is lost by one week [58]. In skeletal muscle, genetic evidence highlights the roles of Pax7 in postnatal skeletal muscle growth. Mice constitutively lacking Pax7 display a normal pool of satellite cells upon birth but their number rapidly declines by 2-3 weeks after birth concomitant with poor postnatal growth and survival [2]. Furthermore, these mice show very poor muscle regenerative capacity, which led initially to the proposal that Pax7 is required for satellite cell maintenance and self-renewal [67] [2]. However, in the context of a conditional Pax7 mutant, it was found that Pax7 was dispensable for satellite cell function when inactivated specifically in the adult [68]. In contrast, when Pax7 was inactivated between birth and 3 weeks of age, postnatal muscle development and regenerative capacity was abrogated, with the most severe phenotypes corresponding to ablating Pax7 expression during the first week of life [68]. Taken together, these results demonstrate that there is a critical period near 3 weeks of age when the muscle stem cell niche undergoes a critical change from ‘juvenile’ to adult. Whether this change is due to progenitors entering a more stable quiescent state or some other unidentified event including changes in the stem cell neighborhood remains to be determined.

Genomic imprinting - a mammalian feature of stem cells

Recent studies have implicated parentally imprinted genes in the regulation of postnatal growth and adult somatic stem cells. Parental imprinting is an epigenetic control specific to mammals that involves the silencing of one allele and occurs in less than 1% of the

human and mouse genome (reviewed in [69] and [70]). Several parentally imprinted genes regulate embryonic development [71, 72]. As a group, they are expressed at high levels in fetal and newborn tissues and decline within the first few weeks after birth in mice [73, 74]. Our laboratory identified the parentally imprinted gene, PW1/Peg3 (paternally expressed gene 3), in a screen designed to isolate early stem cell markers in the skeletal muscle lineage [75]. Subsequent analyses revealed PW1/Peg3 expression was restricted to stem cells in all adult tissues examined, including the skin, blood, bone, testis and the CNS [76]. The expression of a 'universal' adult stem cell marker lends support to the idea that common regulatory mechanisms underlie adult stem cell function regardless of tissue origin, a notion that has been proposed for many years, but which has previously lacked much supporting data [77]. Our findings were followed by the discovery by Goodell and colleagues that a group of parentally imprinted genes including p57, H19, Mest, Peg3/PW1, Dlk1 and IGF2 are expressed in adult somatic stem cells but not in their differentiated progeny [74]. In addition, two groups have recently demonstrated that p57, a well known regulator of the cell cycle, plays a role in maintaining the hematopoietic stem cell pool [78, 79]. Similarly, Dlk1 was shown to be important for skeletal muscle growth and differentiation [80], and neurogenic progression [81]. Moreover these authors demonstrated that neural stem cells selectively undergo a 'relaxation of imprinting' during postnatal development that is required for proper neurogenesis [81]. This finding raises the interesting possibility that bi-allelic expression of imprinted genes may constitute an important regulatory event during stem cell progression and differentiation. As most but not all parentally imprinted genes are shared among all vertebrates, thus it will be of interest to determine whether the same set of genes are expressed in lower vertebrate stem cells or whether this set of imprinted genes is specific to mammalian stem cells.

Future perspectives and concluding remarks

Muscle growth and maintenance during the lifetime of an animal reveals at least three distinct stages after birth (juvenile, adult, and old age) during which stem cells change in behavior and competence. The juvenile period in mice (0 to 3 weeks) is characterized by pronounced growth and a marked increase in muscle mass and size. In this context, stem cells are required to build the muscle in young mice. As stated above, the switch from a Pax7 dependent to Pax7 independent state at 3 weeks old coinciding with a decline in the rate of

postnatal growth provides direct genetic evidence that the stem cells undergo a change in their genetic circuitry during this transition, likely reflecting a change in their role. This change may also reflect the requirement for Pax7 for the myogenicity of PICs during postnatal growth [17]. In the context of muscular dystrophies, this raises the question as to whether it is possible to develop therapeutics that can induce or maintain juvenile stem cells that may represent a more competent and plastic stem cell population as compared to adult stem cells. Whether imprinted gene regulation or tumor suppressor genes underlie the 'more limited' potentials of mammalian stem cells as opposed to those in lower vertebrates may also offer potential therapeutic solutions. Why do mammals and lower vertebrates differ in their regenerative potential is now addressable with the advent of high-throughput 'omic' approaches. Determining how the niche changes, which signals are present during each of the postnatal stages, and what role other cells in the niche play will provide targets for therapeutic development that promise to be more tractable than direct stem cell engraftment. As such, the field of adult stem cells is entering a larger evolutionary context. Should we seek to induce amphibian-like characteristics in mammalian stem cells, we will need to establish a clearer understanding of what distinguishes juvenile postnatal tissue from adult and old tissue as well as fully appreciate why mammals have evolutionarily acquired this more limited potential and what Faustian bargain may have been made along the way.

Acknowledgements

We gratefully thank Drs V. Besson and K. Bismuth for fruitful discussions and critical reading of the manuscript. This work was supported by the French Ministry of Research 'Chaire d'Excellence' and the Muscular Dystrophy Association of America to D.S. and the European Community Seventh Framework Program projects OPTISTEM (Optimization of stem cell therapy for degenerative epithelial and muscle diseases contract number Health-F5-2009-223098) and ENDOSTEM (Activation of vasculature associated stem cells and muscle stem cells for the repair and maintenance of muscle tissue-agreement number 241440). The Myology Group is the beneficiary of a Strategic Plan Support from the Association Française contre les Myopathies (AFM) and is affiliated with the Institute of Myology. A.P. was supported by the French Ministry of Research and the Fondation pour la Recherche Médicale

(Programme “Espoirs de la Recherche”, number FDT20110922527). The authors have no conflict of interest.

Figure Legends

Figure 1. The muscle neighborhood

Skeletal muscle is composed of myofibers, containing myonuclei, and satellite cells (white) that reside beneath the basal lamina and constitute the major muscle stem cell population (Box 1). Blood vessels, composed of endothelial cells, permeate the interstitial space of the muscle fibers and in addition to providing a blood supply, the endothelial cells promote satellite cell proliferation through growth factors secretion and deliver circulating inflammatory cells. Pericytes (purple) actively contribute to postnatal muscle growth and regeneration. The interstitial space is occupied by mesenchymal progenitors (green) as well as connective tissue cells (orange).

Figure 2. Dynamics of stem cell competence with age

At birth, high stem cell numbers are followed by a dramatic decrease within the first days and/or weeks of life depending on the tissue. In skeletal muscle, this juvenile period is characterized by rapid postnatal growth and the presence of a variety of stem cells that are highly plastic and able to contribute to multiple tissue lineages [12, 17, 43]. In the mouse, Pax7 is required for both postnatal muscle growth and regeneration during the first 3 weeks of life [2, 67, 68]. After 3 weeks, regeneration can occur in the absence of Pax7 [68]. During adult life, stem cell number is maintained however their capacity to repair muscle (“stem cell competence”) continues to decrease due to an increase in inhibitory factors in their environment, until about 18 months at which point regenerative capacity is compromised. An additional decline in stem cell competence occurs in aged muscle. Taken together, these results reveal 3 distinct genetic and cellular phases of postnatal skeletal muscle stem cell behavior: a ‘juvenile phase’ (0-3 weeks), adult phase (3weeks to ~18 months), and an ‘aged’ phase (18 months – end of life).

Box1. The satellite cell

Alexander Mauro first identified satellite cells based upon their anatomical location between the basal lamina and the myofiber plasma membrane. Based on this location 'satellite' to the myofiber, he proposed that these cells were resident skeletal muscle progenitors [1]. Since their discovery, extensive effort has been made to understand the origin and role of satellite cells. Satellite cells originate from Pax3-expressing progenitors in the somites of the embryo that migrate to the limb bud where they subsequently up-regulating Pax7 and other myogenic regulatory factors [82, 83]. While the majority of these progenitors undergo differentiation and form primitive nascent myofibers, constituting the basis for the formation of additional muscle during postnatal growth, a small subset of these cells adopt a satellite cell position [5]. In adult muscle, satellite cells express Pax7 and remain quiescent under normal physiological conditions. Following muscle damage, satellite cells re-enter the cell cycle and generate myoblasts that eventually fuse together or with damaged fibers [84]. During this process, satellite cells are replaced by self-renewal through asymmetric division of a small fraction of the satellite cell pool [85]. The satellite cell was a focus for therapeutic applications for muscle diseases and led to several clinical trials in the 1990s however, their poor survival, migration and inability to undergo sufficient self-renewal following engraftment has led the field to seek alternative approaches (reviewed in [86]).

Box 2. Muscle diseases

Muscle diseases are primarily congenital including most notably muscular dystrophies (Duchenne, Emery-Dreyfus, Limb-Girdle), centronuclear myopathies and neuromuscular diseases (Amyotrophic Lateral Sclerosis, Spinal Muscular Atrophy). Muscular dystrophies are characterized by progressive muscle weakness due to mutations in the sarcolemmal proteins (dystrophin, sarcoglycans, dysferlin), nuclear proteins (Emerin, lamin A⁄C) or extracellular proteins (collagen-6, alpha-2 laminin). Although clinical symptoms are well described, no treatment has yet been fully successful.

Most muscular dystrophies involve an eventual exhaustion of the stem cell pool or a change in their fate in which the muscle tissue becomes infiltrated with fibrotic and fat tissue. This

409 evolution is similar to sarcopenia that occurs during physiological aging involving a gradual
410 loss of muscle mass and a decline in regenerative capacity.

411 In the *mdx* mouse model, the satellite cell population is reduced as compared to healthy
412 mice and becomes depleted following repeated cycles of degeneration/regeneration. These
413 repeated cycles of stem cell activation and proliferation leads to telomere shortening [87]
414 and has been proposed to lead to the accumulation of mutations in key regulatory genes
415 required for proper self-renewal and myogenic competence. Therefore while DMD disease
416 progression is driven by dystrophin deficiency, the pathology exacerbates as a result of stem
417 cell dysfunction. In this context, therapies designed to promote stem cell competence may
418 ultimately slow down disease progression.

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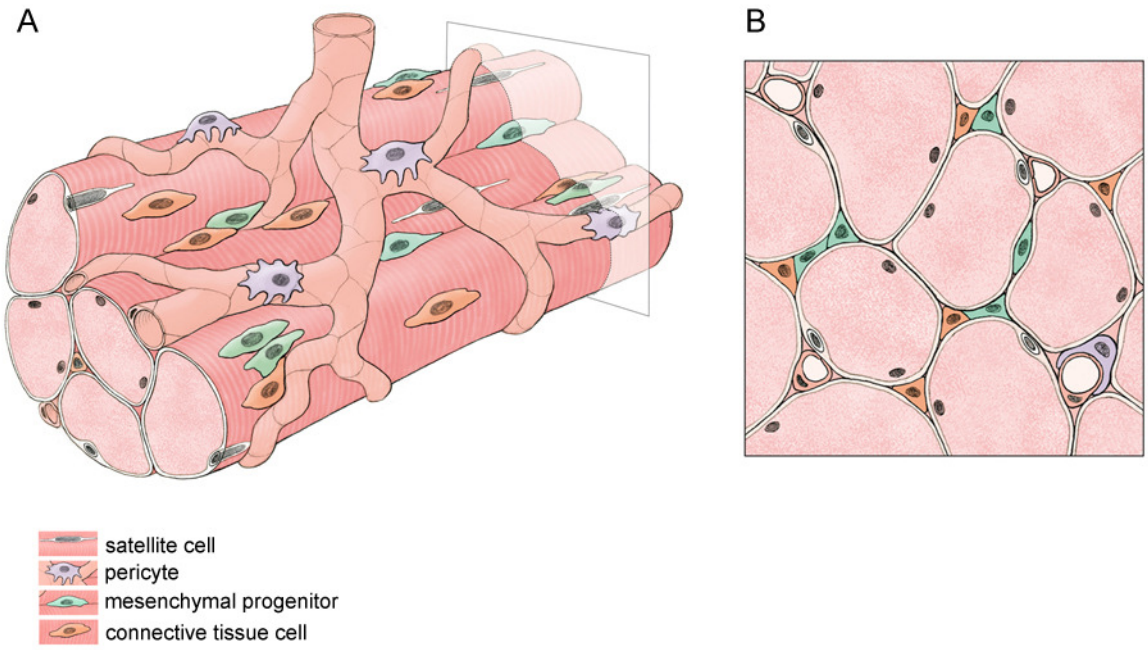
Figure 1


A
B
satellite cell
pericyte
mesenchymal progenitor
connective tissue cell

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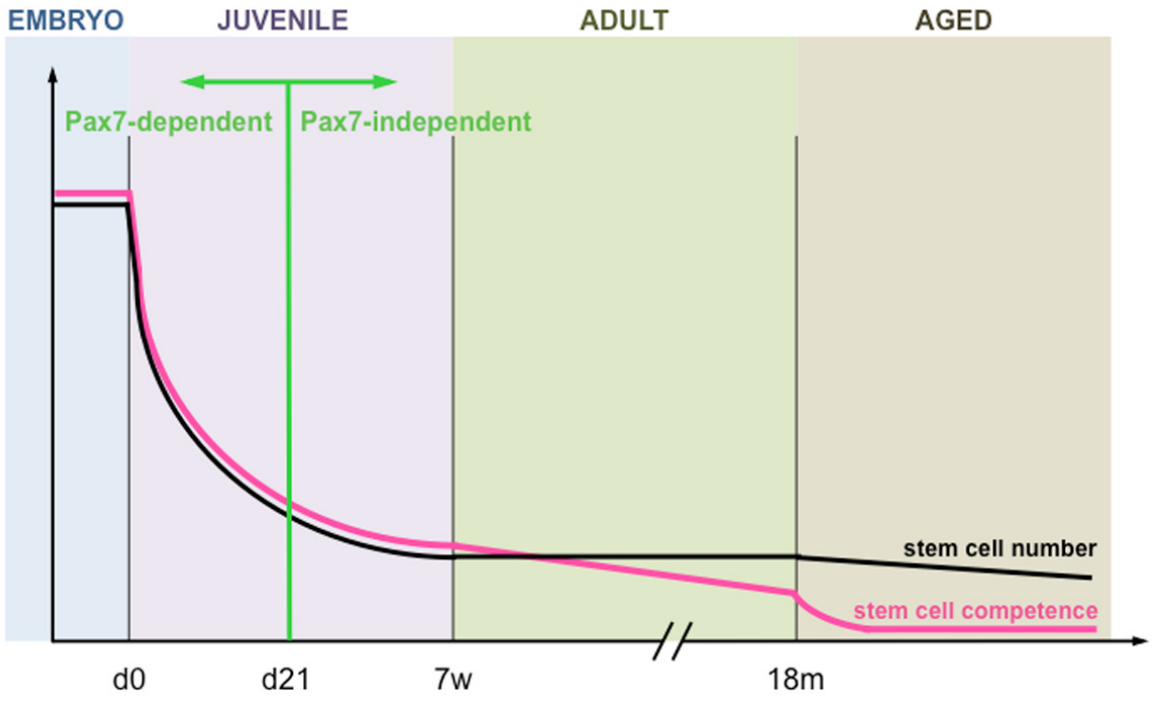
607 **Figure 2**



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