Thèse n°7366

EPFL

A single-cell-based identification and characterisation of Aregs, an inhibitory subpopulation of adipose stem and precursor cells

Présentée le 26 juin 2020

à la Faculté des sciences de la vie Unité du Prof. Deplancke Programme doctoral en approches moléculaires du vivant

pour l'obtention du grade de Docteur ès Sciences

par

Magda ZACHARA

Acceptée sur proposition du jury

Prof. M. De Palma, président du jury Prof. B. Deplancke, directeur de thèse Prof. T. Petrova, rapporteuse Dr Y. Ravussin, rapporteur Prof. C. Wolfrum, rapporteur

 École polytechnique fédérale de Lausanne

2020

Title: A single-cell-based identification and characterisation of Aregs, an inhibitory subpopulation of adipose stem and precursor cells

Name: Magda Zachara SCIPER: 254220

Submitted: 27.04.2020 Final edit: 19.06.2020

Place: Lausanne

Date of exam: 29.05.2020

Thesis Committee:

Thesis Director: Prof. Bart Deplancke, EPFL, Lausanne, Switzerland **President:** Prof. Michele De Palma, EPFL, Lausanne, Switzerland

Panel Experts:

Internal: Prof. Christian Wolfrum, ETHZ, Zurich, Switzerland External: Prof. Tatiana Petrova, UNIL, Lausanne, Switzerland External: Prof. Yann Ravussin, UNIFR, Fribourg, Switzerland

University: École Polytechnique Fédérale de Lausanne (EPFL)
 Institute: Institute of Bioengineering
 Laboratory: Laboratory of Systems Biology and Genetics
 Doctoral Programme: Molecular Life Sciences (EDMS)

In theory, there is no difference between theory and practice. In practice there is. Yogi Berra

> There's a discipline for passion. Stefani Germanotta

Ino nie ustawaj, ino rób dalej. Hanna Jonsson

Acknowledgements

I vividly remember that 9th of April 2015, when Bart and I had our first chat over a cup of tea at the big round table by the window at the SV café. It's gone by so fast! Bart, thank you so much for taking me on, this PhD has been a truly wonderful adventure for me and it has been so thanks to what you had created in your lab. Thank you for always being there ready to mentor, motivate and support. Thank you for your trust and generosity. I am not the first one to say this: your enthusiasm really makes us do things we didn't think we could do. Petra, you were such a lucky strike for me, honestly! I absolutely loved the time we spent together working and not, and how we complemented each other. I think Aregs are a child of that match. I never stopped missing you once you left. Julie, I have no words to thank you for all the help and for supporting all my paranoias. You have been such a fantastic thing for me in this lab. Very few days went by without chats, tea and lots of chocolate, of course! Ah, and thank you for showing me the world of cells. Turned out pretty essential ;-) Wanze, you introduced me to the lab. Thank you for your kindness at the beginning and all the way through my PhD. Your presence and advice have always been so valuable to me. Daniel, we have talked a lot! Thank you for always really listening and really trying to understand. You have been a big support for me. Radiana, I still remember you coming to the lab all these years ago and listening to my ramblings. Well, you still do. You have been a lovely and bright part of my PhD. And thank you for spoiling me with all the delicious Italian food. Masha, you are a queen of so many things! Cakes. Statistics. Massages. Plots. I count myself extremely lucky to have been at the receiving end of all the above. Thank you ever so much for the unforgettable wedding cake! You will forever remain in our photo album. Roel, you felt a friendly soul on day one. And so it remained. Thank you for all the laughter. Keep it going, wherever you go! Pernille, you are such a sunshine of the lab. Thank you for being such a patient (beginner's) R teacher and for all the single-cell hocus pocus! Jörn, thank you for all the morning chats and trying to pass on me a lot of your wisdom about life and science. You seem so passionate about both. AK, with you at Nestlé, I feel we never had enough time together. Thank you for the mutli-topical lunches we did managed to juggle in every now and again. Gerard, they say I am an old soul, but I think you are an even older one. I love that about you! Keep remembering what's really important. And see you in the next life! Vincent, another great teacher! You will never know how much I regret we only ever got to lesson three of bioinformatics. I have always been impressed by your patience and peacefulness while sharing knowledge and skill. Marjan, you are such a generous angel of the lab. Thank you for always being aware and carrying. And for feeding us. Michael, you have been a great colleague. We could chat about anything, and the task would do itself in no time. Johannes, my German neighbour, 3D printers and tape will always make me think of you. Riccardo, thank you for taking me home that day when I thought it was coming down in flames. To all the lab guys: thanks for performing all the amazing sessions of that game I can never remember the name of. I just loved all the noises you were making. Magali, you were so versatile, you could do anything and chat about anything! Thank you for taking care of us. Aurelia, you were an unexpected addition to the lab! Thank you for bringing so much insight into what you are passionate about. Sachi and Judith, we come, we go. This last year has been so busy for me that I did not have time to get to know you as well as I would have liked. I have noticed though what extremely high-quality additions to the lab you both are. I would also like to thank Horia, who was brought to our lab by destiny and worked with me during the last phases of my thesis. You were a brilliant Master student and you helped me a lot. It looks like destiny will be bringing you back to take over Aregs. Best of luck!

Apart from our lab, I have been so extremely lucky to have had countless kindred souls, close by and far away, sharing this path with me personally. Amongst them, there are my Mum and Dad. Thank you for understanding why I wanted to do this and for supporting me in it all the way through and helping however you could. Thanks for all the 5G Mum! ;-) Dad, thank you so much for all the amazing graphic and editorial help all through my PhD and especially at the end - it was a lovely time together! Francesca, we have been chasing something big at the same time. Thank you for understanding that I was not always there for you recently and for going "microtubules" whenever any of us needed it. I wish you heaps of luck and joy doing your thing now! Kip, my wonderful Husband, as cliché as it might sound, you were truly amazing throughout this process, I am completely blown away by your angelic patience, understanding and support. Thank you <3

The work presented below would not have been possible without an extraordinary structure that EPFL represents and the People who create it. By this, I would like to acknowledge and thank numerous people who helped me in the process of my PhD. Whether in a Facility, as a Collaborator, a helpful Colleague or Friend over a coffee, thank you to all who contributed to my being able to present this work.

Finally, I would like to thank my Thesis Committee, Prof. Bart Deplancke, Prof. Michele De Palma, Prof. Christian Wolfrum, Prof. Tatiana Petrova and Prof. Yann Ravussin. I really appreciate the time you spent on my thesis and your feedback. Thank you for the discussion during my defence, it shall remain a very nice memory of the end of my doctoral adventure.

Trélex, 19th June 2020

Abstract

Adipose tissue is an essential element in energy conservation mechanisms. Its unique plasticity is driven by the ability of adipocytes to accumulate and liberate lipids as a function of the energetic status of the organism. Given the recent rise in the global incidence of obesity, there is great interest in understanding the mechanisms behind disturbed energy balance. However, the heterogeneity of adipose tissue and of the somatic stem cells giving rise to mature adipocytes, makes it extremely challenging to characterise the cellular and molecular identity of fat depots. Single-cell RNA sequencing has recently enabled ground-breaking insights into the composition of complex cell populations. Our single-cell-based dissection of adipogenic precursors revealed the existence of distinct cell subpopulations within the murine subcutaneous fat depot. We demonstrated that one of these populations, characterised by a high expression of F3 gene (encoding CD142), showed a completely non-adipogenic phenotype. Moreover, it revealed to be regulatory towards other adipose stem and precursor cells by suppressing their ability to form adipocytes in a paracrine manner. These adipogenic regulatory cells, which we termed Aregs, proved to maintain their inhibitory properties in vivo and were shown phenotypically conserved in humans. We next established the robustness of Aregs as a novel cell sub-type in the context of various isolation strategies, the strength of the adipogenic cue and sex-based differences. Interestingly, we observed that Aregs isolated from the burgeoning subcutaneous depots of new-born mice had high adipogenic propensity, suggesting that the appearance of classical phenotypic and functional properties of Aregs is development-dependent.

In the light of the considerable implications of Aregs in adipose tissue composition and plasticity and, subsequently metabolic health, it is critical to understand the mechanism of their inhibitory nature. Integration of transcriptomic and proteomic datasets allowed us to identify a comprehensive set of highly specific candidates, which we validated in the context of Aregs' identity and function. Our findings revealed a few potentially involved molecular actors. These include secreted factors CD142, GDF10 and MGP, which proved to be directly capable of inhibiting adipogenesis of Areg-depleted adipose stem and precursor cells, and transcription factors PKNOX2 and MEOX2, whose inactivation in Aregs compromised their ability to inhibit adipogenesis of co-cultured differentiating preadipocytes. Collectively, these molecules point to a potential functional relationship of Aregs with vasculature as well as evoke a plausible association of Aregs' phenotype to visceral-like non-adipogenic properties. We are now integrating these findings and completing them by investigating the transcriptional regulation and signalling pathways underlying the elusive mechanism of Aregs' activity.

8

Keywords

Adipose tissue, adipogenesis, adipose stem and precursor cells (ASPCs), Aregs, single-cell RNA sequencing, transcriptomics, cell culture, microscopy

Résumé

Le tissu adipeux est un élément essentiel des mécanismes de conservation de l'énergie. Sa plasticité unique repose sur la capacité des adipocytes à accumuler et à libérer des lipides en fonction de l'état énergétique de l'organisme. Étant donné la récente augmentation du taux d'obésité dans le monde il est très important de comprendre les mécanismes à l'origine des perturbations de l'équilibre énergétique. Cependant, l'hétérogénéité du tissu adipeux et des cellules souches somatiques donnant naissance aux adipocytes matures, rend la caractérisation de l'identité cellulaire et moléculaire des dépôts de graisse extrêmement difficile. Le séquençage de l'ARN à cellule unique a récemment permis d'obtenir des informations inédites sur la composition de populations cellulaires complexes. Notre dissection des précurseurs adipogéniques en utilisant cette méthode, a révélé l'existence de sous-populations cellulaires distinctes au sein du dépôt de graisse sous-cutané murin. Nous avons démontré que l'une de ces populations, caractérisée par une forte expression du gène F3 (codant pour CD142), présentait un phénotype entièrement non adipeux. De plus, elle s'est révélée régulatrice vis-à-vis d'autres cellules souches adipeuses et précurseurs en supprimant leur capacité à former des adipocytes de manière paracrine. Ces cellules régulatrices adipogènes, que nous avons appelées Aregs, ont prouvé qu'elles conservaient leurs propriétés inhibitrices in vivo et se sont révélées phénotypiquement conservées chez l'homme. Nous avons ensuite établi la robustesse des Aregs en tant que nouveau sous-type cellulaire grâce à de diverses stratégies d'isolement, la force du stimulus adipogène et les différences basées sur le sexe. Il est intéressant de noter que les Aregs isolées des dépôts sous-cutanés de souris nouveau-nées ont un fort potentiel adipogénique, ce qui suggère que l'apparition des propriétés phénotypiques et fonctionnelles classiques des Aregs dépend du développement.

Au vu des implications considérables des Aregs dans la composition et la plasticité du tissu adipeux et, par conséquent, dans la santé métabolique, il est essentiel de comprendre le mécanisme de leur nature inhibitrice. L'intégration des ensembles de données transcriptomiques et protéomiques nous ont permis d'identifier un groupe de candidats spécifiques, que nous avons validés dans le contexte de l'identité et de la fonction des Aregs. Nos résultats ont révélé quelques acteurs moléculaires potentiellement impliqués. Il s'agit notamment des facteurs sécrétés CD142, GDF10 et MGP, qul se sont révélés directement capables d'inhiber l'adipogenèse des cellules souches adipeuses et des précurseurs dépourvu d'Aregs, ainsi que des facteurs de transcription PKNOX2 et MEOX2, dont l'inactivation dans les Aregs a compromis leur capacité à inhiber l'adipogenèse des pré-adipocytes différenciés en co-culture. Ensemble, ces molécules mettent en évidence une relation fonctionnelle potentielle entre Aregs et le système vasculaire évoquant une association plausible entre le phénotype d'Aregs et des propriétés non adipogénique de type viscéral. Nous intégrons maintenant ces résultats et les complétons en étudiant la régulation transcriptionnelle et les voies de signalisation qui sous-tendent le mécanisme insaisissable de l'activité des Aregs.

Mots-clés

Tissue adipeux, adipogenèse, cellules souches et précurseurs adipeuses, Aregs, le séquençage de l'ARN à cellule unique, transcriptomique, culture cellulaire, microscopie

Contents

| | Ack | nowled | dgements | 5 | | | |
|--------|-----------------------|--------------------|---|----|--|--|--|
| | Abs | tract . | | 7 | | | |
| | Key | words | | 8 | | | |
| | Résumé | | | | | | |
| | Mots-clés Contents | | | | | | |
| | | | | | | | |
| | List | of Figu | ıres | 13 | | | |
| | List | of Tabl | es | 14 | | | |
| | List | of Sup | plementary Figures | | | | |
| | | 0. 0 dip | | | | | |
| Chapt | er 1 | | | | | | |
| Introd | luctio | on | | 1/ | | | |
| | 1.1 | Energ | y homeostasis | | | | |
| | | 1.1.1 | Adipose tissue as a centre of energy conservation mechanisms | | | | |
| | | 1.1.2 | Adipose tissue expansion mechanisms | | | | |
| | | 1.1.3 | Disturbed energy balance and obesity | 22 | | | |
| | 1.2 | 1.2 Adipose tissue | | | | | |
| | | 1.2.1 | Physiological role and types of adipose tissue | 22 | | | |
| | | 1.2.2 | Brown and beige adipose tissues | 23 | | | |
| | | 1.2.3 | White adipose tissue | | | | |
| | | 1.2.4 | White adipose tissue as an endocrine organ | | | | |
| | | 1.2.5 | Visceral and subcutaneous adipose tissue in health and disease | 30 | | | |
| | 1.3 | Adipo | se tissue development and adipose progenitors | 32 | | | |
| | | 1.3.1 | Adipose tissue composition | 32 | | | |
| | | 1.3.2 | Molecular mechanisms of adipogenesis | 33 | | | |
| | | 1.3.3 | White adipose precursors | 34 | | | |
| | | 1.3.4 | Adipose precursor markers | 35 | | | |
| | | 1.3.5 | Subcutaneous <i>versus</i> visceral ASPCs | | | | |
| | | 1.3.6 | The undissected heterogeneity of adipose stem and precursor cells | | | | |
| | 1.4 | Metho | odology | 39 | | | |
| | | 1.4.1 | Single-cell RNA sequencing | | | | |
| | | 1.4.2 | Adipose progenitors' single-cell RNA sequencing | 40 | | | |
| | | 1.4.3 | In vitro model of adipogenesis | | | | |
| | 1.5 | The so | cope of this thesis | 42 | | | |

| 2.1 Introduction. .47 2.2 Results .47 2.2.1 ScRNA-seq reveals the heterogeneity of subcutaneous ASPCs in the mouse .47 2.2.2 Aregs show a paracrine adipogenic inhibitory capacity .50 2.2.3 Aregs and their inhibitory capacity are conserved in humans. .52 2.2.4 Aregs locate proximal to the vasculature and inhibit adipogenesis <i>in vivo</i> . .53 2.3 Conclusion .55 |
|---|
| 2.2 Results |
| 2.2.1 ScRNA-seq reveals the heterogeneity of subcutaneous ASPCs in the mouse |
| 2.2.2 Aregs show a paracrine adipogenic inhibitory capacity |
| 2.2.3 Aregs and their inhibitory capacity are conserved in humans |
| 2.2.4 Aregs locate proximal to the vasculature and inhibit adipogenesis <i>in vivo</i> |
| 23 Conclusion 55 |
| 2.5 Conclusion |
| 2.4 Miscellaneous |
| 2.5 Supplementary Figures |
| Chapter 3 In-depth characterisation of Aregs and first insights into the mechanism of their activity71 |
| 3.1 Introduction |
| 3.2 Results |
| 3.2.1 Aregs defined by different anti-CD124 antibodies present a robust phenotype74 |
| 3.2.2 Aregs isolated with different strategies and treated with various differentiation cocktails |
| remain non-adipogenic |
| 3.2.3 Sex-based differences in the context of ASPCs and Aregs |
| 3.2.4 Aregs derived from new-born mice give rise to adipocytes <i>in vitro</i> |
| 3.2.5 Genomic data integration |
| 3.2.6 Proteomic analysis of Aregs |
| 3.2.7 Recombinant CD142 and GDF10 inhibit <i>in vitro</i> adipogenesis |
| 3.2.8 siRNA-mediated knockdown of specific genes alters the phenotype and activity of Aregs . 90 |
| 3.3 Conclusion |
| 3.4 Miscellaneous |
| 3.5 Tables |
| 3.6 Supplementary Figures |
| Chapter 4 |
| Discussion |
| 4.1 The discovery of Areas, a novel cell sub-type 113 |
| 4.2 In search of Areas' mechanism |
| 4.3 Areas' role in biology |
| |
| Chapter ک Methods |
| Bibliography141 |
| Curriculum Vitae |

List of Figures

| Figure 1.1 | Principles of energy homeostasis |
|------------|---|
| Figure 1.2 | Adipose tissue expansion mechanisms: hyperplasia versus hypertrophy21 |
| Figure 1.3 | Distinct developmental origins of white, brown and beige adipocytes 25 |
| Figure 1.4 | Factors promoting development and activity of brown and beige adipocytes27 |
| Figure 1.5 | Obese subcutaneous and visceral white adipose tissue |
| Figure 1.6 | Molecular mechanisms of adipogenic differentiation |
| Figure 1.7 | Single-cell RNA sequencing |
| Figure 2.1 | ScRNA-seq reveals the heterogeneity of subcutaneous ASPCs in the mouse |
| Figure 2.2 | Aregs (CD142+ABCG1+ ASPCs) show a paracrine adipogenic inhibitory capacity51 |
| Figure 2.3 | Aregs and their inhibitory capacity are conserved in humans |
| Figure 2.4 | Aregs locate proximal to the vasculature and inhibit adipogenesis in vivo |
| Figure 3.1 | Aregs sorted with different antibodies present a robust non-adipogenic phenotype \dots 76 |
| Figure 3.2 | Aregs present a robust non-adipogenic phenotype upon various adipogenic cues78 |
| Figure 3.3 | Male and female Aregs are phenotypically and functionally similar |
| Figure 3.4 | Murine pups-derived Aregs show a high adipogenic propensity |
| Figure 3.5 | Single-cell- and bulk RNA-seq-based Areg-specific candidate genes |
| Figure 3.6 | Mass-spectrometry confirms many Areg-specific candidate genes |
| Figure 3.7 | CD142 and GDF10 recombinant proteins inhibit <i>in vitro</i> adipogenesis |
| Figure 3.8 | siRNA-mediated knockdown of specific genes alters the phenotype and activity of Aregs |

List of Tables

| Table 3.1 | Comparison of experimental strategies between Schwalie et al. and Merrick et al 96 |
|-----------|---|
| Table 3.2 | Genes overlapping between the top 200 P3 (C1) and the top 200 G3 (10x) hits97 |
| Table 3.3 | Genes overlapping between the top 200 P3 (C1), the top 200 G3 (10x) hits and the top 200 differentially expressed genes in Aregs vs. CD142– ASPCs in bulk RNA-seq |
| Table 3.4 | Aregs' proteomic hits overlap with the top 200 P3 (C1) genes |
| Table 3.5 | Aregs' proteomic hits overlap with the top 200 G3 (10x) genes 100 |
| Table 3.6 | Aregs' proteomic hits overlap with the top 200 bulk RNA-seq-derived DE Areg-specific genes |

List of Supplementary Figures

| Supplementary Figure 2.1 | ScRNA-seq reveals the heterogeneity of ASPCs (Fluidigm C1) |
|--------------------------|---|
| Supplementary Figure 2.2 | ScRNA-seq reveals the heterogeneity of ASPCs (10x Genomics) |
| Supplementary Figure 2.3 | Three ASPC subpopulations with distinct adipogenic differentiation capacity |
| Supplementary Figure 2.4 | Aregs (CD142+ABCG1+ ASPCs) show adipogenic inhibitory capacity 63 |
| Supplementary Figure 2.5 | The adipogenic inhibitory capacity of Aregs is paracrine |
| Supplementary Figure 2.6 | Aregs and their inhibitory capacity are conserved in humans |
| Supplementary Figure 2.7 | Aregs locate proximal to the vasculature and inhibit adipogenesis <i>in vivo</i> |
| Supplementary Figure 2.8 | Supplementary methods for the scRNA-seq analyses |
| Supplementary Figure 3.1 | Aregs show a bigger size than the total ASPCs and CD142– ASPCs \ldots 102 |
| Supplementary Figure 3.2 | Differentiation and proliferation of ASPCs with various adipogenic cues and culture media |
| Supplementary Figure 3.3 | Pups- and adult-derived ASPCs present phenotypic differences 104 |
| Supplementary Figure 3.4 | Pups- and adult-derived ASPCs show different proliferation rates 105 |
| Supplementary Figure 3.5 | Single-cell and bulk RNA-seq-based Aregs-specific candidate genes 106 |
| Supplementary Figure 3.6 | Mass-spectrometry confirms most Areg-specific candidate genes 107 |
| Supplementary Figure 3.7 | CD142 and GDF10 recombinant proteins inhibit in vitro adipogenesis . 108 |
| Supplementary Figure 3.8 | siRNA-mediated knockdown of specific genes alters the phenotype and activity of Aregs |

Chapter 1 Introduction

Note: The brown adipose tissue section of this chapter is based on the published article: "A systems perspective on brown adipogenesis and metabolic activation. 2017, Obesity Reviews (18):65–81"

Authors: Rachana Pradhan*, Magda Zachara,* & Bart Deplancke *These authors contributed equally: Rachana Pradhan and Magda Zachara.

Contributions of Magda Zachara: Wrote article sections 1.1 (Metabolic activity of brown adipose tissue), 1.2 (Known therapeutic interventions in humans), 1.3 (Secreted factors for brown adipose tissue recruitment) and prepared Figure 1.1 (Schematic representation of factors that promote development and activity of brown and beige adipocytes).

1.1 Energy homeostasis

1.1.1 Adipose tissue as a centre of energy conservation mechanisms

Energy homeostasis mechanisms evolved in order to ensure continuous availability of energetic supplies in the changing environment and constitute a crucial determinant of survival of all species (Hwang and Kim, 2019; Spiegelman and Flier, 1996). In higher eukaryotes, including mammals, these mechanisms are a complex interplay of various physiological and anatomical systems in response to the internal energy status and external conditions. They involve major regulatory inputs from the nervous system dictating behavioural responses but also highly regulated capacity of storing and releasing energy in a form of triglycerides, with Gibb's free energy ultimately governing the order and direction of all cellular metabolic processes (Fig. 1.1, Spiegelman and Flier, 1996; Vergara et al., 2019). In vertebrates, the majority of calories are stored in the form of lipid droplets inside specialised cells recognised as adipocytes (Ottavianl et al., 2011; Rosen and Spiegelman, 2014). Within adipocytes, specific physiological mechanisms exist allowing stabilisation of adipose energy stores during extended periods of limited or abundant nutritional availability. The rationale of this remarkable system implies a set of simple, yet robust rules: during extended nutritional deprivation, diminishing energy stores are sensed, which leads to decreased energy expenditure and enhanced food-seeking behaviours, while continued nutritional abundance causes increased energy expenditure and reduced food intake (Spiegelman and Flier, 1996). In order for such a system to work in equilibrium and avoid unbalanced body weight by either allowing excessive expenditure resulting in starvation and underweight or favouring excessive energy storage leading to overnutrition and obesity, it must be tightly regulated. The body weight regulation initially relies on the ability of adipocytes to readily release and store the energy as a function of energetic status of the organism. This regulation constitutes a complex integration of genetic, physiological and behavioural factors, which are communicated through a myriad of transcriptional, metabolic and neural cues (Dulloo, 2008). Several levels of signal transmission are required for such a system to effectively control the whole-body homeostasis. First of all, the information of the energy store status in adipose tissue must be sensed and conveyed to regulatory sites of the organism being able to recognise such a signal (Spiegelman and Flier, 1996). This information can travel in a form of secreted factors, including adipokines, cytokines, hormones and neural transmitters exerting endocrine regulation on the responsive organs and the whole-body



Figure 1.1 Principles of energy homeostasis

A scheme depicting transformation of energy from food to heat through the organism, considering energy losses. Intrinsic or extrinsic disequilibrium within this system results in perturbing the physiological and anatomical state of the organism (adapted form Dulloo et al., 2010).

energy homeostasis (Meissburger et al., 2016; Rosen and Spiegelman, 2014). The second requirement is the recognition and uptake of these cues by regulatory sites, which implies the existence of specific receptors within the receiving structures. One of such organs is hypothalamus being capable of assimilating signals generated by adipose tissue (Chen et al., 1996; Lee et al., 1996). Finally, the third condition of an effective regulation of energy homeostasis is a mechanism capable of integrating signals from periphery as well as higher brain centres, and responding in the appropriate manner to the integrated cues, modulating behavioural and physiological phenomena, that is, regulating the energy intake *versus* energy expenditure by applying a multitude of mechanisms including, for example, hunger and satiety (Spiegelman and Flier, 1996).

1.1.2 Adipose tissue expansion mechanisms

With the particular role the adipose tissue has to play, comes its remarkable plasticity and capacity to change dimensions. It is indeed the only non-neoplastic tissue with such expansion capacity (Krotkiewski et al., 1983; Rosen and Spiegelman, 2014). Paradoxically, this unique competence lies at the very basis of one of the most prevalent human pathologies, obesity with its underlying co-morbidities. During times of positive energy balance, adipose tissue has an ability of increasing its size following one of two main processes: hypertrophy, where existing adipocytes increase in size, or hyperplasia, with the formation of *de novo* adipocytes from the pool of resident adipogenic precursors, through the process of adipogenic differentiation (Fig. 1.2, Wang et al. 2013). The mechanism through which the adipose depots expand, is a pivotal determinant of the metabolic outcome of the expansion process. As much as differentiated adipocytes possess an outstanding potential for hypertrophy and are able to increase in size to a diameter of several hundred micrometres, during this process they experience elevated mechanical stress and hypoxia, when they expand to dimensions reaching



Figure 1.2 Adipose tissue expansion mechanisms: hyperplasia versus hypertrophy

In states of overnutrition, storage of energy surplus occurs if caloric input exceeds energy expenditure. Adipose tissue can expand either by hyperplasia – through differentiation of resident ASPCs, or through hypertrophy – by enlargement of existing adipocytes. Hyperplastic expansion is usually considered the healthy storage, as the tissue can maintain proper vascularisation and insulin sensitivity, hypertrophy on the other hand, is at the origin of unhealthy storage, with oversized adipocytes unable to maintain angiogenesis resulting in hypoxia and fibrosis. Sometimes, hypoxic adipocytes undergo necrosis, which leads to infiltration of immune cells, altogether resulting in systemic insulin resistance and metabolically unhealthy obesity (adapted from Ghaben and Scherer, 2019).

the limits of oxygen diffusion, all of which ultimately contributes to adipose tissue inflammation (Ghaben and Scherer, 2019; Halberg et al., 2009). In the mid-20th century already it was understood that the increased adipocyte size was associated with the increased insulin resistance, i.e. impaired cellular responsiveness to insulin and defective glucose clearance from the blood, one of the components of metabolic syndrome (Ghaben and Scherer, 2019; Salans et al., 1968). The hypoxic response of the hypertrophic tissue, mediated through hypoxia-inducible factor 1, is not sufficient to induce vascularisation of the expanding organ, which subsequently triggers expression of pro-fibrotic genes, leading to tissue fibrosis (Ghaben and Scherer, 2019). This occasionally, causes fibrotic cells to undergo necrosis, which recruits immune cells eventually leading to tissue inflammation.

The alternative mechanism of hyperplasia was demonstrated in lineage-tracing models of rodents, where, during prolonged overnutrition the tissue expansion occurred by *de novo* adipocytes emerged from the differentiating adipose progenitors (Wang et al., 2013b). It was later suggested that the adipose tissue expanded through hyperplasia, is capable of maintaining proper vascularisation as well as appropriate levels of the insulin-sensitizing and anti-inflammatory adipokines and hormones, which is important for counteracting obesity-associated metabolic deterioration (Lundgren et al., 2007). Small adipocytes, were also correlated with a diminished susceptibility to developing diabetes and

suggested to have the propensity to offset the negative metabolic effects of obesity, making hyperplasia an interesting therapeutic target (Ghaben and Scherer, 2019).

1.1.3 Disturbed energy balance and obesity

Potential mechanisms of counteracting detrimental consequences of obesity have become critical in recent years, given the alarming rise in the global incidence of obesity and its associated co-morbidities comprising insulin resistance, type 2 diabetes, cardiovascular and fatty liver disease, which are collectively termed the metabolic syndrome (Pradhan et al., 2017). These pathologies constitute a severe burden on health system and exert a huge economic cost, estimated in the 2014 McKinsey Global Institute report to be one of the top three social burdens generated by humans, just after the cost of smoking and armed war and adding up to \$2 trillion annually (McKinsey Global Institute, 2014). Estimates provided by WHO describe that the global obesity has virtually tripled between 1975 and 2016, with 1.9 billion adults, 18 years and older, overweight, of which over 650 million obese in 2016. Moreover, while less than 1% of children and adolescents aged 5-19 were obese in 1975, 6% of girls and 8% of boys were obese in 2016, adding up to more 124 million. It was also reported that 65% of the world's population live on territories where obesity and overweight kills more people than underweight (WHO, 2018).

The pandemic of obesity is believed to be an outcome of interactions of diverse causal factors including greatly perturbed energy homeostasis resulting from an unhealthy diet and prevalent lack of physical activity, but also genetic predisposition as well as environmental factors, all together leading to metabolic and endocrine deregulation (Pradhan et al., 2017). As much as preventive approaches comprising global awareness, healthy lifestyle and exercise are crucial for societal health, for individuals presenting advanced and severe obesity such conduct modifications may no longer be possible, with the latter needing targeted and effective therapies. The earnestness of the situation has created an urgency for actively researching the biological mechanisms behind the damaging energy imbalance phenomena, as these remain vastly ununderstood.

1.2 Adipose tissue

1.2.1 Physiological role and types of adipose tissue

Apart from its major contribution to the energy homeostasis regulation as a highly adjustable endocrine organ, adipose tissue plays an important and complex role in reproduction, with the gonadotropin releasing hormone (GnRH) oscillation subsequently assuring normal function of the reproductive hypothalamus-pituitary-gonadal axis both in men and women, and by providing substrates for other regulatory hormonal signals (Michalakis et al., 2013). Adipose tissue also provides mechanical protection to fragile and delicate organs such as heart, adrenal glands, ovaries and the eye, but also to anatomical structures exposed to high levels of mechanical stress, like the heel, palm and buttocks (Ghaben and Scherer, 2019; Rosen and Spiegelman, 2014). In certain times of our civilisation, voluminous adipose depots were also associated with wealth as they were during the Middle Ages and Renaissance (Bloomgarden, 2003). Additionally, localised fat depots on the other hand, are also used as displays of sexual selection, as the cheek pads in male orangutans and human female buttocks in certain cultures (Rosen and Spiegelman, 2014). To address this versatile organ in the context of the therapeutic targets with the aim of alleviating obesity and its damaging effects, it is necessary to gain advanced understanding concerning the healthy and functional system in order to aim at reverting the system to its normal physiological state. Contrary to what was believed until the mid-20th century, adipose tissue turns out to not only be a flexible and resourceful organ in its function, but also comprised of numerous highly heterogenous anatomical and histological structures.

The most traditional classification divides fat into white and brown adipose tissue (Harms and Seale, 2013). This categorisation is based on the macroscopic appearance, which itself is determined by the differences between the cellular units of the tissues, that is adipocytes. White adipocytes, forming white adipose tissue (WAT), are big in diameter and contain a unilocular lipid droplet filling virtually the entire cell, which confers its whiteish or, in humans, yellowish aspect. White adipocytes constitute the energy storage unit of the organism. Brown adipocytes, on the other hand, are smaller in diameter and contain a few smaller lipid droplets as well as a large number of iron-rich mitochondria, rendering them, and subsequently the brown adipose tissue (BAT), brown in colour. Mitochondria in brown adipocytes are functionalised by the high content of uncoupling protein 1 (UCP1), *via* which brown adipocytes regulate energy expenditure through non-shivering thermogenesis. This mechanism makes brown adipocytes attractive therapeutic targets in the fight with obesity.

1.2.2 Brown and beige adipose tissues

Brown adipocytes are believed to be an outcome of a single and a relatively recent evolutionary event, which probably secured the evolutionary success of mammals enabling neonatal thermogenesis and enhancing survival in cold surroundings (Cannon and Nedergaard, 2004). The existence of brown adipose tissue (BAT) was first described in hibernating animals as early as the mid-sixteenth century and was then referred to as the hibernating gland (Lidell, 2018). The name was soon challenged, as tissue of the corresponding aspect was also discovered in various non-hibernating mammals like rats and cats and, at the beginning of 20th century, also in human foetuses (Lidell, 2018), in both cases localised interscapularily. Later studies corroborated these findings mapping the human infant location of BAT to the shoulder and neck area but also to other sites like subpleural, axillary and perirenal regions, while in adult humans describing these depots persisting as small and residual (Cypess et al., 2009).

BAT-mediated thermogenesis

Recent research conducted in rodents in the context of brown adipose tissue shows that, next to its function of maintaining core body temperature, BAT has a significant and positive role in wholebody metabolism including glucose and fatty acids homeostasis (Celi et al., 2015; Sidossis and Kajimura, 2015). Consistently with this assumption, it has been demonstrated that in humans, BAT activity correlates with leanness, decreased prevalence of developing insulin resistance and type 2 diabetes as well as improved glycaemic and lipid profiles (Cypess et al., 2009). The evoked properties of BAT have been linked to its capacity to burn glucose and lipids and produce heat (Cannon and Nedergaard, 2004). In this process, called thermogenesis, chemical energy liberated from substrate combustion is not incorporated into molecules such as ATP or creatine phosphate, or transformed into work but rather dissipated as heat, which is then distributed within the body (as a sort of secreted paracrine factor), a process mediated by UCP1 (Cohen and Spiegelman, 2015). Activation and deactivation of thermogenesis has been shown to be rapid and consistent with an "on-demand" process balancing between conserving constant temperature and limiting substrate expenditure (Celi et al., 2015). It has been demonstrated that the activation of brown adipose tissue (which is itself a small organ) increases the tissue perfusion and could result in quadrupling of energy expenditure, as not only the resident fat storage is utilised but also circulating glucose and free fatty acids (Halpern et al., 2014), which makes it an extremely interesting therapeutic target in the battle against morbid obesity and the metabolic syndrome associated conditions. It is therefore crucial to understand the physiology of BAT, its activation, metabolic outputs as well as its potential recruitment.

Beige adipocytes

Whilst in mice the classical brown adipocytes reside within the interscapular BAT, the subcutaneous inguinal fat pad presents a mixture of white and "beige" or "brite" (brown in white), recruitable brown adipocytes, the proportion of which is a function of the feeding and energetic status of the animal (Bartelt and Heeren, 2014). In human adults, the brown adipocytes can be found in a small residual depot of BAT deep in the neck, while the beige adipocytes localise to a subclavicular depot, which holds a mixture of all three types of adipocytes: classical white, classical brown and beige (Bartelt and Heeren, 2014).

Beige adipocytes display similar characteristics as brown adipocytes, with the multilocular lipid droplets and high content of mitochondria functionalised by the presence of the BAT marker UCP1. Nevertheless, they originate from different cellular lineages (Fig. 1.3, Sidossis and Kajimura, 2015). The classical brown adipocytes residing in BAT, develop from precursor cells in the somites, which also yield skeletal myocytes, dorsal dermis and a subset of white adipocytes. This somatic population of pluripotent precursors shows to be positive for transcription factors which will later determine their fate including, Pax7, Engrailed-1 (En1) and myogenic factor 5 (Myf5) (Kajimura et al., 2015). Lineage tracing analysis showed that this is not the case for the beige fat cells, which together with the white adipocytes, originate from Myf5 negative populations of progenitors (Seale et al., 2008). It was further demonstrated that the transcriptional profile of a classical brown adipocyte resembles more the one of a skeletal muscle cell than the white or a beige adipocyte one, as does its mitochondrial proteome



Figure 1.3 Distinct developmental origins of white, brown and beige adipocytes

Brown adipocytes derive from a Pax7+/Myf5+ lineage that they share with skeletal muscle, while white adipocytes come from Pax7-/Myf5- progenitors. Beige adipocytes differentiate by being activate through cold, sharing the white lineage (adapted from Rosen and Spiegelman, 2014).

(Sidossis and Kajimura, 2015). While investigating the Myf5 negative population of progenitors from the murine inguinal WAT depots, the clonal analysis showed that the beige and white adipocytes present distinct molecular profiles and that only the beige clones supported *Ucp1* expression upon β -adrenergic stimulation (Wu et al., 2012).

Effects of BAT activation

Multiple studies (in rodents and in humans) demonstrated that activation of BAT improves whole-body homeostasis. The outcome of this activation encompasses increased energy expenditure, reduced adiposity, decreased plasma glucose and lipid levels (Betz and Enerbäck, 2015; Wang et al., 2015). In BAT transplantation experiments, recipient mice housed at room temperature presented improved glucose tolerance, increased insulin sensitivity and showed to be protected from diet-induced weight-gain (Bartelt and Heeren, 2014; Enerbäck et al., 1997). A series of studies on UCP1 depleted mice further supported the evidence of BAT in regulating metabolic health. Improved glucose uptake activity of BAT results in ameliorated systemic glucose homeostasis and insulin sensitivity (Chondronikola et al., 2014), which makes BAT a potential tool in the fight against type 2 diabetes. As clinical interest in BAT grows, its endocrine functions are starting to be investigated and appreciated as BAT's non-thermogenic role. Heat being one of the secreted factors, there seem to be a collection of molecules secreted by BAT and termed "batokines" as opposed to adipokines, factors secreted by WAT (Kajimura et al., 2015). Irisin and METRNL have been recently proposed as batokines as they were shown to be secreted by BAT upon cold exposure. The same stands for FGF21 and BMFb2, which

were previously demonstrated to signal to BAT and their secretion following BAT activation would suggest their potential paracrine and/or autocrine mode of action (Kajimura et al., 2015). One of the proposed candidates for a batokine is neuregulin-4 (NRG4), a member of the epidermal growth factor family (EGF), which was shown to inhibit lipogenesis in the liver and therefore regulate hepatic lipid homeostasis (Wang et al., 2013a). BAT metabolic function/role has been compared to a "metabolic sink" for glucose, fatty acids and potentially for other toxic metabolites (Bartelt and Heeren, 2014). The implications of this expanding scope of BAT's physiological competency make it potentially interesting in fighting not only obesity but also conditions such as diabetes and fatty liver disease (Kajimura et al., 2015).

Function and physiology of beige adipocytes

The relative contribution of beige adipocytes to the over-all UCP1-dependent thermogenic capacity was important to evaluate, as the total amount of UCP1 in the beige cells is estimated at 10% of the amount found in the classical brown adipocytes (Kajimura and Saito, 2014; Nedergaard et al., 2001). A number of studies could show that despite this modest quantity of UCP1 in beige cells, they present significantly positive contribution to the whole-body energetic homeostasis.

Mice with a high propensity for beigeing within the retroperitoneal WAT *via* β -adrenoreceptor agonists were shown to be more resistant to diet-induced obesity (Xue et al., 2015), which stands also for several transgenic mouse models with an increased number of beige adipocytes in their white adipose depots (Cederberg et al., 2001). Finally, in the absence of classical brown adipocytes, induced beige cells contribute to non-shivering adaptive thermogenesis (Schulz et al., 2013). As beige adipocytes are recruitable and their development within WAT is highly inducible, the mechanism of their differentiation is of great interest for clinical molecular signature of beige rather than brown adipocytes (Wu et al., 2012), which makes it even more attractive to investigate.

Beigeing, transcriptional and epigenetic regulators

A few powerful transcriptional activators of beiging in WAT were described (Kajimura et al., 2015), including PRDM16, which also controls a brown fat to skeletal muscle transition (Seale et al., 2008), as well as its transcriptional partners: PGC-1 α , C/EBP- β and Euchromatic histone-lysine N-methyltransferase 1 (EHMT1). It was shown, that the activity of these factors, not only supports the brown-like transcriptional programme, but also supresses the white fat gene programme (Seale et al., 2008). FoxC2 activity increases the sensitivity of the β -adrenergic-dependent PKA signalling pathway thus amplifying the NE signal transduction, a potent activator of beiging (Cederberg et al., 2001).

Recent efforts to dissect the core regulatory network underlying beiging with the systems-genetic-based approach lead to demonstrating extensive transcriptional changes arising during beiging revealing candidate regulators (Li et al., 2019). The identified candidate genes were classified as playing a potential role in transcriptional regulation of *Ucp1*, as functional components of beige adipocytes or specific markers for beige adipocytes. As the study was conducted in various murine strains presenting different degree of beiging, genes showing differential expression between highly beiging strains as opposed to lowly beiging strains were inquired, revealing a number of transcription factors,



Figure 1.4 Factors promoting development and activity of brown and beige adipocytes

Shown are factors with therapeutic potential as BAT potentiators. Physiological stimull originating at the systemic level can be divided into 1) cold exposure-derived factors namely norepinephrine (NE) that is secreted by the SNS; 2) exercise-induced myokines including irisin, Meteorin-like (METRNL), interleukin 6 (IL-6), lactate and β - aminoisobutyric acid (BAIBA); and 3) insulin as a post-prandially secreted factor. In addition, endocrine stimull are also visualized including thyroid hormone T3, natriuretic peptides (NP), fibroblast growth factor 21 (FGF21), bone morphogenetic protein 7 (BMP7), bone morphogenetic protein 8b (BMP8b), orexin (OX), vascular endothelial growth factor (VEGF) and prostaglandins (PG). Finally, several synthetic molecules are also shown that can act as BAT inducers but that are no longer used given their adverse secondary effects: mitochondrial uncoupler: 2,4-dinitrophenol, β 3-adrenoreceptor agonist: CL 316,243 and PPAR γ activators: thiazolidinediones (TZDs). The presented list of factors activating BAT is not exhaustive. A few organ schemes originate from Servier Medical Art. Figure legend: Organs from top (clockwise): Brain, Skeletal muscle, WAT, Heart, Liver, Thyroid, BAT and Pancreas (from Pradhan et al., 2017).

including PKNOX2, PLAGL1 and ZFP521 all showing higher expression in undifferentiated samples and being therefore potential candidates for *Ucp1* regulators (Li et al., 2019).

BAT, with its established capacities to improve whole-body energetic homeostasis is an obvious target of therapies. While the discussed and other beiging modulators can be targeted by drug-based approach, the latter did not show the most promising to date, with numerous side effects during the use of, i.e. chemical uncouplers such as dinitrophenol, β 3 AR-agonists or thiazolidinediones (TZDs) (Tamucci, 2018). It has been proposed that physiological approaches including cold exposure, exercise and a defined diet, factors intuitively linked with overall health and good condition, become established therapeutic tools to support or even replace pharmaceutics (for more details see review Pradhan, Zachara, and Deplancke, 2017).

1.2.3 White adipose tissue

Despite the remarkable physiological functions of brown adipose tissue and its promising potential in the fight with obesity, white adipose tissue is far more prevalent (Berry and Rodeheffer, 2013). WAT coordinates systemic metabolism and it is its expansion (to a remarkable maximum of 15-fold) that is responsible for perturbing the whole-body energy homeostasis. It is therefore indispensable to gain profound understanding of the physiology of WAT and identify mechanisms, which while deregulated, are at the basis of white adipose tissue pathologies including overweight, hyperglycaemia, hyperlipidaemia, hypertension, liver disease, diabetes and increased risk of carcinogenesis (Berry and Rodeheffer, 2013).

1.2.4 White adipose tissue as an endocrine organ

As an established whole-body energy homeostasis modulator, adipose tissue needs an efficient way of communicating its energetic status and requirements with the rest of the body, as well as has to be capable of receiving and reacting to executive signals from higher structures. This crosstalk can be classified into three main categories: nutritional signalling, neural signalling and secretion-based signalling, involving autocrine, paracrine and endocrine factors collectively termed adipokines (Rosen and Spiegelman, 2014). Nutritional mechanisms consist of adipocytes liberating free fatty acids by lipolysis during fasting. Free fatty acids are then released into the circulation through which they reach skeletal muscles and other organs and where they are readily utilised, while sparing glucose to be used by the central nervous system and red blood cells (Rosen and Spiegelman, 2014). Neural pathways rely on the rich innervation of adipose tissue both by sympathetic and parasympathetic fibres, mediating lipolysis during fasting and lipid accumulation after feeding respectively (Bartness et al., 2010). The most widespread and complex system however is signalling *via* adipokines.

Leptin

A number of molecules and molecular mechanisms have been described concerning the feedback signalling system reflecting the status of adipose energy stores, one of the best understood being leptin, encoded by the ob gene and identified in the mid-90's thanks to cloning of the ob gene (Zhang et al., 1994). This discovery was an inflection point in the adipose biology field, showing that adipose tissue was an active endocrine organ and demonstrating the power of secreted factor-mediated signalling. The protein hormone leptin, produced predominantly in adipocytes, is expressed and secreted in the fed state in proportion to their triglyceride content. It was shown that leptin is released into the blood stream, where its levels correlate with the extent of obesity to a remarkable level (Considine et al., 1996; Frederich et al., 1995; Maffel et al., 1995). Leptin has been long described as a hormone inhibiting hunger and subsequently reducing body weight by decreasing food intake and increasing energy expenditure (Rosen and Spiegelman, 2014). It is however recently regarded more as a signal to the brain that there is enough "fuel" for normal physiological function and its absence (in case of weight loss or during fast) signals to the brain that countermeasures must me taken including increase in food intake and decrease in metabolic rate (Ravussin et al., 2014). These effects are mediated centrally through hypothalamic nuclel including the arcuate, lateral, dorsomedial and ventromedial nuclel expressing Leptin receptors. Leptin receptors are also present in the nucleus of the solitary tract of the hindbrain, where is it believed to affect dopaminergic reward mechanisms modulating hedonic feeding behaviours (Leinninger et al., 2009). Apart from exerting its effects centrally it also does so at the periphery, an example of which is its promoting inflammation by enhancing cytokine production, macrophage activity and the CD4+ T helper cells response (Carbone et al., 2012). Transcriptionally, Lep expression is regulated by a complex interplay and opposing activity of C/EBPa and PPARya as well as of FOSL2 regulating adipogenic differentiation-dependent expression of Lep (Wrann et al., 2012).

Adiponectin

Adiponectin is highly adipose tissue-specific and constitutively secreted circulates in the serum at high concentrations (2-10 µg/ml) in the form of trimers, hexamers as well as higher order structures, most likely representing the most active forms (Turer and Scherer, 2012). It exerts its action mainly through two classes of receptors, ADIPOR1 and ADIPOR2 (Yamauchl and Kadowaki, 2013). The receptors, being widespread throughout the body, allow transducing adiponectin's effects to many tissues affecting many physiological processes, including promoting metabolic health by inducing fatty acid oxidation in the liver, suppressing hepatic glucose production and increasing peripheral insulin sensitivity (Goldstein et al., 2009). It was however shown incapable of reducing body weight. Interestingly, adiponectin expression and secretion are decreased in individuals presenting visceral obesity, regardless of increased fat mass, which allowed the use of adiponectin as a marker of insulin resistance and metabolic dysfunction (Turer et al., 2011).

FGF21

FGF21 is another signalling secreted protein, produced by liver, adipose tissue and other tissues and exerting numerous beneficial effects on metabolic function including improved glucose and lipid homeostasis and weight loss (Iglesias et al., 2012). FGF21 was shown to directly enhance the production of adiponectin in adipose tissue. In the absence of adiponectin, on the other hand, FGF21 is still capable of reducing body weight but this is no longer accompanied by improved glucose homeostasis and diminished insulin resistance (Holland et al., 2013).

Other adipokines

A number of other adipokines have been identified as molecular actors in the adipose tissue crosstalk with the organism including resistin (although to date demonstrated to be more relevant in rodents), RBP4 and LCN2 (lipocalins expressed at high level in obesity with RBP4 preferentially present in VAT), adipocyte fatty-acid binding protein *aP2* (encoded by *Fabp4* gene, elevated in obesity and promoting hepatic insulin resistance and gluconeogenesis) as well as later identified chemerin, omentin, vaspin (Bremer and Jialal, 2013).

These and many more adipose tissue-specific molecules are involved in a complex network of signalling between fat and the rest of the body with the ultimate aim of maintaining and regulating energy equilibrium, nutrient balance and tissue homeostasis but also communicating occurring perturbations emphasising the tremendous importance and power of a secreted signal.

1.2.5 Visceral and subcutaneous adipose tissue in health and disease

There are two main types of white adipose tissue: subcutaneous (SAT) and visceral (VAT) (Hwang and Kim, 2019). While originally separated based on their anatomical location, in recent years it became clear that this classification is oversimplified and that they comprise a variety of essential differences

| | Immune responses | | Adipogenic characteristics | | | Others |
|-----|------------------------------|------------------------------|----------------------------|---|---|---------------------------|
| | Fibrosis | Inflammation | Progenitor no. | <i>Ex vivo</i> adipogenic potential | <i>In vivo</i> adipogenic potential | Vasculature in obesity |
| SAT | ↑ | Ť | Ť | $\uparrow \uparrow \uparrow$ | None | 1 |
| VAT | $\uparrow \uparrow \uparrow$ | $\uparrow \uparrow \uparrow$ | \downarrow | 1 | $\uparrow \uparrow$ | \downarrow |

Figure 1.5 Obese subcutaneous and visceral white adipose tissue

Murine SAT and VAT respond differently to obesity in the context of immune response, adipogenic properties as well as vasculature. *In vivo* observations reveal obese VAT presenting substantially increased fibrosis, inflammation and in situ adipogenic propensity as well as reduced vascularization, as opposed to SAT (adapted from Hwang and Kim, 2019).

including developmental timing, biological functions, macroscopic appearance as well as molecular and transcriptional signature (Berry and Rodeheffer, 2013). Moreover, particular depots do not seem to present interspecies correlation, with the main human depot being the visceral omental fat, a large peritoneal fatty fold covering and connecting the stomach to intestines and other visceral organs, with subcutaneous fat redistributed to numerous subdermal regions, while in rodent models, the subcutaneous inguinal and visceral epididymal (or gonadal) depots being the predominant ones (Ghaben and Scherer, 2019).

Most adipocytes rise during development and their emergence is tightly associated with angiogenesis, but the developmental timing of different depots varies between species (Birsoy et al., 2011; Greenwood and Hirsch, 1974). In rodent models, subcutaneous white adipose tissue is formed embryonically with the expression of adipose-specific markers in subcutaneous regions during embryonic days 16.5-17.5 and the appearance of lipid-filled subcutaneous adipocytes one day after that (Birsoy et al., 2011; Greenwood and Hirsch, 1974). The visceral depots become visible by post-natal day 7 and develop during the first 2 post-natal weeks (Han et al., 2011; Wang et al., 2013b). In humans, adipose tissue develops early during the course of the second trimester, with the proliferation tending to diminish later on during gestation followed by an increase primarily by feeling the existing adipocytes up until the age of approximately 10 years. By that time the number of total adipocytes is set for each individual and, in healthy conditions, stays roughly the same throughout adulthood, with the average yearly turnover of approximately 8-10% (Knittle et al., 1979).

Another difference between subcutaneous and visceral adipose tissue is their histological appearance, with subcutaneous fat being more heterogeneous containing large mature adipocytes mixed with small multilocular adipocytes while visceral fat presenting a more homogeneous aspect consisting mainly of large unilocular adipocytes (Tchernof et al., 2006; Tchkonia et al., 2013).

There is also growing evidence suggesting that subcutaneous and visceral adipose tissues have different functional roles in metabolic regulation. It was shown, that in obese humans, expanded visceral fat was highly correlated with increased susceptibility of metabolic disorders, a correlation which was not observed in obese individuals with predominant subcutaneous adipose tissue expansion (Hwang and Kim, 2019). On the contrary, the metabolic parameters of obese people with a higher fraction of subcutaneous adipose were shown to be closer to the norm (Popa et al., 2016). In experiments in which obese mice underwent tissue transplantation, direct evidence of subcutaneous adipose tissue being metabolically beneficial was shown. In these animals, intra-abdominal graft of subcutaneous fat improved insulin resistance, glucose intolerance as well as decreased body weight gain (Fig. 1.5). It was also demonstrated that long-term obesity resulted in reduced vasculature in expanded visceral fat while in obese subcutaneous fat vasculature was denser and maintained. Moreover, obese VAT presented increased fibrosis and inflammation together with elevated in vivo adipogenic potential (Hocking et al., 2015). It has been proposed that hypertrophic adipocytes prevalently observed in VAT during obesity contribute to systemic insulin resistance while in contrast the higher fraction small multilocular adipocytes in SAt improve glucose uptake (Hwang and Kim, 2019; Kim et al., 2015). However, contradictory findings have been reported showing that in diet-induced

obesity, gonadal fat expands *via* both adipocyte hypertrophy and hyperplasia, while inguinal depots in the corresponding animals expand predominantly by hypertrophy (Wang et al., 2013b).

As much as hypertrophy seems to be the initial mechanism of adipose tissue expansion in times of chronic energy surplus, followed by hyperplasia only at the certain critical threshold of hypertrophic adipocytes (~ 0.7-0.8 µg/cell), the exact interplay of the two systems is yet to be elucidated (Krotk-iewskl et al., 1983). Further, it is still to be shown whether the divergent metabolic outcomes in the context of subcutaneous and visceral adipose tissue regulation are a simple correlation or constitute an actual causal relationship (Hwang and Kim, 2019).

1.3 Adipose tissue development and adipose progenitors

1.3.1 Adipose tissue composition

Although mature adipocytes constitute the great majority of adipose tissue when normalized to volume, cell number-wise they compose only 20-30% of fat tissue (Müller et al, 2016). The remaining 70-80% are the stromal vascular fraction (SVF) cells including red blood cells, hematopoietic and endothelial cells, macrophages, pericytes, fibroblasts as well as adipose stem cells and preadipocytes (Bunnell et al., 2008). The latter, collectively termed adipose stem and precursor cells (ASPCs), and presenting a still undissected molecularly and functionally heterogenous population, is at the origin of developing adipocytes.

Historically, studies of adipocyte development, dating back to the late 19th century, presented contradictory hypotheses, stating, on one hand, that adipocytes rise within loose connective tissue with all fibroblast-like cells having the capacity of accumulating lipids, and on the other implying that emerging adipocytes followed discrete developmental patterns and originate from specific progenitors (Hepler et al., 2017). Less ambiguously, it was also postulated that rising adipocytes first appear in connection with blood vessels networks forming a so called "primitive organ", a theory confirmed in recent years by demonstrating the dependence between adipogenesis and angiogenesis (Han et al., 2011). Nowadays, it is well established that adipose tissue develops in highly controlled spatiotemporal manner. In the context of embryonic development, adipocytes emerge from mesenchyme, which is primarily of mesodermal origin. However, in the cephalic region, mesenchyme derives from the neuroectoderm and therefore adipocytes in that location are of ectodermal origin. Lineage tracing assays in mice indicated that the subcutaneous and intra-abdominal depots emerge however from distinct mesenchymal lineages and that adipocytes in visceral but not subcutaneous and brown fat originate from cells expressing mesothelial cell marker Wilms tumor protein homolog (*Wt1*) (Hepler et al., 2017).

1.3.2 Molecular mechanisms of adipogenesis

During development but also during adulthood, adipocytes arise from adipogenic progenitors, making the mechanisms governing their emergence, division and differentiation propensity critical in understanding the resulting adipose biology.

Adipogenesis can be regarded as a two-step process: first, the mesenchymal precursors, characterised by high expression of platelet-derived growth factor receptor alpha and/or beta (*Pdgfra* and/ or *Pdgfrb*), define their fate and commit to the adipogenic lineage (losing thereby their multilineage potential and no more capable of differentiating into chondroblasts, osteoblasts and myoblasts), forming preadipocytes, second, the committed preadipocytes undergo growth arrest, start accumulating nutrients and differentiate into lipid-filled, insulin-responsive mature adipocytes (Ghaben and Scherer, 2019).

Adipocyte commitment

Bone morphogenetic protein 2 (BMP-2) and 4 (BMP-4) were one of the first identified drivers of adipogenic commitment, being sufficient for the cultured fibroblasts to commit and required for the subsequent adipogenesis (Bowers et al., 2006; Huang et al., 2009). The signalling occurs through the BMP receptors, which activate the SMAD4 transcription factor, which subsequently is able to stimulate the terminal differentiation by promoting transcription of peroxisome proliferator-activated receptor-gamma (PPARy), the master regulator of adipogenesis. BMP-2 was also proposed to compete with BMP-3b/GDF10 in the context of adipogenic *versus* osteogenic regulation, where it was suggested



Figure 1.6 Molecular mechanisms of adipogenic differentiation

Schematic differentiation of mechanistics of adipogenesis. Mesenchymal precursors being marked by the expression of *Acta2*, *Pdgfra* and *Pdgfrb* and capable of giving rise to other cell types including chondroblasts, osteoblasts and myoblasts, serve as a pool of adipogenic precursors. During adipocyte commitment, BMP-mediated signaling next restricts the fate of mesenchymal progenitors to adipocyte lineage and, while still of fibroblast-like morphology, they start expressing *Zfp423*. Once the committed preadipocyte undergoes growth arrest, master regulator *Pparg* expression is activated followed by the expression of or transcriptional co-activators *Cebpa* and *Cebpb*. Continuing lipid accumulation activates expression of early (fatty-acid binding protein (*Fabp4/Ap2*), the insulin-sensitive transporter (*Glut4*)) followed by late (adiponectin (*Adipoq*), leptin (*Lep*), lipases adipose triglyceride lipase (*Atgl*), lipoprotein lipase (*LpI*) and perilipin (*Plin*)). De-differentiation of a mature adipocyte back to the committed preadipocyte stage was also shown to be possible (adapted from Ghaben and Scherer, 2019). that the inhibitory activity that BMP-3b plays in the process of osteoblastic differentiation, might occur through itself and BMP-2 being mutually antagonistic and competing for the availability of *Smad4* (Matsumoto et al., 2012).

A couple of zinc-finger transcription factors were shown to be implicated in the regulation of early adipogenesis, with the ASPC-specific ZFP423 sensitizing mesenchymal precursors to pro-BMP signalling and ZFP467 supressing osteogenesis and promoting adipogenesis by enhancing the expression of another *bona fide* regulator of adipogenic gene transcription, co-activator CCAAT/enhancer-binding protein alpha (C/EBPα) (Gupta et al., 2012; Quach et al., 2011). Additionally, activator Krueppel-like factor 5 (KLF5) acts as a co-activator of C/EBPβ, which in turn enhances *Pparg* and *Cebpa* transcription (OishI et al., 2005). *Cebpb* transcription is also regulated by cAMP-response element-binding protein (CREB), which itself is modulated by EGR1 and EGR2 respective inhibitor and activator of adipogenesis (Ghaben and Scherer, 2019). Finally, GATA-binding protein 2 and GATA-binding factor 3 inhibit adipogenic differentiation by preventing *Pparg* transcription (Tong, 2000).

Adipocyte differentiation

Historically, PPARγ was identified as a transcription factor regulating the expression of an adipose-specific gene *Fabp4*, coding for Fatty acid-binding protein (Ghaben and Scherer, 2019). Later, it was characterised as the master regulator of adipogenesis as it is indispensable for *in vitro* and *in vivo* adipogenesis (Barak et al., 1999; Rosen and Spiegelman, 2000) even though the physiological ligand of PPARγ is still unknown (Schupp and Lazar, 2010). The crucial downstream effect of PPARγ in the context of adipogenesis is the activation of the transcriptional factor C/EBPα (Ghaben and Scherer, 2019). *In vitro* overexpression of *Cebpa* is sufficient to drive adipogenic differentiation, although still requiring the PPARγ-mediated regulation, the two being functionally synergistic and determining adipogenesis through a positive feedback loop. Subsequently, a second positive feedback loop reinforces the decision to undergo adipogenesis does not require C/EBPα most probably thanks to the functional redundancy shared with C/EBPβ. This is however not the case for adult adipogenesis, which requires C/EBPα activity (Ghaben and Scherer, 2019).

1.3.3 White adipose precursors

The identification and characterization of adipose progenitors is crucial to understanding adipocyte and adipose tissue development, function as well as its underlying perturbations and pathologies. Using a plethora of cell culture models, including mouse embryonic fibroblasts (MEFs), immortalized "determined" preadipocyte cell line 3T3-L1 derived from murine MEFs in the 1970s as well as primary adherent cells originating from stromal vascular fraction, allowed expanding our understanding of the nature of adipose stem and precursor cells (Hepler et al., 2017, Rosen and Spiegelman, 2014).

In these *in vitro* models, the adherent (and therefore devoid of red blood cells as well as of the endothelial (CD31+) and hematopoietic (CD45+) compartments) cells at confluence, respond

to incubation in a powerful differentiation cocktail of pro-adipogenic cues including: insulin, a glucocorticoid agonist and a phosphodiesterase inhibitor, by readily accumulating lipids within intracellular vacuoles and acquiring several characteristics of adipocytes, together allowing them to have been used as a primary model of adipogenesis (Rosen and Spiegelman, 2000). 3T3-L1 cellular model especially contributed a significant advance in the elucidation of cellular and biological events that take place as well as the transcriptional cascade that occurs during the conversion on the fibroblast-like cells to *in vitro* adipocytes. Specifically, PPARγ, a nuclear hormone receptor, the "master regulator" of this transcriptional hierarchy, which is necessary and sufficient for *in vitro* adipogenesis, was originally discovered through the use of the 3T3-L1 cell line (Ghaben and Scherer, 2019).

Thanks to these inquiries, candidates previously described to mark stem cell populations in other tissues could be interrogated in the context of *in vitro* adipogenesis, including CD29 (β1-integrin), CD34 and SCA1 (mesenchymal and stem cell markers respectively) and showed to enrich for adipogenic precursors (Cawthorn et al., 2012). However, it had been long established that the population of ad-ipose stem and precursors cells presents high heterogeneity and recent years showed lots of effort in order to dissect this complex cell population. Numerous novel markers have been identified including: CD24, PDGFRα, (Berry and Rodeheffer, 2013; Rodeheffer et al., 2008), ZFP423 (Gupta et al., 2010) and PREF1(DLK1) (Hepler et al., 2017; Smas and Sul, 1993). Nevertheless, none of them is a universally accepted marker specific for a pre-adipocyte type nor uniformly marks a particular pre-adipocyte sub-population. In particular, despite efforts using classical approached such as fluorescence-activated cell sorting (FACS), lineage tracing methods as well as cell population-based transcriptional studies, there is still no clear picture of the lineage relationships within the fat pad (Harms and Seale, 2013).

1.3.4 Adipose precursor markers

Although adipogenesis is one of the best-studied cell differentiation paradigms, we currently still have an incomplete knowledge of the *in vivo* origin and nature of adipose stem and precursor cells, from which mature adipocytes develop. This is partially due to the high cellular and molecular heter-ogeneity of ASPCs and mature adipocytes (Berry et al., 2014; Cristancho and Lazar, 2011; Sanchez-Gurmaches and Guertin, 2014; Jeffery et al., 2014; Lee at al, 2019). Dissecting and understanding this heterogeneity and transcriptional regulation of ASPCs will be crucial for a better understanding of the dynamics of adipose tissue biology in health as well as in obesity. A few of currently employed pre-adipogenic markers and the context of their discovery and use are described below.

PDGFRa

PDGFRα was an important marker in identifying a muscle-resident pool of precursors for adipocytes and fibrocytes emerging during an injured muscle healing (Uezuml et al., 2010). They were termed "fibroadipogenic progenitors" (FAPs) and shown to be distinct from the muscle progenitors and could be identified by the expression of *Pdgfra* together with *Cd34* and *Ly6a*. Their adipogenic potential was demonstrated in transplantation into an injured and unhealthy muscle emphasizing the importance of the microenvironment in modulating adipogenic differentiation (Hepler et al., 2017).

The equivalent, proliferative, CD34+ SCA1+ PDGFR α + population within adipose tissue was shown to be responsive to β 3 adrenergic receptor (β 3AR) signalling and bipotent *in vitro*, able of differentiating both into white and beige adipocytes (Hepler et al., 2017; Lee et al., 2012). These PDGFR α + progenitors are located peri-vascularily and lineage tracing approach indicated that newly rising adipocytes induced by high fat diet, emerge from cells expressing *Pdgfra*. Furthermore, it has been suggested that nearly all mature adipocytes within the organism descend from the *Pdgfra* lineage (targeted by *Pdgfra-Cre*) together with CD24– and CD24+ ASPCs also being PDGFR α +, making it a suitable marker for ASPC selection (Berry and Rodeheffer, 2013).

CD24

CD24 was proposed to play an important role in marking preadipocyte population in a study aiming at prospectively isolating a pure population of ASPCs from primary murine stromal vascular fraction (Rodeheffer et al., 2008). The rationale of isolation followed a, to date, golden standard of FACSbased selection of ASPCs depleted from endothelial and hematopoietic markers (CD31 and CD45 respectively) and enriched for commonly accepted mesenchymal stem cell markers existing in different tissues, namely CD34, SCA1, and CD29 (Rodeheffer et al., 2008). Such defined ASPCs were then selected for the expression of CD24, which was previously identified as a cell-surface protein expressed on stem cell populations in other tissues (Hepler et al., 2017). Remarkably, CD24+ ASPCs revealed to be a highly proliferative and stem-cell like adipose progenitor population, while CD24– ASPCs appeared to constitute a more committed preadipocyte population expressing genes such as *Pparg2* and *Cebpa*. It was later shown, that *in vivo*, CD24+ ASPCs gave rise to CD24– ASPCs in the axis of adipogenic differentiation, showing the existence of hierarchical subpopulation structure within the pool of adipose precursor cells displaying different levels of commitment (Hepler et al., 2017; Rodeheffer et al., 2008). Recent lineage tracing assays utilizing BrdU-based "pulse-chase" approach indicated that CD24+, despite being relatively rare *in vivo*, contribute to adipocyte hyperplasia in obesity (Jeffery et al., 2015).

ZFP423

In the efforts to identify and localize the ASPCs, the expression of the lineage-determining factor *Pparg* was traced in mice and showed to associate with cells residing within the blood vessel walls of mature adipose tissue and cells positive for *Pparg* expression resembled mural cells (pericytes and vascular smooth muscle cells) (Tang et al., 2008). These cells expressed mural markers including *Pdgfrb*, *Ng2* and *Acta2*, but also showed a uniform expression of *Cd34* and *Ly6a* (coding for SCA1) stem markers. These observations were in line with the premise of the adipocyte precursor niche being formed by the microenvironment of the vasculature (Hepler et al., 2017), an assumption further supported by the identification of the mural-specific (in addition to endothelial cells) presence of ZFP423 a multi-zinc finger transcription factor regulating preadipocyte expression of *Pparg* and subsequently adipogenic differentiation *in vitro* and *in vivo* (Gupta et al., 2010). These non-endothelial ZFP423 positive cells also showed expression of the mural marker *Pdgfrb*, but also the ASPC-specific genes including
Pdgfra, *Cd34* and *Ly6a*. Interestingly, ZFP423 positive mural cells, feature expression of *Pparg* and other adipogenic lineage-selective markers and were demonstrated to be devoid of *Cd24* expression, indicating their potential committed character (Tang et al., 2008; Vishvanath et al., 2016).

DLK-1

One of the first preadipocyte-specific markers identified was a secreted EGF repeat-containing protein DLK-1 (Hudak et al., 2014; Smas and Sul, 1993). *Dlk1/Pref1* is expressed exclusively by preadipocytes and not mature adipocytes and was shown to act as an inhibitor of adipogenesis (Hudak et al., 2014). Lineage tracing assays using *Dlk1-CreERT2* mice revealed that dermal adipocytes emerged from DLK-1 positive cells. When isolated, these cells were also shown positive for *Cd24* and negative for *Zfp423* and *Pparg* expression and presented a high adipogenic propensity *in vitro* in line with the premise of CD24+ ASPCs being early, non-committed adipogenic precursors (Rodeheffer et al., 2008). Physiologically, mice deficient in *Dlk1*, failed to undergo expected white adipose tissue expansion upon high fat diet, with inguinal depots expanding exclusively by hypertrophy and failing to undergo hyperplastic event (Hudak et al., 2014).

While Dlk1+ precursors were shown to give rise to both inguinal and gonadal adipocytes (Hudak et al., 2014), differences within subcutaneous and visceral ASPCs and their regulation has been of interest given the remarkable phenotypic and metabolic differences between these tissues.

1.3.5 Subcutaneous versus visceral ASPCs

Subcutaneous and visceral adipose tissue depots present distinct features and contribute differently to metabolic health with increased visceral rather than subcutaneous fat during the onset of obesity being associated with higher risk of developing metabolic diseases. However, it has been demonstrated numerous times that the adipogenic capacity in the presence of identical adipogenic cues differs considerably between subcutaneous and visceral ASPCs. Counterintuitively for many in the field, subcutaneous ASCPs, as well as stromal vascular fraction cells, show a higher adipogenic propensity under ex vivo cell culture conditions (Meissburger et al., 2016; Tang et al., 2008; Wang et al., 2013a). Meanwhile, lineage tracing approaches allowed identifying a higher fraction of murine Pdafra expressing mural cells within the gonadal WAT than in the inguinal WAT of lean mice, correlating with the previously demonstrated potential of these depots to undergo hyperplasia when challenged with high fat diet (Vishvanath et al., 2016). Corroborating this finding, it was also shown that high fat diet in mice rapidly and transiently induced proliferation of ASPCs within white adipose tissue (which were at the origin of new adipocytes), the activation described specific for the gonadal depots in male mice and stated consistent with the obesogenic WAT expansion in human obesity (Jeffery et al., 2015). Contrary, obese individuals treated with thiazolidinediones (PPARy agonists, prescribed in type 2 diabetes as enhancers of insulin sensitivity), showed expansion of subcutaneous adipose tissue by hyperplasia while the relative mass of visceral depos was reduced (Miyazakl et al.; Spiegelman, 1998).

These inconsistencies between ex vivo cell culture models and in vivo assays might reflect the importance of the tissue environment of ASPCs and the signalling occurring within the ASPC niche in situ. In an approach aiming at addressing the dramatic differences in the adipogenic potential between the visceral and subcutaneous precursor cells, the respective murine stromal vascular fractions, arguably containing supplementary elements of the ASPC niche, and not only ASPCs themselves, were interrogated (Meissburger et al., 2016). It was demonstrated that the decreased adipogenic potential of murine gonadal versus inguinal SVF was not due to the decreased number of ASPCs within. Notably, an even higher fraction of ASPCs, defined as lineage negative Lin- (CD31- CD45- TER119-) CD29+ CD34+ SCA1+, was found within the gonadal as opposed to inquinal SVF, suggesting reduced pro-adipogenic capacity of gonadal ASPCs themselves. The hypothesis of the modulatory role of the ASPC niche was then tackled by investigating the factors secreted by the respective SVFs after demonstrating the inhibitory potential of the gonadal SVF conditioned medium. An in-depth analysis of the gonadal SVF-secreted factors and their subsequent functional in vitro validation showed a number of candidate molecules potentially responsible for the inhibitory dynamics postulated within the visceral ASPC niche, including, amongst others, Decorin (Dcn), Sparc-like 1 (Sparcl1), biglican (Bqn) and matrix gla protein (Mqp) (Meissburger et al., 2016). It remains to be appreciated whether the identified secreted factors are specific to visceral adipogenesis and whether they operate in concert with each other as well as with intrinsic properties of visceral ASPCs.

Human visceral ASPCs, presenting the same compromised adipogenic propensity *in vitro*, were interrogated in a global gene expression study, which identified many genes involved in retinoic acid (RA) synthesis as potentially involved in their non-adipogenic phenotype (Takeda et al., 2016). Visceral and subcutaneous adipose tissue as well as ASPCs from adult human individuals without diabetes showed RA-associated metabolism gene expression highly specific for the visceral *versus* subcutaneous compartment. This encompassed a significantly higher expression of a number of genes involved in the *bona fide* RA synthesis including *RDH10*, *CRBP1* and *ALDH1A2*, but also genes reportedly modulated by RA signalling such as *RARRES1/2*, *MGP* and *F3* (Takeda et al., 2016). Indeed, RA was previously reported as a potent inhibitor of adipogenic differentiation in mice (Schwarz et al., 1997) and endogenous levels of RA were subsequently shown higher in visceral *versus* subcutaneous ASPCs (Takeda et al., 2016). It was also proposed that the involved RA signalling pathway was under the control of Wilms tumor protein, through its binding site within the promoter region of *ALDH1A2* gene, which lead to early stage inhibition of visceral but not subcutaneous adipogenesis (Takeda et al., 2016).

Interestingly, a study dissecting the cellular heterogeneity within different adipose depots using a clone-based approach, identified a population of ASPCs marked by the expression on *Wt1*, only present in visceral depots and presenting significantly lower adipogenic propensity *in vitro* comparing to other ASPC types within the depot (Lee et al., 2019).

1.3.6 The undissected heterogeneity of adipose stem and precursor cells

The universally accepted heterogeneity of adipose stem and precursor cells has proven challenging to dissect phenotypically and molecularly (Cristancho and Lazar, 2011). The molecular mechanisms driving the phenotypic differences between ASPCs within the same depot as well their variation across subcutaneous and visceral compartments are yet to be elucidated (Berry et al., 2014; Meissburger et al., 2016; Takeda et al., 2016). Existing and ongoing studies are a fascinating interplay of investigations researching whether this variation is determined by the intrinsic properties of the ASPCs or/and a para- or endocrine activity of the ASPC niche environment. This and many other questions regarding the dynamics and regulation of adipose tissue development and maintenance in health and in disease, may be answered through gaining a clear picture of the increasingly appreciated heterogeneity of ASPCs. To this end, a number of assays employing single-cell RNA-sequencing, a cutting-edge technology in the field of dissecting cellular heterogeneity, were conducted in recent years in the context of adipose stem and precursor cells, with the study from our laboratory published in 2018, being the first in the field of murine adipogenesis (Burl et al., 2018; Hepler et al., 2018; Merrick et al., 2019; Schwalie et al., 2018, the latter being the content of Chapter 2).

1.4 Methodology

1.4.1 Single-cell RNA sequencing

Comprehensive measurement of transcriptomes of single cells, has been possible only for a few years (Tang et al., 2009) but already revolutionised the field of transcriptomics and sees a constant and rapid development (Chen et al., 2019; Hwang et al., 2018). Gene expression differences between individual cells may have profound functional consequences both in unicellular and multicellular or-ganisms, which classical bulk RNA sequencing methods do not permit to reveal (Fig. 1.7.a and b). Starting from sequencing and analysing the transcriptome of one single cell (Tang et al., 2009), single-cell RNA sequencing (scRNA-seq) technology at this point allows evaluating hundreds of thousands of single cells within one assay. This tremendously fast advancement in upscaling the number of analysed cells lies essentially in the cell isolation and capture methodology, which started at manual separation of single cells and FACS-based isolation, followed by integrated fluidic circuits (i.e. Fluidigm C1, first automated solution for single cell genomics) and nanodroplet based technologies (i.e. Drop-seq (Macosko et al., 2015), 10x Genomics), which increased the number of handled cells to tens of thousands (Fig. 1.7.c, Svensson et al., 2018).



Figure 1.7 Single-cell RNA sequencing

a, Represented are six single cells with heterogeneous expression of three genes. scRNA-seq reveals heterogeneity in gene expression and co-expression. **b**, Using bulk RNA sequencing would reveal uniform expression of all three genes (adapted from Macaulay and Voet, 2014). **c**, Scaling up scRNA-seq: cell numbers reported in publications corresponding to indicated dated and technologies used (adapted from Svensson et al., 2018). **d**, and **e**, Reconstruction of retinal cell types from almost 45,000 single-cell transcriptional profiles prepared using Drop-seq, **d**, Schematic representation of retinal cell sub-types within the murine retina. **e**, tSNE representing clustering of almost 45,000 cells as a function of their global relative gene expression relationships, revealing 39 cell populations, the clusters are coloured by cell types represented in d (adapted from Macosko et al., 2015).

One of the early benchmarking examples of scRNA-seq studies is the remarkable, Drop-seq-based analysis of almost 45,000 murine retinal cells and identification of 39 transcriptionally distinct cell populations, subsequently creating a molecular atlas of gene expression of known retinal cell classes as well as uncovering novel cell subtype candidates (Fig. 1.7.d and e, Macosko et al., 2015).

Being under constant development as much technologically as in the context of analytical pipelines, scRNA-seq is becoming a routine discovery assay, with extensive projects being undertaken such as Tabula Muris (The Tabula Muris Consortium, 2018) and its single-cell transcriptomics of 20 murine organs encompassing 100,000 sequenced cells or Human Cell Atlas, an initiative to create unique ID card for each human cell type, a three-dimensional map of how cells work together within tissues and finally insights on how changes within this map underlie health and disease (humancellatlas.org).

1.4.2 Adipose progenitors' single-cell RNA sequencing

The dynamically evolving scRNA-seq technology applied to adipocyte and ASPC research could allow uncovering the molecular identity of adipocytes and fat cell precursors, map them on adipocyte lineage trees as well as determine cellular differences between subcutaneous, visceral and brown adipose tissues (Burl et al., 2018; Hepler et al., 2018; Merrick et al., 2019; Schwalie et al., 2018). ScRNA-seq thus has the potential of answering many fundamental questions including: how heterogeneous adipose-derived stem cells within the SVF are; are there distinct structural or/and functional cell subpopulations of ASPCs governing and fine-tuning adipose proliferation and differentiation interplay in health and disease; can these sub-types be distinguished using specific molecular markers; is perturbed balance of diverse ASPCs implicated in obesity and its associated pathologies.

A number of these issues were addressed by the recent scRNA-seq-based studies. In one of them, the authors identified three main subpopulations within visceral ASPCs (Helper et al., 2018). A highly adipogenic visceral adipogenic precursors were identified as LY6C– CD9– PDGFR β + cells (including committed preadipocytes) presenting a high expression of *Pparg*, *Fabp4* and *Lpl*. LY6C– CD9+ PDGFR β + were shown to express *Msln* and *Upk3b* and characterised as mesothelial-like cells (MLCs). Finally, a LY6C+ PDGFR β + ASPCs were shown to be devoid of adipogenic capacity. These cells, termed fibro-inflammatory progenitors (FIPs), were further demonstrated to display pro-fibrogenic and pro-inflammatory phenotypes and exert an anti-adipogenic effect on the remaining ASPCs.

A different study reports single-cell based investigation of subcutaneous and visceral adipogenic progenitors and identifies distinct ASPC subpopulations differentially poised to enter the adipogenic differentiation pathway (Burl et al., 2018).

Finally, Merrick and colleagues interrogated the subcutaneous adipose stem and precursor cells in the context of untangling the mesenchymal progenitor cell hierarchy within adipose tissue (Merrick et al., 2019). Once again, three main sup-groups of ASPCs were identified one of which corresponded to highly proliferative and multipotent DPP4+ progenitors, the second showed high expression of known pre-adipogenic markers including *Pparg* and was classified as committed preadipocytes, and the third, distinct pre-adipogenic population was characterised by a high expression of *F3* and *Clec11a* (Merrick et al., 2019).

The above-described studies, alongside our own efforts, strive to dismantle the complex heterogeneity of ASPCs, with the ultimate aim of identifying distinct functional subpopulations differentially contributing to the overall adipogenic potential of the pool of progenitors *in vitro* and *in vivo*.

1.4.3 In vitro model of adipogenesis

In order to investigate the cellular and molecular mechanisms of adipogenesis, adipose stem and precursor cells are cultured in *in vitro* models optimised to mimic, to their best abilities, the *in vivo* conditions. The golden standard ASPCs are FACS-isolated and defined by lack of expression of endothelial and hematopoietic markers (CD31 and CD45 respectively) as well as positive for one or a few of the generally accepted surface stem and mesenchymal markers including SCA1, CD34 and CD29 (Rodeheffer et al., 2008). Furthermore, this selection might be expanded to markers such as PDGFRa, CD29 and CD24, defining what is considered more pre-adipogenic state (Ambrosi and Schulz, 2017; Rodeheffer et al., 2008). Although, as discussed before, none of these markers is universally accepted as being specific for a preadipocyte type nor uniformly marks a particular preadipocyte subpopulation (Harms and Seale, 2013). As a function of a designed assays, it is therefore to be decided whether to remain less strict in the context of employed markers and deal with a more heterogenous population or to restrict the phenotype and handle a less divergent but maybe incomplete cell population.

Once the cultured ASPCs attain confluence, they are ready to receive the adipogenic cue, which triggers transformation they undergo during adipogenesis, becoming therefore a dynamic study model. Although there exist various versions of the adipogenic signal composition, the most physiological one encompasses three basic compounds: insulin, 3-isobutyl-1-methylxanthine (IBMX) and dexamethasone (Moseti et al., 2016). Insulin regulates the transcription of the *Cebpa*, *Cebpb*, and *Cebpg* genes in fully differentiated 3T3-L1 adipocytes, it represses the expression of *Cebpb* and *Cebpg*. It promotes a change in the rate of intracellular facilitated glucose transporter member 4 (GLUT-4) recycling and trafficking (Moseti et al., 2016).

Glucocorticoid dexamethasone is an anti-inflammatory steroid molecule stimulating osteogenic and adipogenic differentiation in a cell-, time-, and concentration-dependent manner (Moseti et al., 2016). It reciprocally regulates expression of the *Cebpa* and *Cebpg* genes in 3T3-L1 adipocytes and white adipose tissue. Dexamethasone also regulates *Fabp4* gene expression and its combination with IBMX regulates *Pparg*, promoting adipogenesis (Moseti et al., 2016).

IBMX is a competitive, nonselective phosphodiesterase inhibitor, raising intracellular cAMP and protein kinase A (PKA). PKA signalling pathway is required for transcriptional activation of *Pparg* and thus adipogenic gene expression of *Cebpa* and *Cebpb* (Moseti et al., 2016).

1.5 The scope of this thesis

This thesis is built on the dissection of the heterogeneity of murine subcutaneous adipose stem and precursor cells (ASPCs) with the use of single-cell RNA sequencing. Its set aim has been to resolve the diversity of cells within the stromal fraction of the murine subcutaneous fat depot, identify potential groups existing within and finally characterise these groups in the molecular and physiological context.

In Chapter 2, I report published findings where, using the resolving power of single-cell transcriptomics, we revealed distinct subpopulations of adipose stem and precursor cells in the stromal vascular fraction of murine subcutaneous adipose tissue. Based on these results, I identified one of these subpopulations as a CD142+ positive fraction of cells presenting a non-adipogenic phenotype and capable of supressing adipocyte formation *in vitro*, an activity which was also confirmed by *in vivo* assays. I demonstrated that these adipogenesis-regulatory cells, which we termed Aregs, are refractory to adipogenesis and functionally conserved in humans. We also performed the first transcriptomic characterisation of Aregs, which I complemented with revealing the *in situ* localisation of Aregs in the murine subcutaneous fat pad.

In Chapter 3, I present the in-depth characterisation of murine subcutaneous Aregs, through which I demonstrated the robustness of their non-adipogenic phenotype and identity in the context of various isolation strategies, modulated adipogenic cues and sex-based differences. Through this approach, I also discovered that surprisingly, Aregs do not exhibit their adult-specific phenotype in the fat pad of new-born mice, where I showed their highly adipogenic propensity accompanied by increased cell proliferation. Subsequently, I report the process of identification of Areg-specific markers determined through the integration of three different genomic datasets corroborated by preliminary proteomic assays. I inferred a set of ten Aregs-specific candidates encompassing secreted proteins, transcriptional factors and an enzyme, which I interrogated experimentally in the context of murine subcutaneous Areg identity and function.

Chapter 2 A stromal cell population that inhibits adipogenesis in mammalian fat depots

Note: This chapter is based on the published article:

"A Stromal Cell Population That Inhibits Adipogenesis in Mammalian Fat Depots. 2018, Nature 559 (7712): 103–8."

Authors: Petra C. Schwalie*, Hua Dong*, Magda Zachara,* Julie Russeil, Daniel Alpern, Nassila Akchiche, Christian Caprara, Wenfei Sun, Kai-Uwe Schlaudraff, Gianni Soldati, Christian Wolfrum & Bart Deplancke

*These authors contributed equally: Petra C. Schwalie, Hua Dong, Magda Zachara.

Contributions of Magda Zachara: Designed the study and wrote the manuscript, performed the single-cell experiments, conducted FACS, qPCR, cell culture and related imaging assays, conducted all histological assays, performed all experiments related to human cells.

2.1 Introduction

Adipocyte development and differentiation have an important role in the aetiology of obesity and its co-morbidities (Rosen and Spiegelman, 2014). Although multiple studies have investigated the adipogenic stem and precursor cells that give rise to mature adipocytes (Billon et al., 2007; Crisan et al., 2008; Gupta et al., 2012; Jiang et al., 2014; Majka et al., 2010; Tang et al., 2008; Tran et al., 2012; Vishvanath et al., 2016; Zannettino et al., 2008), our understanding of their in vivo origin and properties is incomplete (Jiang et al., 2014; Rosen and Spiegelman, 2014; Sanchez-Gurmaches and Guertin, 2014). This is partially due to the highly heterogeneous and unstructured nature of adipose tissue depots (Cristancho and Lazar, 2011), which has proven difficult to molecularly dissect using classical approaches such as fluorescence-activated cell sorting and Cre-lox lines based on candidate marker genes (Berry et al., 2014; Sanchez-Gurmaches and Guertin, 2014). Here, using the resolving power of single-cell transcriptomics (Kolodziejczyk et al., 2015) in a mouse model, we reveal distinct subpopulations of adipose stem and precursor cells in the stromal vascular fraction of subcutaneous adipose tissue. We identify one of these subpopulations as CD142+ adipogenesis-regulatory cells, which can suppress adipocyte formation in vivo and in vitro in a paracrine manner. We show that adipogenesis-regulatory cells are refractory to adipogenesis and that they are functionally conserved in humans. Our findings point to a potentially critical role for adipogenesis-regulatory cells in modulating adipose tissue plasticity, which is linked to metabolic control, differential insulin sensitivity and type 2 diabetes.

2.2 Results

2.2.1 ScRNA-seq reveals the heterogeneity of subcutaneous ASPCs in the mouse

To study the molecular characteristics and the subpopulation structure of adipogenic stem and pre-cursor cells (ASPCs), we performed single-cell RNA sequencing (scRNA-seq) on Lin–(CD31–CD45–TER119–)CD29+CD34+SCA1+ cells from the subcutaneous stromal vascular fraction (SVF) of transgenic

mice, in which red fluorescent protein is induced in cells that express *Dlk1* (Fig. 2.1a and Methods). CD29, CD34 and SCA1 are expected to enrich for stem cells, and DLK1 has previously been suggested to mark preadipocytes (Hudak et al., 2014; Rodeheffer et al., 2008). We obtained 208 high-quality cells (Fig. 2.1a, Suppl. Fig. 2.1a–d and Methods) and confirmed that the expression of all fluorescence-activated cell sorting (FACS) markers corresponded to the enrichment strategy that was used (Suppl. Fig. 2.1e).

The cells grouped into three clusters, referred to as populations (P1, P2 and P3) (Fig. 2.1b, Supplementary Table 1 and Methods), each of which was characterized by the specific expression of hundreds of genes (Fig. 2.1c and Supplementary Table 2). Notably, only P2-specific genes enriched for adipogenesis-related functionality, including the adipogenic master regulator *Pparg* (Fig. 2.1c, Suppl. Fig. 2.1f, g and Supplementary Tables 2, 3). We further assessed the individual and combined expression of commonly used lineage marker genes (Supplementary Table 4 and Methods). We found that only *Fabp4* which is associated with adipogenesis—was significantly differentially expressed across cells, showing high expression in P2 (Suppl. Fig. 2.1h and Supplementary Table 19). Consistently, P2 cells scored significantly higher on the adipogenic scale than did P1 or P3 cells (Suppl. Fig. 2.1i, Supplementary Table 19 and Methods). Virtually all cells scored high on the stem cell scale, as also exemplified by *Cd34* expression (Suppl. Fig. 2.1h, j and Supplementary Table 19), consistent with the selection of an ASPC-enriched cell pool. Finally, among genes previously used to mark pre-adipogenic populations, only *Pparg* and *Prdm16* expression was significantly correlated with *Fabp4* expression and enriched in P2 cells (Suppl. Fig. 2.1k, I and Supplementary Tables 5, 19).

To independently validate the three cell populations we detected, we assessed a larger number of Lin- SVF cells using the 10x Genomics Chromium platform. We considered 1,804 high-quality single cells (Fig. 2.1d, Suppl. Fig. 2.2a, b and Methods) and found that most of them reflected an ASPC state, on the basis of their high Itab1 (encoding CD29), Cd34 and Ly6a (encoding SCA1) expression (Suppl. Fig. 2.2c). Consistently, mature adipocyte markers such as Adipog, Retn and Cidec were virtually absent. We found that despite differences in cell stratification, capture and molecular assessment, these data largely recapitulated our initial findings (Fig. 2.1e, f, Supplementary Table 6 and Methods). The four clusters (G1–G4) that we detected could all be mapped back to the three previously described populations: G1 and G4 together approximately corresponded to P1, G2 to P2, and G3 to P3, with over 30% of the top 100 markers overlapping (Fig. 2.1f, Suppl. Fig. 2.2d, e, Supplementary Table 7 and Methods). This included the top-ranking transcription factors Creb5 (in P1 and G1), Peg3 (in P2 and G2) as well as Meox2 (in P3 and G3). Consistently, G2-specific genes enriched for pathways related to adipogenesis (Supplementary Table 8), scored significantly higher on the adipogenic scale (Suppl. Fig. 2.2f and Supplementary Table 19) and included Fabp4 (Fig. 2.1g, h and Suppl. Fig. 2.2g). Pparg was again strongly positively correlated with Fabp4, in contrast to most of the other (pre-)adipogenic markers with the notable exception of Pdgfrb (Fig. 2.1h, Suppl. Fig. 2.2g, h and Supplementary Tables 9, 19). Cd34 and Ly6a were expressed at higher levels in G1 and G4 cells, and were significantly negatively correlated with Pdgfrb, Fabp4 and Pparg expression (Fig. 2.1h and Supplementary Tables 9, 19). In summary, by using two distinct scRNA-seq-based approaches, we identified at least three subpopulations among Lin- SVF cells. The two major ones lie at opposite poles of stem cell-specific (Cd34 and Ly6a) and pre-adipogenic (Fabp4, Pparg and Cd36) gene expression. These two subpopulations make up





a, Schematic of FACS-based selection of ASPCs (Fluidigm C1). **b**, t-distributed stochastic neighbour embedding (t-SNE) 2D cell map (C1). n = 208 cells total, 3 biological replicates; populations P1, n = 83; P2, n = 96; P3, n = 29 cells. **c**, Heat map (blue-to-red) of expression of top 10 population markers and transcription factors among the top 100 population markers (in colour); hierarchically clustered rows, mean gene expression (white-to-red, left). n = 208 cells, 3 biological replicates. **d**, Schematic of FACS-based selection of Lin– SVF cells (10x Genomics). **e**, t-SNE 2D cell map (10x Genomics); populations G1, n = 699; G2, n = 664; G3, n = 122; and G4, n = 319 cells. **f**, Percentage of the top 100 10x Genomics population markers that overlap with the top 100 C1 population markers; transcription factors and lineage markers are highlighted. **g**, t-SNE 2D cell map (10x Genomics) highlighting the expression of an adipogenic (*Fabp4*) and a stem cell (*Cd34*) marker (black). **h**, Correlation (Spearman's rho) between the expression of (pre-)adipogenic markers and *Fabp4* (left) or *Cd34* (right). In e–h, n = 1,804 cells, 1 biological replicate. See Suppl. Figs. 2.1, 2.2 and 2.8 for related results.

over 90% of the cells (Suppl. Fig. 2.2d) and can be further subclassified in increasingly homogenous cell groups, all of which are molecularly separated from a third and unrelated subpopulation (G3 or P3).

2.2.2 Aregs show a paracrine adipogenic inhibitory capacity

To assign adipogenic functionality to the three major subpopulations that we detected, we identified marker genes that encode surface proteins: *Cd55* and *ll13ra1* for P1 (and G1 and G4), *Aoc3* (en-coding VAP1) and *Adam12* for P2 (and G2), and *F3* (encoding CD142) and *Abcg1* for P3 (and G3) (Fig. 2.2a and Suppl. Fig. 2.3a). Using FACS (Suppl. Fig. 2.3b), we isolated cellular fractions that were enriched for these respective markers and validated each subpopulation by quantitative PCR (Fig. 2.2b, Suppl. Fig. 2.3c and Supplementary Table 10). We then quantified lipid accumulation in response to a white fat differentiation cocktail for all ASPCs, marker-positive ASPCs and marker-negative ASPCs (Methods). We did not observe a difference in adipogenesis for P2 cells, whereas P1 cells showed an increased propensity and P3 cells a strongly decreased propensity to form adipocytes in response to the same stimulus (Fig. 2.2c, d and Suppl. Fig. 2.3d, e). Consistently, we measured significantly higher levels of expression of adipogenic markers in P1 cells compared to ASPCs depleted of P1 cells, and the reverse was true for P3 cells (Suppl. Fig. 2.3f and Supplementary Tables 10, 19). None of the populations exhibited differences in nuclei number, suggesting that the adipogenic differences we observed were not due to distinct proliferative properties (Suppl. Fig. 2.3g and Supplementary Table 19).

CD142–ABCG1– and CD142– ASPCs (hereafter CD142–(ABCG1–) ASPCs) showed markedly higher differentiation compared to all ASPCs, which suggests that P3 cells inhibit adipogenesis (Fig. 2.2c, d and Suppl. Fig. 2.3d–f). To test this hypothesis, we performed a titration experiment in which CD142+ABCG1+ ASPCs were mixed with CD142–ABCG1– ASPCs in ratios ranging from 0 to 100 per cent. We observed a nonlinear relation that showed a greater decrease in adipogenic differentiation than expected on the basis of a simple dilution effect (Fig. 2.2e, f). Again, we did not observe significant differences in nuclei numbers, which suggests that proliferation remained unaltered (Suppl. Fig. 2.4a and Supplementary Table 19). Thus, a fraction of ASPCs that is characterized by high *CD142* and *ABCG1* expression is refractory to adipogenesis and negatively regulates the adipogenic capacity of other ASPCs. A negatively modulatory function such as this is conceptually reminiscent of T regulatory cells or 'Tregs' that use their immunosuppressive capacity to maintain immune homeostasis and mediate peripheral toler-ance (Vignali et al., 2008); therefore, we suggest that these 'adipogenesis-regulatory' cells be named 'Aregs'.

To characterize Aregs, we profiled the transcriptomes of all ASPCs, CD142+ ASPCs and CD142– ASPCs at four time points: *ex vivo*, upon culture (after 5 and 24 h) and after adipogenic differentiation (Suppl. Fig. 2.4b–f, Supplementary Table 11 and Methods). This transcriptomic data further molecularly confirmed the non-adipogenic character of Aregs. Genes that have previously been associated (Rosen and Spiegelman, 2014) with adipogenesis—such as *Pparg, Fasn, Fabp4, Lpl* and *Fabp12*—were expressed at significantly lower levels in Aregs that were subjected to differentiation, when compared to both all ASPCs and to CD142– ASPCs (Suppl. Fig. 2.4g–i and Supplementary Tables 11, 12, 19). The majority (64% of the differentially expressed genes) of Areg-specific expression patterns were maintained upon plating and only a further 20% of the differentially ex-pressed genes changed their expression after culture for another day (Suppl. Fig. 2.4j). The genes that maintained their expression included those encoding the two surface markers used for the isolation of Aregs (*F3* and *Abcg1*), the early osteogenic marker



Figure 2.2 Aregs (CD142+ABCG1+ ASPCs) show a paracrine adipogenic inhibitory capacity

a, Expression of the P3 and G3 FACS marker F3 (CD142) assessed using scRNA-seg; P1, n = 83 (green); P2, n = 96 (red); P3, n = 29 (blue) cells; G1, n = 699; G2, n = 664; G3, n = 122; and G4, n = 319 cells. **P ≤ 0.01, Wilcoxon rank-sum test. b, Fold-changes (CD142+ versus CD142– ASPCs) of population-specific genes assessed using gPCR. c, Microscopy images of ASPC fractions after adipogenesis. d, Fraction of differentiated cells per ASPC type shown in c, n = 4-5 independent wells. M–, marker-negative, M+, marker-positive. In c, d, experiments were repeated at least three times, yielding similar results. e, Microscopy images of distinct ratios of Lin–SCA1+CD142+ABCG1+ (P3) and Lin–SCA1+CD142–ABCG1- cells after adipogenesis. f, Mean and s.d. of the percentage of differentiated cells per titrated fraction shown in e; n = 4independent wells. g, Heat map (blue-to-red) of expression of genes that show significantly higher expression levels in CD142+ than in CD142– ASPCs after sorting (day 0, D0), plating (5 h) and culture (day 1, D1); expression levels upon adipogenesis (day 12, D12) are also included; lineage (osteogenic Alpl and inflammatory II6) and genes among the top 10 scRNA-seq-derived markers (Fig. 2.1c) are highlighted; n = 4-8, 4 biological replicates, 1–3 independent wells each. h, Microscopy images after adipogenesis of Areg-depleted ASPCs co-cultured with Aregs with Fgf12, Rtp3, Spink2, Vit and control siRNA-mediated knockdown. i, Fraction of differentiated cells measured in the CD142– ASPCs underneath Aregs with the knockdowns shown in h. In e, f, h, i, experiments were repeated two times, yielding similar results. In h, l, n = 6, 2 biological replicates, 3 independent wells each. In all panels, nuclei are stained with DAPI (blue) and lipids are stained with LD540 (yellow). Scale bars, 50 μ m (d, i). *P \leq 0.05, **P \leq 0.01, t-test. See Suppl. Figs. 3–5 for related results.

Alpl and the inflammation-associated cytokine *ll6* (Fig. 2.2g). Forty-seven genes retained their specific expression across plating, culture and adipogenic differentiation, including those genes that encoded the transcription factor MEOX2, the secreted factor DKK3 and the enzyme FMO2, all among the top genes associated with P3 in the scRNA-seq data (Figs 2.1c, 2.2g). Consistent with the scRNA-seq results

(Suppl. Fig. 2.1f, g), we found that genes that were expressed at high levels in Aregs immediately after sorting are characteristic of blood vessels and enrich for the 'Hedgehog signalling' pathway (Suppl. Fig. 2.4k–m and Supplementary Tables 3, 12). However, we did not observe consistently higher expression of endothelial marker genes in Aregs (Suppl. Fig. 2.4n).

We further investigated the mechanism by which Aregs exert their adipogenesis-suppressive effect by testing whether the inhibition requires direct cell-cell contact. Transwell experiments in which ASPCs, Aregs or Areg-depleted ASPCs were inserted above ASPCs revealed that adipogenesis differences were maintained in the absence of direct contact (Suppl. Fig. 2.5a, b), which suggests a paracrine signalling mechanism. To identify genes that may mediate this signal, we performed a loss-of-function screen targeting 23 genes that showed high expression in Aregs compared to Areg-depleted ASPCs and were expressed only at low levels in mature adipocytes (Supplementary Table 13 and Methods). We subjected plated SVF cells to short interfering RNA (siRNA)-mediated reduction in expression of each of these genes, reasoning that knockdown would be effective only in Aregs. Thus, any changes in differentiation would be attributable to alterations in Areg activity. We found that the knockdown of Rtp3, Spink2, Fgf12 and Vit significantly increased the differentiation of plated SVF cells, but not cell number (Suppl. Fig. 2.5c-f and Supplementary Table 19), which suggests that these genes modulate the inhibitory potential of Aregs. To validate these findings, we used transwells comparing the anti-adipogenic effect of wild-type Aregs to the anti-adipogenic effect in Aregs in which each of these genes was knocked down. Loss of Rtp3 and, to a lesser extent, Spink2 and Vit lowered the paracrine inhibitory potential of Aregs (Fig. 2.2h, i and Suppl. Fig. 2.5q-j). Our findings therefore support a paracrine mechanism of action, in which these genes may act up-stream of one or several factors secreted by Aregs or, in the case of *Spink2*, as a direct signalling molecule.

2.2.3 Aregs and their inhibitory capacity are conserved in humans

To investigate whether Aregs constitute a conserved cell population that functions similarly in humans, we used a FACS isolation strategy based on CD142 that was analogous to the strategy we used for the mouse model (Suppl. Fig. 2.6a and Methods). Across three individuals, CD142+ cells exhibited higher levels of *F3* gene expression when compared to CD142– cells (Suppl. 2.Fig. 6b and Supplementary Tables 14, 19), validating our approach. We observed only a mild enrichment of *ABCG1* expression (Suppl. Fig. 2.6b), which may point to species-specific gene expression differences. Similar to mouse CD142+ ASPCs, human CD142+ ASPCs were refractory to adipogenic differentiation, as assessed by lipid accumulation (Fig. 2.3a, b and Methods) and marker gene expression (Suppl. Fig. 2.6b and Supplementary Table 14). Consistent with the mouse data, *F3* expression increased during adipogenesis, which negated the difference in expression between the CD142+ and CD142– populations (Suppl. Fig. 2.6b). We further examined whether the adipogenic inhibitory property is maintained after culture by separating the CD142+ population from the CD142– population in cultured (passage 1) ASPCs (Suppl. Fig. 2.6c), and then inducing adipogenesis. Population differences were consistent across four individuals (Fig. 2.3c, d). We observed significantly larger numbers of mature adipocytes among



Figure 2.3 Aregs and their inhibitory capacity are conserved in humans

a, Microscopy images of *ex vivo* human ASPCs, CD142– ASPCs and CD142+ ASPCs after adipogenesis. **b**, Adipogenic differentiation (arbitrary units, AU) per each fraction shown in a (individual 1, n = 7 fields of view) and another individual (individual 5, n = 20 fields of view). –, CD142–; +, CD142+. **c**, Microscopy images of *in vitro* human (individual 1) ASPCs, CD142– ASPCs and CD142+ ASPCs after adipogenesis. In **a**, **c**, experiments were repeated at least three times (distinct individuals), yielding similar results. **d**, Adipogenic differentiation per each fraction shown in **c**, and in three other individuals (individuals 1 to 4, n = 8 fields of view). –, CD142–; +, CD142+. **e**, Expression of genes that were most-highly expressed in CD142– *versus* CD142+ ASPCs upon adipogenesis (day 12, D12); n = 4 biological replicates (distinct individuals). In all panels, nuclei are stained with Hoechst (blue) and lipids are stained with Bodipy (yellow). Scale bars, 50 µm, *P ≤ 0.05, **P ≤ 0.01, Wilcoxon rank- sum test. See Suppl. Fig. 2. 6 for related results.

CD142– cells than among all ASPCs, which suggests that human CD142+ ASPCs also have an inhibitory capacity (Fig. 2.3c, d and Supplementary Table 19). Transcriptomic profiling of induced cells revealed hundreds of differentially expressed genes among the three fractions, with CD142– ASPCs exhibiting the highest number of differences (Suppl. Fig. 2.6d and Supplementary Table 15).

The adipocyte-like phenotype of CD142– cells was globally confirmed by the high expression of genes associated with adipogenic functionality (Fig. 2.3e, Suppl. Fig. 2.6e–i and Supplementary Table 16), including the adipogenic markers *ADIPOQ*, *CIDEC* and *LPL*. Finally, we performed a cross-species experiment in which human Lin– SVF cells were co-cultured with mouse Aregs or Areg-depleted ASPCs. We observed a reduction in adipogenesis in the transwells containing Aregs (Suppl. Fig. 2.6j, k), which suggests that the paracrine signal is conserved across species. Taken together, our data suggest that Aregs are functionally conserved in humans.

2.2.4 Aregs locate proximal to the vasculature and inhibit adipogenesis *in vivo*

Having characterized Aregs based on cell-culture experiments, we next aimed to study their distribution and effect *in vivo*. Immunofluorescence staining of cryosections of the subcutaneous adipose tissue of mice revealed a CD142 signal that indicated a peri-vascular sheath, which featured small individual cells and was located within the adipose parenchyma but not the lymph nodes (Fig. 2.4a and Suppl. Fig. 2.7a–d, arrows). By combining staining for CD142 with staining for the stem cell marker SCA1, we identified a small fraction of CD142+SCA1+ cells that are also located peri-vas-cularly (Fig. 2.4b–d and Suppl. Fig. 2.7e–g, arrows). This is consistent with our gene expression data, which suggest that Aregs constitute a small fraction of all ASPCs as well as a link to blood vessels (Suppl. Figs 2.1g, 2.2d and 2.4l). We therefore hypothesize that the CD142+SCA1+ cells we detected correspond to Aregs.

Using a FACS strategy analogous to the one used above, we found that Aregs were also present in visceral fat depots (Fig. 2.4e and Methods). The visceral SVF contained a significantly higher proportion of CD142+ABCG1+ cells, when compared to the subcutaneous SVF (Fig. 2.4e and Supplementary Table 19). Furthermore, we found that obese *ob/ob* mice have significantly more Aregs than do lean mice, in both the subcutaneous and the visceral adipose depots (Fig. 2.4e and Supplementary Table 19). To assess whether the presence of Aregs exerts an anti-adipogenic effect *in vivo*, we embedded (1) total SVF or CD142–ABCG1– SVF cells and (2) Lin–SCA1+ or CD142–Lin–SCA1+ SVF cells in Matrigel, injecting them into the left and right subcutaneous layer of the mouse abdomen



Figure 2.4 Aregs locate proximal to the vasculature and inhibit adipogenesis in vivo

a–**d**, Microscopy images of the *in vivo* localization of CD31 (orange), CD142 (green) and SCA1 (pink) in subcutaneous white adipose tissue in mice. Nuclei are stained with Hoechst (blue). Scale bars, 50 μ m. Experiments were repeated at least three times, yielding similar results. Arrows in a indicate CD142+ around big vessels within the adipose parenchyma (left), CD142+ absence in the lymph nodes (middle) and CD142+ in individual cells (right); arrows in **c**, **d** indicate individual cells presenting both CD142 and SCA1 staining (white). **e**, Fraction of Aregs (CD142+ABCG1+ ASPCs) in the subcutaneous and visceral SVF from lean and obese mice; n=4 biological replicates. **f**–**h**, Total SVF and Areg-depleted (CD142–ABCG1–) SVF cells were implanted into the subcutaneous adipose depot in Matrigel. **f**, Image of implantation sites after three weeks of high-fat diet. **g**, Microscopy images of Matrigel plugs with total SVF or CD142–ABCG1– SVF cells. Scale bar, 100 μ m. **h**, Mean and s.d. of the percentage of mature adipocytes that developed within the plugs (n = 5 biological replicates) analysed using CellProfiler25. *P ≤ 0.05, **P ≤ 0.01 paired t-test. See Suppl. Fig. 2.7 for related results.

(Methods). After implantation, the pad-bearing mice were fed a high-fat diet for three weeks to induce adipogenesis. Consistent with our *in vitro* data, we observed a notable difference between the Matrigel pads for all mice (Fig. 2.4f–h and Suppl. Fig. 2.7h–j). The absence of Aregs either from total SVF cells or from ASPCs led to a significantly higher number of mature adipocytes being formed in the implants, as also revealed by the much whiter appearance of Matrigel pads containing CD142–(ABCG1–) cells (Supplementary Table 19). The staining of implant cryosections for endothelial cells using isolectin IB4 revealed a similar degree of vascularisation (Suppl. Fig. 2.7k, I). These findings strongly suggest that Aregs control the formation of mature adipocytes *in vivo*.

2.3 Conclusion

Our single-cell transcriptomics-based approach using a mouse model revealed at least three cell populations in the subcutaneous ASPC-enriched SVF. The largest one was CD55+, and showed enrichment of the adipogenic stem cell markers Cd34 and Ly6a as well as a high adipogenic differentiation capacity in vitro. A recent study has identified a homologous subpopulation in the human SVF, which exhibited an enhanced regenerative potential and was dysfunctional in diabetes (Rennert et al., 2016). These CD55+ cells may therefore exhibit the highest plasticity of all Lin- SVF cells in both mice and humans, a hypothesis that warrants further investigation. We determined that a small (less than 10% of Lin- SVF cells) CD142+ subpopulation suppresses adipocyte formation in vitro and in vivo. To our knowledge, we provide the first molecular characterization and functional follow-up of this CD142+ subpopulation and show that the mechanism of action of this subpopulation possibly involves the genes Spink2, Rtp3, Vit and/or Fgf12. Our findings suggest that depot- and condition-specific differences in hyperplasia might not be solely attributable to alterations in the number of ASPCs, but instead might also be influenced by the number of Aregs, as these latter cells may control the de novo adipogenic capacity of adipose tissue. As adipocytes can also arise in tissues such as bone marrow and muscle (Shook et al., 2016), it is tempting to speculate that *de novo* adipogenesis in these tissues is also controlled through the presence of Areg-like cells. Together, our findings point to a potentially critical role for Aregs in modulating the plasticity and metabolic signature of distinct fat-cell-containing systems, within which they may constitute essential components of the elusive adipogenic precursor niche.

2.4 Miscellaneous

Acknowledgements:

We thank J. Auwerx, W. Chen, E. Dorcey, N. Gheldof, O. Naveiras and K. Schoonjans for constructive discussions and carefully reading the manuscript. This research was supported by the Human Frontier Science Program LT001032/2013 (to P.C.S.), the Swiss National Science Foundation Grants (#31003A_162735 and 31003A_162887), the Kristian Gerhard Jebsen Foundation for Metabolic Research (to B.D.) and by institutional support from the Swiss Federal Institute of Technology in Lausanne (EPFL) and Zürich (ETH). We thank the EPFL FCCF, BIOP (especially R. Guiet and O. Burri), HCF and GECF for cell sorting, imaging, histology and sequencing support, as well as the VITAL-It platform (University of Lausanne) for computational support.

Reviewer information:

Nature thanks O. Stegle and the other anonymous reviewer(s) for their contribution to the peer review of this work.

Author contributions:

B.D., C.W., P.C.S., M.Z. and H.D. designed the study and wrote the manuscript. P.C.S. conducted all analyses related to transcriptomics. N.A., J.R., H.D. and M.Z. performed the single-cell experiments. H.D. and M.Z. conducted all FACS, qPCR, cell culture and related imaging assays. H.D. and W.S. performed all experiments related to transplants. H.D. performed all siRNA- based knockdown experiments. M.Z. and J.R. conducted all histological assays. C.C., K.U.S. and G.S. provided access to human samples and helped with processing them. M.Z. and D.A. performed all experiments related to human cells. All authors read and approved the final manuscript.

Competing interests:

The authors declare no competing interests.

Additional information:

Suppl. is available for this paper at https://doi.org/10.1038/s41586-018-0226-8. Supplementary information is available for this paper at https://doi.org/10.1038/s41586-018-0226-8. Reprints and permissions information is available at http://www.nature.com/ reprints. Correspondence and requests for materials should be addressed to C.W. or B.D.

2.5 Supplementary Figures



Supplementary Figure 2.1 ScRNA-seq reveals the heterogeneity of ASPCs (Fluidigm C1)

a, Number of aligned reads per cell for each of the three Fluidigm C1 (C1) scRNA-seq experiments. R1, n = 74; R2, n = 71; R3, n = 63 single cells from three independent biological replicates, each derived from a pool of mice. **b**, Correlations (corr, Spearman's rho) between expression in single cells across all genes or only the artificial RNA spike-ins (ERCC). **c**, Correlation (Spearman's rho) between expression in merged single cell (per individual biological replicate, R1–R3) and

58

bulk population (n = 4 biological replicates) samples. d, Number of expressed genes/sample in each of the three biological scRNA-seq replicates (R1–R3), as well as the merged individual replicates (SC, n = 3) or the bulk population control samples (bulk, n = 4). e, Expression of ubiquitous *Actb*, negative (*Ptprc* and *Pecam1*) and positive (*Cd34*, *Ly6a* and *Itgb1*) FACS markers. f, g, Pathways significantly enriched (f) and top 10 significantly enriched GTEx tissue samples (g) among the top 200 genes specific to one of the three populations (P1, green; P2, red; P3, blue). Full enrichment results are listed in Supplementary Table 3. h, t-SNE 2D maps of all analysed cells (C1), highlighting the expression of the stem cell marker *Cd34* and the adipogenic marker *Fabp4* (black). i, Bean plots showing the distribution of adipogenic scores across cells be-longing to one of the three populations (P1, n = 83; P2, n = 96; P3, n = 29 cells). **P ≤ 0.01, Wilcoxon rank-sum test. j, Stem cell (lower panel) and adipogenic (upper panel) score distributions across all analysed cells, highlighting a single outlier cell not expressing—or expressing very low levels of—*Itgb1* (CD29), *Cd34* and *Ly6a* (SCA1) as well as very high levels of the mature adipogenic markers *Adipoq, Restn* and *Cidec*. **k**, Correlation (Spearman's rho) between the expression of 12 (pre-)adipogenesis-related genes and the expression of *Fabp4*; significant (Bonferroni multiple testing adjusted P ≤ 0.01) correlations in black. I, t-SNE 2D maps of all analysed cells, highlighting the expression of RFP and various genes previously used to mark adipogenic precursors or preadipocytes (black); microscopic RFP status also displayed. In e, h, j, l, n = 208 cells from three independent biological experiments, each based on a pool of mice.



Supplementary Figure 2.2 ScRNA-seq reveals the heterogeneity of ASPCs (10x Genomics)

a, **b**, Number of aligned reads per cell (a) and number of expressed genes per cell (b) for the scRNA-seq experiment performed using the 10x Genomics Chromium instrument. **c**, Expression of ubiquitous *Actb*, negative (*Ptprc* and *Pecam1*), positive (*Cd34*, *Ly6a* and *Itgb1*) FACS markers and adipocyte markers (*Adipoq, Retn* and *Cidec*). **d**, Number of cells in each of the four 10x Genomics populations (G1–G4). **e**, Percentage of the top 100 marker genes of the 10x Genomics populations (G1 and G4, G2, and G3) that overlap the top 100 marker genes of the C1 cell populations (P1–P3). f, Bean plots showing the distribution of adipogenic scores across cells that belong to one of the four 10x Genomics populations (G1–G4). Only P values for G2 comparisons are marked. **g**, Box plots showing the distribution of log(normalized expression) values of pre-adipogenic and ASPC markers across the four 10x Genomics populations (G1–G4). *P ≤ 0.05, **P ≤ 0.01, Wilcoxon rank-sum test. In e–g, G1, n = 699; G2, n = 664; G3, n = 122; and G4, n = 319 cells. **h**, t-SNE 2D maps of all analysed cells (10x Genomics), highlighting the expression of the pre-adipogenic markers *Pdgfra* and *Pdgfrb* (black). In c, h, n=1,804 cells from one biological experiment based on a pool of mice.





ASPCs M- M+

ASPCs M- M+

Supplementary Figure 2.3 | See next page for caption

Supplementary Figure 2.3 Three ASPC subpopulations with distinct adipogenic differentiation capacity

a, Box plots showing the distribution of log(normalized expression) values for surface markers with available FACS-grade antibodies selected for subpopulation follow-up: CD55 and IL13RA1 (P1, G1 and G4), VAP1 and ADAM12 (P2 and G2) and ABCG1 (P3 and G3). *P \leq 0.05, **P \leq 0.01, Wilcoxon rank-sum test. P1, n = 83; P2, n = 96; P3, n = 29; G1, n = 699; G2, n = 664; G3, n = 122; and G4, n = 319 cells. **b**, FACS-based sorting strategy to isolate the three identified populations. **c**, qPCR-based expression fold-changes (log2(fold- change (FC) marker-positive *versus* marker-negative ASPCs)) for a panel of P1-, P2- and P3-specific genes. **d**, Microscopy images of distinct ASPC fractions after adipogenic differentiation; CD55+ (P1 or G1 and G4), VAP1+ (P2 or G2), and ABCG1+ (P3 or G3). Nuclei are stained with DAPI (blue) and lipids with LD540 (yellow). Scale bar, 50 µm. **e**, Bean plots showing the distribution of the fraction of differentiated cells (Fr. diff.) per each ASPC subpopulation shown in d, n = 4 independent wells. M–, marker negative; M+, marker positive. *P \leq 0.05, **P \leq 0.01, t-test. **f**, qPCR-based expression fold-changes (log2(fold-change, marker-positive *versus* marker-negative ASPCs)) upon adipogenic differentiation for a panel of adipogenic marker genes. **g**, Bean plots showing the distribution of the mean nuclei number for the four differentiated ASPC fractions shown in d, e and Fig. 2c, d. n = 4 or 5 independent wells. In d, e, g, the experiments were repeated independently three times, yielding similar results; representative images are shown. All panels, population 1 (P1, green), population 2 (P2, red), population 3 (P3, blue).

















а

Mean Nuclei +/- SD (103)

2.8 2.9 3.0 3.1 3.2

0 20 40

60

% P3

100

80

Supplementary Figure 2.4 Aregs (CD142+ABCG1+ ASPCs) show adipogenic inhibitory capacity

a. Mean and s.d. of number of nuclei per experiment; x axis represents the percentage of Lin–SCA1+CD142+ABCG1+ cells (P3, Aregs) mixed in with Lin–SCA1+CD142–ABCG1– cells; n = 4 independent wells. b, Schematic of sample collection aiming at a detailed characterization of CD142+ ASPCs versus CD142- ASPCs and all ASPCs, after sorting (D0), upon plating (5 h), after culturing (D1) and after adipogenic differentiation (D12). c, Number of aligned reads (top) and number of expressed genes per sample (bottom) across barcoded RNA-seg samples. d, Correlation (Spearman's rho) between the barcoded mRNA-seq samples after sorting, and the merged scRNA-seq P3 cells; n = 4 biological replicates (top). Example of the correlation (Spearman's rho) between the log(normalized expression) estimates across merged scRNA-seq P3 cells and one barcoded mRNA-seq replicate (bottom). e, Number of genes (×103) that were significantly differentially expressed (FDR 0.05, fold-change 2) in all comparisons between all ASPCs, CD142+ ASPCs and CD142-ASPCs, after sorting (D0), upon plating (5 h), after culture (D1) and after adipogenic differentiation (D12) (left) as well as upon adipogenic induction (D0 versus D12, D0-D12, and 5 h versus D12, 5 h-D12), in all ASPCs, CD142+ ASPCs and CD142– ASPCs (right). f, Venn diagram showing overlaps between significantly differentially expressed genes (FDR 0.05, fold-change 2) in CD142+ ASPCs (+) versus CD142- ASPCs (-) and all ASPCs (ASPC), after sorting (D0) and after adipogenic differentiation (D12). g, Heat map of the expression (row-wise z-scores of log(normalized expression), blue-tored)of genes + significantly differentially expressed (FDR 0.05, fold-change 2) in CD142 ASPCs versus CD142 – ASPCs and versus all ASPCs upon adipogenic differentiation (D12). Adipogenic marker genes are highlighted. h, i, Pathways (h) and top 10 significantly enriched GTEx tissue samples (i) that are significantly enriched among the genes displayed in f. j, Venn diagram showing overlaps between significantly differentially expressed genes (FDR 0.05, fold-change 2) in CD142+ ASPCs versus CD142– ASPCs at all four time points that were assessed. k, Heat map of the expression (row-wise z-scores of log(normalized expression), blue-to-red) of genes that were expressed at significantly higher levels (FDR 0.05, fold-change 2) in CD142+ ASPCs versus CD142– ASPCs after sorting (D0). Genes encoding transcription factors (TFs) and secreted factors (Secr.) are highlighted. I, Top 10 significantly enriched GTEx tissue samples among the genes that were expressed at significantly higher levels (FDR 0.05, fold-change 2) in CD142+ ASPCs versus CD142- ASPCs after sorting, plating and culture (D0, 5 h and D1, respectively) (left) as well as after sorting only (D0) (right). m, Pathways significantly enriched among the genes displayed in k. For h, i, l, m, full enrichment results are listed in Supplementary Table 12. n, Heat map of the expression (row-wise z-scores of log(normalized expression), blue-to-red) of a panel of endothelial marker genes. In g, k, n, n = 4-8, 4 biological replicates, 1–3 independent wells each.



Supplementary Figure 2.5 The adipogenic inhibitory capacity of Aregs is paracrine

a, Microscopy images (after adipogenic differentiation) of mouse ASPCs co-cultured in transwells with all ASPCs, CD142– ASPCs or CD142+ ASPCs. Nuclei are stained with Hoechst (blue) and lipids with Bodipy (yellow). Scale bar, 50 µm. The experiments were repeated independently two times, yielding similar results; representative images are shown. b, Bean plots showing the extent of differentiation (arbitrary units, a.u.) per each ASPC fraction shown in a, as well as an additional independent biological replicate. **P ≤ 0.01, Wilcoxon rank-sum test. Experiment S1: ASPCs and CD142– ASPCs, n = 8; CD142+ ASPCs, n = 7 fields of view; experiment S2: ASPCs and CD142– ASPCs, n = 5; CD142+ ASPCs, n = 7 fields of view. c, qPCR-measured gene expression (Rel. expr.) of knockdowns of Fgf12, Rtp3, Spink2 (n = 4) and Vit (n = 6) in total SVF. Two biological replicates, 2 or 3 independent wells each. d, Microscopy images of plated SVF cells with knockdowns of Fqf12, Rtp3, Spink2 and Vit, as well as control (scramble siRNA) knockdowns, after adipogenesis. Nuclei are stained with DAPI (blue) and lipids are stained with LD540 (yellow). Scale bars, 50 µm. e, Bean plots showing the distribution of the fraction of differentiated SVF cells with knockdowns of Fgf12, Rtp3, Spink2 and Vit, as well as control knockdowns. f, Bean plots showing the distribution of the mean number of nuclei for the differentiated fractions shown in e. In e, f, for Faf12, Rtp3 and Spink2 in experiment S1: n=7, 2 biological replicates, 3 or 4 independent wells each; in experiment S2: n = 8, 2 biological replicates, 4 independent wells each; for Vit: n = 6, 2 biological replicates, 3 independent wells each. q, qPCR-measured gene expression of Faf12, Rtp3, Spink2 and Vit in Aregs of the experiments shown in Fig. 2h, i; n = 6, 2 biological replicates, 3 independent wells each. **h**, Bean plots showing the distribution of the fraction of differentiated cells, as measured in the CD142– ASPCs underneath Aregs with the knockdowns of Fqf12, Rtp3,

Spink2, and control; n = 6, 2 biological replicates, 3 independent wells each (independent replication of the experiment shown in Fig. 2h, i). **i**, Bean plots showing the distribution of the mean number of nuclei for the differentiated fractions shown in Fig. 2h, i (experiment S1) and Suppl. Fig. 5h (experiment S2). n=6, 2 biological replicates, 3 independent wells. **j**, Expression of adipogenic markers measured using qPCR in the CD142– ASPCs underneath Aregs with knockdown of genes shown in Fig. 2h, i; n = 6: 2 biological replicates, 3 independent wells each; Experiments were repeated two times, yielding similar results. In c–j, *P ≤ 0.05, **P ≤ 0.01, t-test.



Supplementary Figure 2.6 Aregs and their inhibitory capacity are conserved in humans

a, FACS-based strategy to isolate human (one individual is shown) *ex vivo* CD142+ ASPCs and CD142– ASPCs. **b**, qPCR-based expression fold-changes (log2(fold change)) of CD142+ ASPCs *versus* CD142– ASPCs for *F3* and *ABCG1* after sorting (left) and *F3*, *PPARG* and *FABP4* after differentiation (right). n = 3 biological replicates (distinct individuals). *P \leq 0.05, one-sided paired t-test. **c**, FACS-based strategy to isolate the human (one individual is shown) *in vitro* CD142+ ASPCs and CD142– ASPCs. **d**, Venn diagram showing overlaps between significantly differentially expressed genes (FDR 0.1, fold-change 2) in CD142+ ASPCs *versus* CD142– ASPCs and all ASPCs after adipogenic differentiation (D12). **e**, **f**, Pathways (e) and top 10 GTEx tissue samples (f) significantly enriched among the genes that were expressed at significantly higher levels (FDR 0.1, fold-change 2) in CD142– ASPCs *versus* CD142+ ASPCs after adipogenic differentiation (Fig. 3e). **g**, Heat map of the expression (row- wise z-scores of log(normalized expression), blue-to-red) of genes that were expressed at significantly higher levels (FDR 0.1, fold-change 2) in CD142+ ASPCs *versus* CD142– ASPCs after adipogenic differentiation (D12). **h**, **i**, Pathways (h) and top 10 GTEx tissue samples (i) significantly enriched among the genes that were expressed at significantly higher levels (FDR 0.1, fold change 2) in CD142+ ASPCs *versus* CD142– ASPCs after adipogenic differentiation (D12). **h**, **i**, Pathways (h) and top 10 GTEx tissue samples (i) significantly enriched among the genes that were expressed at significantly higher levels (FDR 0.1, fold change 2) in CD142+ ASPCs *versus* CD142– ASPCs after adipogenic differentiation (D12) and displayed in g. For e, f, h, i, full enrichment results are provided in Supplementary Table 16. In d–i,

n = 4 biological replicates (distinct individuals). j, Microscopy images (after adipogenic differentiation) of human *ex vivo* ASPCs co-cultured with mouse Areg- (CD142+) or Areg-depleted (CD142–) ASPCs. Nuclei are stained with Hoechst (blue) and lipids with Bodipy (yellow). Scale bar, 50 μ m. The experiment was performed once. k, Bean plots showing the extent of adipogenic differentiation (arbitrary units, a.u.) per each ASPC fraction shown in j. n = 35 fields of view. All panels except b: *P \leq 0.05, **P \leq 0.01, Wilcoxon rank-sum test.



Supplementary Figure 2.7 Aregs locate proximal to the vasculature and inhibit adipogenesis in vivo

a-g, Microscopy images of the *in vivo* localization of CD31 (orange), CD142 (green) and SCA1 (pink) markers in mouse subcutaneous white adipose tissue. Nuclei are stained with Hoechst (blue). Scale bar, 50 µm. The experiments were repeated independently at least three times, yielding similar results; representative images are shown. Negative control (a) or tissue cryosections stained with the indicated secondary antibodies only (b). In **c**, perfused (left) *versus*

non-perfused (right) tissue stained for the indicated markers. In **d**, arrows indicate CD142+ around big vessels within the adipose parenchyma (left), out-side the lymph nodes (middle) and within individual cells (right); in **e**–**g**, arrows indicate individual cells presenting both CD142 and SCA1 staining (white). **h**, Total SVF and Areg-depleted SVF cells (CD142–ABCG1–) were implanted into the subcutaneous adipose depot in Matrigel. Microscopy images of Matrigel plugs with total SVF or CD142–ABCG1– SVF cells. Scale bar, 100 μ m. Individuals 3 to 5 are shown, and individuals 1 and 2 are presented in Fig. 4g. **i**, Mean and s.d. of the percentage of mature adipocytes developed within the plugs composed of either Lin–SCA1+ or Lin–SCA1+CD142– ASPCs (n = 7 biological replicates) analysed using CellProfiler25. *P ≤ 0.01, paired t-test. Mean and s.d. are shown. **j**, Microscopy images of Matrigel plugs corresponding to i. Scale bar, 100 μ m. **l**, Quantification of vascularisation within the plugs, composed of either Lin–SCA1+ or Lin–SCA1+CD142– ASPCs (n = 6 biological replicates) analysed using CellProfiler25.





Supplementary Figure 2.8 Supplementary methods for the scRNA-seq analyses

a, b, t-SNE 2D maps of all analysed cells (Fluidigm C1), highlighting the four subpopulations (P1–P4) obtained when clustering analysis was performed with k = 4 (a) and cells belonging to one of three biological replicates (b) (pink, R1; green, R2; blue, R3). c, For each of the four clusters shown in a, the number of cells stemming from each biological replicate is highlighted. **d**, Silhouette analysis results for k = 3 and k = 4. **e**, Venn diagram showing the overlap between the 527 significantly differentially expressed genes identified using M3Drop in the Letter, and the 1,827 genes showing high variability (HVG). f, For each cluster shown in Fig. 2.1b, we determined the number of cells contained in the alternative clustering obtained by considering 1,827 highly variable genes for SC3 analysis. The vast majority of cells are similarly attributed, irrespective of gene set choice. q, Percentage of the top 100 marker genes of the three subpopulations described in the Letter that overlap with the top 100 marker genes of the subpopulations derived from the analysis based on highly variable genes. h, t-SNE 2D maps of all analysed cells (C1) considering only the highly variable genes, highlighting the three subpopulations (P1–P3) identified by cluster analysis in the Letter (M3Drop, right) and using the highly variable genes (HVG, left). In a, b, h, n = 208 cells from three independent biological experiments, each based on a pool of mice. i, Marker gene expression across all 10x Genomics cells, including a housekeeping gene (Actb), the negative FACS markers Ptprc (Cd45) and Pecam1 (Cd31), the positive FACS markers Cd34, Ly6a (Sca1) and Itgb1 (CD29), and the mature adipogenic markers Adipog, Retn and Cidec. j, t-SNE 2D maps of all 10x Genomics cells highlighting the expression of Xist and the cell division marker Mki67 (black). k, Sum of squares for distinct number of clusters (2 to 15) in the 10x Genomics data. In i, j, n = 2,919 cells from one biological experiment based on a pool of mice, before G1, Xist, Krt18 or Krt19 and Epcam filtering. I, Silhouette width for distinct number of clusters (2 to 13) in the 10x Genomics data. m, Percentage of the top 100 marker genes of the 10x Genomics populations (G1–G4) that overlaps with the top 100 marker genes of the populations (P1–P3) derived from the C1 data based on the highly variable genes.

Chapter 3 In-depth characterisation of Aregs and first insights into the mechanism of their activity

Note: This chapter is based on a study in preparation for publication.

Authors: Magda Zachara, Horia Hashimi, Julie Russeil, Radiana Ferrero, Pernille Y. Rainer, Maria Litovchenko, Romain Hamelin, Florence Armand & Bart Deplancke

Contributions of Magda Zachara: Designed the study, preformed the experiments and analysis, wrote the manuscript.
3.1 Introduction

The discovery of Aregs has intriguing implications in the domain of adipose biology and the single-cell transcriptomic resources that we generated, greatly contribute to the constantly ongoing investigation of the heterogeneity of adipose stem and precursor cells. A plethora of questions however remain unanswered in the context of the composition of adipose tissue and the resident progenitor cells, regarding their transcriptional regulation, well defined molecular fingerprints as well as secretory competence. Understanding the regulatory properties of Aregs could provide answers as to how the dynamics within the ASPCs might be modulated and how it might affect the development and changes of adipose tissues in health and disease. The inhibitory activity of Aregs was shown to occur through the paracrine way, but the molecular actors and the processes behind this regulation are yet to be uncovered. In this part of the study, the existing transcriptomic and initial proteomic data have been integrated in the search for approved molecular markers of Aregs. Subsequently, a few candidates, including secreted proteins and transcription factors, have been experimentally interrogated in the context of Aregs' non-adipogenic character as well as the mechanisms underlying their anti-adipogenic activity, providing first insights into Areg-specific regulation.

The course of investigation of Areg modulatory processes was afflicted by an emergence of a study reporting conflicting observations regarding Aregs (Merrick et al., 2019). To address this discrepancy, an unexpected detour was taken to conduct a series of assays thoroughly reassessing the phenotypic and functional aspects of Aregs, which allowed us to reinforce the robustness of our discoveries, as well as to provide novel and exciting revelations.

In-depth characterisation of the phenotype and functionality of Aregs

A recent study (Merrick et al., 2019) challenged the phenotypic properties of Aregs (Schwalie et al., 2018). This study also used single-cell RNA sequencing (scRNA-seq) to characterize the adipose progenitor-containing cell population from the developing subcutaneous adipose depot of 12-day old C57BL/6J mice with the aim of identifying and profiling the mesenchymal progenitor cell hier-archy in adipose tissue. The reported computational analysis of the Lin– compartment overlapped to a great extent with our previous findings (Schwalie et al., 2018) and led to the identification of three major clusters of adipose stem and precursor cells that highly corresponded to clusters identified in our study, even though the Lin– cells in our assays originated from adult animals.

As in our analysis, these groups (P1 to P3) delineated predominant expression of canonical mesenchymal progenitor markers including *Cd34*, *Ly6a* and *Pdgfra*. Group 1 was termed "interstitial progenitors" and featured expression of *Dpp4*, *Pi16* (present as the 8th and 4th most specific markers for the corresponding population (G1) in our analysis) and *Wnt2* and did not present the expression of preadipocytic markers. Group 2 cells showed high expression of *lcam* together with a few preadipocyte identity markers, including *Pparg*, *Fabp4* and *Cd36*, all of which were specific to the corresponding Population 2 across our datasets (P2 and G2), suggesting that this group corresponds to what might be more committed adipocytes. Group 3 cells were reported to be characterised by the high expression of *F3* (coding CD142), *Clec11a* and *Fmo2*, all of which specifically mark the identity of our Population 3, Aregs (Merrick et al., 2019). Interestingly, overlapping the top cluster-defining genes of Group 3 and Aregs yielded an assembly of highly Aregs-specific genes including, amongst others, *Meox2*, *Mgp*, *Gdf10* and *Bgn* (Merrick et al., 2019). This high consistency is even more striking considering that the assessed animals were from different developmental stages.

The discrepancy appeared when the authors reported different phenotypic behaviour of ASPCs from the subcutaneous adipose tissue of adult CD1 mice isolated by population-specific markers. Group 1 cells isolated with the surface marker DPP4, showed to be resistant to *in vitro* adipogenic differentiation, while the two other populations, Group 2 ASPCs isolated with the use of ICAM1 marker and Group 3 cells defined by the CD142 marker showed high differentiation propensity upon receiving a complete as well as a minimal (insulin only) adipogenic stimulus (Merrick et al., 2019).

These observations are contradictory to our findings, especially considering the completely non-adipogenic character of CD142+ ASPCs isolated from subcutaneous depots of adult C57BL/6J mice that we demonstrated. Having consulted the experimental procedures of the study in question in order to understand where the presented difference might stem from, we discovered that a number of important parameters were modified, including FACS-based gating strategies, adipogenic cocktail composition as well as the age and sex of animals (Table 3.1). Determined to clarify the issue and to re-establish the robustness of the Areg phenotype, we set out to verify if changing any of the parameters could alleviate the non-adipogenic character of Aregs.

3.2 Results

3.2.1 Aregs defined by different anti-CD124 antibodies present a robust phenotype

It was proposed that the observed differences in the functional properties between the CD142+ ASPC population isolated by Merrick and colleagues, and our Aregs were due to the utilized FACS strategies for cell purification and our use of ABCG1 as an additional Areg-specific marker (Merrick et al., 2019, Hwang-Kim, 2019). We therefore decided to address the FACS-based strategy of isolating Aregs and assayed various anti-CD142 antibodies. Merrick and colleagues used the same clone of the anti-CD142 antibody (a rabbit monoclonal clone #001, SinoBiological (SB), Methods) than the one used in our published study. The antibody used was however coupled to a fluorophore in-house, while we used a PE-conjugated clone. We resolved to reproduce this approach, isolating Aregs also using an conjugated clone. In order to add robustness to this assay, we included two more anti-CD142 antibodies (mouse monoclonal clone HTF-1 PE-conjugated, BiOrbyt (BO) and a goat polyclonal PE-conjugated, R&D Systems (R&D), Methods).

Examining the flow cytometry (FC) profiles of the tested antibodies namely, 1) SinoBiological anti-CD142-PE antibody (SB), 2) SinoBiological anti-CD142 antibody in-house conjugated to a PE fluorophore with the use a Lightning-Link kit (SB-LL, Methods), 3) R&D Systems anti-CD142 antibody (R&D) and 4) BiOrbyt anti-CD142-PE antibody (BO), we consistently observed for all clones that the anti-CD142 antibody does not allow a clear determination of a negative and a positive cell population as it exhibits a distribution rather than a separation of events corresponding to the degree of CD142 marker presence on the cell membrane (Fig. 3.1a). The gating strategy for isolation of Aregs based on this rather challenging profile had been determined empirically in the early stages of the project and it had been set to around 5-7% of cells, as those were considered the most positive for CD142 (Schwalie et al., 2018). The accuracy of this approach was verified by assessing specific gene expression in the sorted subpopulations (Fig. 2.2b). For the tested anti-CD142 antibodies, we observed a similarity of the FC profiles spanning more than 5 orders of magnitude of the fluorescence intensity for all antibodies except the BiOrbyt/HTF-1 clone (Fig. 3.1a – bottom panel). However, given the varying emplacement of the maximal fluorescence intensity, the gating strategy had to be adapted to reproduce our initially validated isolation approach, with 5-7% of cells being defined as the most positive for CD142 and therefore identified as Aregs and the majority of events with the maximum of intensity corresponding to CD142-ASPCs, the molecular counterparts of Aregs. Such gating strategy was therefore used to sort Aregs defined by the four distinct antibodies for downstream phenotypic assessment.

Remarkably, the tested antibodies yielded highly consistent results with Aregs being completely non-adipogenic and CD142– ASPCs presenting a significantly (except the BO antibody) higher adipogenic potential than the total ASPCs (Fig. 3.1b and c). This is in agreement with our previous findings (Schwalie et al., 2018) and confirms the inability of Aregs, CD142+ ASPCs defined as shown here, to give rise to *in vitro* adipocytes. In addition, it points to their anti-adipogenic character with the CD142– ASPCs differentiating to a substantially higher degree than the total ASPCs. This assay, using different clones of antibodies from different sources, shows the phenotypic robustness of Aregs. We also observed that all the isolated Aregs show a lower cell number than their molecular counterparts as well as the total ASPCs post-differentiation (Fig. 3.1d). This might be caused by a diminished survival, lower proliferation rate but also by Aregs being bigger in size, thus attaining contact inhibition at a lower total cell number per field of growth. The back-gating analysis of Aregs points to the latter, with Aregs localising to an area within the scatter plot corresponding to bigger cells compared to CD142– ASPCs (Suppl. Fig. 3.1a). Interestingly, we have previously observed a bigger size of CD142+ human ASPCs in an Image Stream assay involving cells from three individuals in two biological replicates (Suppl. Fig. 3b-e, Methods). In this flow cytometry-based method equipped with visualising



Figure 3.1 Aregs sorted with different antibodies present a robust non-adipogenic phenotype

a, FACS-based gating strategy to isolate Aregs, defined as 5-7% CD142+ ASPCs, and CD142– ASPCs defined as around 50% cells negative for CD142 marker, with the use of four different antibodies "SB" – SinoBiological anti-CD142–PE, "SB-LL" SinoBiological anti-CD142 coupled to PE in house (Methods), "R&D" – R&D Systems anti-CD142–PE and "BO" – BiOrbyt anti-CD142–PE; the experiment was repeated n=4 with similar results, represented is one biological replicate. **b**, Representative florescence microscopy images of total ASPCs, CD142– ASPCs and Aregs isolated with the use of the respective anti-CD142 antibodies described in a, after *in vitro* adipogenic differentiation; the presented images correspond to 25 tiled and thresholded 20x images containing 7-8 z-stacks in order to capture the majority of the well surface (Methods), n=4, as above. **c**, Boxplots showing the distribution of the lipid accumulation (Adipogenic score - arbitrary unit) shown in b. **d**, Boxplots showing the distribution of the normalised nuclei number for cellular populations shown in b. In all images, nuclei are stained with Hoechst (blue) and lipids are stained with Bodipy (yellow). For c and d all values in each independent experiment were normalised to ASPCs, represented are n=4 biological replicates, with n=3 technical replicates in each; for statistics details see Methods.

devices we could consistently observe that, while the circularity of all gated cell populations was comparable (Suppl. Fig. 3c), the area of CD142+ ASPCs was higher compared to the CD142– and total ASPCs (Suppl. Fig. 3d), and corresponded to a higher intensity of the fluorescence coming from the fluorophore of the CD142 marker (Suppl. Fig. 3e). Moreover, the analysis of an average size of nuclei inferred from the total area of fluorescence derived from the Hoechst signal *versus* the total number of nuclei per field of growth, revealed a significantly higher average size of nuclei for Aregs than for the CD142– ASPCs and total ASPCs, and lower for CD142– ASPCs (Suppl. Fig. 3.1f), an observation corroborating with the corresponding size profiles of Aregs and CD142– ASPCs in the flow cytometry back-gating analysis.

Together, these observations eliminate the potential gating strategy as well as the source of the antibody used for Areg isolation as a cause for the discrepancy of the Areg phenotype. Interestingly,

the isolation strategy of Merrick and colleagues was validated by bulk RNA sequencing of CD142+ ASPCs, in which numerous markers overlapping with Aregs were identified including highly ranked *F3*, *Clec11a*, *Gdf10*, *Bgn*, *Mgp* and *Meox2*, and confirming therefore that the segregated population corresponds to Aregs at the molecular level.

3.2.2 Aregs isolated with different strategies and treated with various differentiation cocktails remain non-adipogenic

Another dissimilarity we remarked while analysing the differences potentially underlying the discrepancy in the context of adipogenic potential of Aregs was the fact that Merrick and colleagues isolated CD142+ ASPCs following a different strategy as reported by us. Up to 20% of ASPCs were isolated by FACS as CD142+ ASPCs (Merrick et al., 2019). To our understanding, a cell population defined in such a way can no longer be considered as Aregs, which are defined as a small subpopulation of ASPCs (corresponding to our scRNA-seq data) highly expressing CD142 marker. Moreover, we discovered that the adipogenic cocktails with which the isolated cells were treated in the discussed study greatly differed from ours. The complete cocktail used by Merrick and colleagues, besides insulin (being delivered at a lower concentration of 20 nM *versus* 170 nM in our case), IBMX (3-isobutyl-1-methylxanthine) and dexamethasone (at equal concentrations), also contained T3 (triiodothyronine) and indomethacin, while the minimal cocktail contained insulin (20 nM) only. Furthermore, the induction cocktail was delivered to the cells cultured in DMEM/F12 (ratio 1:1) cell culture medium as opposed to DMEM only, used in our study (Table 3.1, Methods).

After having conducted a set of assays in which we implemented all the above-described conditions, we could demonstrate that sorting CD142+ ASPCs with the less stringent gating (20% as opposed to 5-7%, Fig. 3.2a) did not result in their potential to give rise to in vitro adipocytes (Fig. 3.2b). We next treated the isolated cell populations with all versions of induction cocktails including the classical complete medium (Insulin, IBMX, and dexamethasone) in DMEM, the complete medium used by Merrick and colleagues supplemented with T3 and indomethacin in DMEM/F12, as well as two versions of minimal cocktails containing either DMEMD with 170 nM or DMEM/F12 with 20 nM of insulin. The maintenance conditions also presented differences, with the classical maintenance cocktail consisting of 170 nM insulin in DMEM and the one used by Merrick and colleagues containing 20 nM insulin and T3 in DMEM/F12, with no maintenance compounds used in both minimal conditions (Table 3.1, Methods). Again, neither Aregs, nor CD142+ ASPCs sorted at 20% (CD142+ ASPCs), showed adipogenic propensity in vitro. A slight increase in the lipid accumulation, in the form of a few more differentiated cells, could be observed for CD142+ ASPCs compared to Aregs for all tested differentiation cues, but none of the differences were significant (Fig. 3.2c). The cell proliferation was also found comparable between Aregs and CD142+ ASPCs with the classical versions of the cocktails. Cocktails delivered in DMEM/F12 however, showed to lower the number of CD142+ ASPCs but not Aregs (Suppl. Fig. 2a). CD142- ASPCs tested alongside revealed interesting differences upon the assayed conditions. The differentiation potential of this highly adipogenic ASPC population was significantly higher





a, FACS-based gating strategy to isolate Aregs, 20% CD142+ ASPCs and CD142– ASPCs; the experiment was repeated at least n=4 with similar results, represented is one biological replicate. **b**, Representative florescence microscopy images of Aregs, 20% CD142+ ASPCs and CD142– ASPCs isolated following the sorting strategy shown in a, after *in vitro* adipogenic differentiation with the indicated adipogenic differentiation cocktails (for details see Methods); the presented images correspond to 25 tiled and thresholded 20x images containing 7-8 z-stacks in order to capture the majority of the well surface (Methods), n=4, as above. **c**, Bar plots showing lipid accumulation (Adipogenic score - arbitrary unit) shown in b, the height of the bar indicating the mean of the measurements averaged across all replicates, represented are n=4 biological replicates, with n=3 technical replicates in each; for statistical details see Methods. In all images, nuclei are stained with Hoechst (blue) and lipids are stained with Bodipy (yellow).

when exposed to the complete cocktail supplemented with T3 and indomethacin in DMEM/F12 medium, with no significant differences between complete and minimal versions of the cocktails (Fig. 2c). The CD142– ASPC cell numbers were also negatively affected by the use of cocktails administered in DMEM/F12 (Suppl. Fig. 2a). Upon further investigation, we could also demonstrate that the degree of differentiation of CD142– ASPCs seemed to be determined by the medium in which the differentiation cocktails were delivered, with higher adipogenesis for all versions of induction administered in DMEM/F12 *versus* DMEM. (Suppl. Fig. 2b-c), which might be explained by the presence in F12 but not in DMEM of freely available fatty acids potentially facilitating or accelerating intracellular lipid accumulation (Mamontova et al., 2018).

We could therefore show that as much as the dissimilarities in the tested differentiation conditions manifest phenotypically in the context of *in vitro* adipogenesis of CD142– ASPCs, this was not the case for Aregs nor for CD142+ ASPCs, which remain undifferentiated even when being exposed to the more "aggressive" pro-adipogenic stimuli.

3.2.3 Sex-based differences in the context of ASPCs and Aregs

Our above-presented exploratory single-cell-based study leading to the discovery of Aregs as well as its experimental validation were deliberately conducted in both male and female adipose stem and precursor cells in order to render the study more unbiased. It is however generally accepted that sex-determined differences might influence numerous *in vitro* assays. We therefore resolved to examine ASPCs from male and female animals separately in the context of Aregs and their adipogenic differentiation potential. Our motivation was reinforced by the lack of consistency of sex-composition of experiments presented by Merrick and colleagues, reporting certain experiments performed with both male and female animals and others with male animals only (Merrick et al., 2019).

Male and female SVF cells proved similar in the context of flow cytometry-based phenotypic profiles regarding mesenchymal and pre-adipogenic markers with the only difference residing in the significantly higher fraction of SCA1+ cells within the male stromal cells (Fig. 3.4a-c). No difference was apparent in the profile or the percentage of CD142 distribution within the ASPC pool between the two sexes (Fig. 3.4a-c). Upon adipogenic differentiation, we could observe a significantly higher adipogenic propensity of male ASPCs as well as CD142– ASPCs, potentially linked to the higher portion of SCA1+ cells within the SVF, but no difference was apparent for Aregs, which showed no lipid accumulation for neither male or female, replicating the Areg phenotype established in the mixed population (Fig. 3d and e). Interestingly, we could observe a significantly lower cell number of cultured male compared to female Aregs (Fig. 3.3f). These preliminary observations lay ground for investigating interesting sex-based differences in the context of phenotype, adipogenic propensity and cell proliferation. Importantly though, our analyses revealed no obvious differences with regard to Aregs, confirming their robust identity across both sexes.





a and **b**, FACS-based gating strategy of a, male and b, female Lineage negative, SCA1+ (ASPCs), CD142– and CD142+ ASPCs (Aregs) cellular fractions within the SVF; the experiment was repeated n=4 with similar results, represented is one biological replicate. **c**, Bar plots showing the percentage of cellular fractions gated as shown in a and b; the experiment was repeated n=4. **d**, Representative florescence microscopy images male and female ASPCs, CD142– ASPCs and Aregs, isolated following the sorting strategy shown in a and b after *in vitro* adipogenic differentiation; the presented images correspond to 25 tiled and thresholded 20x images containing 7-8 z-stacks in order to capture the majority of the well surface (Methods), n=4, as in a. **e**, Bar plots showing lipid accumulation (Adipogenic score - arbitrary unit) shown in d; represented are n=4 biological replicates, with n=3 technical replicates in each. **f**, Bar plots showing normalised nuclei number of cell fractions shown in d, represented are n=4 biological replicates, with at least n=3 technical replicates in each. In c, e and f the height of the bar indicates the mean of the measurements averaged across all replicates; for statistical details see Methods. In all images, nuclei are stained with Hoechst (blue) and lipids are stained with Bodipy (yellow).

3.2.4 Aregs derived from new-born mice give rise to adipocytes *in vitro*

An additional condition we decided to interrogate was the age of animals from which the cells were isolated, as a number of experiments reported by Merrick and colleagues were performed on new-born mice or on 8-12-day old pups.

In order to address this, burgeoning subcutaneous adipose depots were dissected from newborn mice (Methods) and the stromal vascular fraction was interrogated in the context of the expression of the lineage and pre-adipogenic markers. Interestingly, the overall flow cytometry profile was similar to the adult one (Fig. 3.4a-b), with, nevertheless, significant differences in the percentages of various populations. The lineage negative (CD31- CD45- TER119-) fraction constituted 90.3% (+/- 3.7%) within the stromal vascular fraction of new-born pups compared to 62.1% (+/-10.6%) of the Lin- population within the adult SVF (Fig. 3.4c). In contrast, the proportion of SCA1+ cells, which in adult animals constitutes an important part of the Lin- population (69.2% +/-11.3%), was significantly lower (25.4% +/-5.4%), suggesting that the SVF of new-born pups contains Lin– cells that have an identity that is likely non-pre-adipogenic, which can also be appreciated when back-gating the SCA1+ cells (Suppl. Fig. 3.3a). Interestingly, a unique population could be observed within the SVF cells of new born mice, which was not observed in the adult SVF (Fig. 3.4a-b SCA1 and CD142 profiles). Indeed, a back-gating analysis of this pup-specific, partially SCA1+ cell population revealed that it contains cells that occupy the part of the Lin- fraction that is not positive for the SCA1 marker (Suppl. Fig. 3.3b). We also observed that the profile representing granularity and size of the analysed cells, did not seem to show the aspect and emplacement that the mesenchymal cells tend to present (Suppl. Fig. 3.3b, top panel, Scatter). Lack of a bigger choice of markers as well as the lineage antibodies being all conjugated to the same fluorophore, does not allow us to further investigate the identity of this peculiar pup-specific cell population within this set-up. Another interesting observation was the smaller size of Aregs compared to what we could observe within the adult SVF (Suppl. Fig. 3.1a and 3.3c), with in fact CD142- ASPCs appearing to be slightly bigger. Moreover, all considered pup-derived populations, namely Aregs, CD142+ ASPCs and CD142- ASPCs showed to be largely negative for SCA1, while they are mainly SCA1+ in adult SVF (Suppl. Fig. 3.1a and 3.3c). The marked dissimilarities observed concerning the aspect of the ASPCs in regards to expression of mesenchymal and pre-adipogenic marker between these two sources might stem from their very different developmental stages and the ongoing ASPC hierarchy establishment that occurs within the new-born fat depots.

In order to examine the pups-derived ASPC populations of interest in downstream functional assays, we next isolated Aregs, CD142+ ASPCs (20%) and CD142- ASPCs and interrogated them phenotypically in the context of adipogenic propensity in vitro, using the set of differentiation cocktails described above (Fig. 3.2, Methods). Remarkably, we observed that pups-derived Aregs and CD142+ ASPCs underwent a robust adipogenic differentiation upon the treatment of both complete cocktails, to an extent that was comparable to the one of CD142– ASPCs, an unprecedented observation in our hands (Fig. 3.4.d-e, Suppl. Fig. 3.4a-b, Aregs were differentiated with complete cocktails only given their limited numbers in pups). The minimal stimulus in the form of insulin was however not sufficient to assure the differentiation of any of the populations, with colonies of cells undergoing differentiation upon insulin delivered in DMEM/F12 in the case of CD142- ASPCs (Fig. 3.4d). The adipogenic effect of the complete cocktail used by Merrick and colleagues was still more pronounced for all assayed populations (Fig. 3.4d-i). Interestingly, we did not observe marked differences in proliferation capacity of the considered ASPCs populations, which is clearly the case for adult animals. Rather, we noticed that minimal cocktails resulted in lower cell numbers most possibly due to the lack of dexamethasone and IBMX (Suppl. Fig. 3.4b). Under a higher magnification, we made a striking observation regarding pup-derived Aregs, CD142+ ASPCs and CD142- ASPCs in that cells from all of these populations underwent adipogenesis within virtually every cell. This was true for both conditions and with a slight gradient





a and **b**, FACS-based gating strategy of a, pups- and b, adult-derived Lineage negative, SCA1+ (ASPCs), CD142–, 20% CD142+ ASPCs and Areg cellular fractions within the SVF; the experiment was repeated n=4 with similar results, represented is one biological replicate. **c**, Bar plots showing the percentage of cellular fractions gated as shown in a and b; the experiment was repeated n=4. **d**, Representative florescence microscopy images pups- and adult-derived 20% CD142+ ASPCs and CD142– ASPCs, isolated following the sorting strategy shown in a and b after *in vitro* adipogenic differentiation with the indicated adipogenic cocktails (Methods); the presented images correspond to 25 tiled and thresholded 20x images containing 7-8 z-stacks in order to capture the majority of the well surface (Methods), n=4, as in a. **e**, Bar plots

of the overall degree of differentiation increasing from Aregs towards CD142– ASPCs and with the Merrick complete medium allowing the cells to accumulate more lipid droplets with a clearly higher lipid content (Fig. 3.4f and h). This phenotype dramatically contrasts with the adult cells, where only CD142– ASPCs efficiently accumulate lipids, with still more efficient differentiation observed with the Merrick complete cocktail (Fig. 3.4.g and i). We could also observe remarkable differences regarding cell proliferation between pups- and adult-derived ASPC populations with significantly lower cell numbers for all adult populations in all tested conditions (Fig. 3.4g and Suppl. Fig. 3.4c-e). These striking phenotypic properties of all considered pup-derived ASPC populations could be considered intuitive given the developmental stage of these cells. It is curious however to observe these dramatic differences between the new-born and adult pools of ASPCs, especially since the corresponding single-cell-based profiles correlated to a large degree (Merrick et al., 2019). We now intend to investigate transcriptomic signatures of these populations in their native, freshly isolated as well as adipogenic with the aim of identifying relevant markers and regulators.

Although we could, for the first time, observe Aregs undergoing differentiation, and this in the context of new-born mice, we believe that the conundrum caused by Merrick and colleagues reporting "adipogenic Aregs" is still not entirely resolved. In their study, the authors work with various genetic backgrounds including C57BL/6J, the *bona fide* strain for *in vivo* and *in vitro* metabolic studies (used in all our experiments), but also the outbred CD1 strain as well as the 129S6/SvEv line. It will be interesting, and necessary for the consistency in the field, to also address the above-discussed in the various strains.

Investigation of the mechanism of the inhibitory activity of Aregs

Aregs, capable of negatively inhibiting adipogenesis, were demonstrated to exert their action *via* a secreted principal (Schwalie et al., 2018). In order to understand how this inhibition occurs mechanistically, it is necessary to inquire about the specific molecular identity of Aregs. In the course of the study, a few preliminary candidates including *Spink2*, *Vit* and *Fgf12* were identified as presenting high expression in Aregs compared to CD142– ASPCs as well as not being expressed in mature adipocytes. These candidates were experimentally followed-up by our collaborators.

To address a broader spectrum of candidates, a number of previously and newly generated datasets were integrated qualitatively with the aim of delineating the shared most Areg-specific genes identified by various approaches.

showing lipid accumulation (Adipogenic score - arbitrary unit) shown in d; represented are n=3 biological replicates with n=2 or 3 technical replicates. **f** and **g**. Representative florescence microscopy images of f, pups- and g, adult-derived Aregs, 20% CD142+ ASPCs and CD142– ASPCs after *in vitro* adipogenic differentiation with the indicated adipogenic cocktails (Methods), the images represent a blow-up of images shown in d for pups and Fig. 3.2d for adult cells. **h**, Bar plots showing lipid accumulation (Adipogenic score - arbitrary unit) shown in f; represented is one biological replicate with n=2 or 3 technical replicates. **i**, Bar plots showing lipid accumulation (Adipogenic score - arbitrary unit) shown in f; represented is one biological replicate with n=1-6 technical replicates. In all images, nuclei are stained with Hoechst (blue) and lipids are stained with Bodipy (yellow). In c, e, h and i the height of the bar indicates the mean of the measurements averaged across all replicates; for statistical details see Methods.

84

3.2.5 Genomic data integration

First, the results generated by the two single-cell RNA sequencing experiments (Fluidigm C1 and 10x Genomics, Schwalie et al. 2018), were integrated by inferring the common most representative candidate genes for population P3 in the C1 dataset and population G3 in the 10x dataset corresponding to Aregs. The top 200 genes from both datasets were overlaid, yielding 65 common markers. These were then ranked as a function of their relevance for Aregs across the two datasets (Table 3.2). Next, we overlapped the list of such identified genes with the Areg-specific candidates identified by the bulk RNA-sequencing experiment involving freshly isolated ASPCs, CD142– ASPCs and Aregs (Schwalie et al., 2018). As much as integrating datasets from two scRNA-seg assays (even though different in terms of capture and processing technology) is fully justifiable and yields over 30% overlap, merging these data with a bulk RNA sequencing dataset is less obvious. Major differences between these two kinds of datasets must be considered, including the fact that genes detected using a single cell approach might be masked in a bulk assay. Conversely, lowly expressed genes might be detected using bulk RNA-sequencing, given the abundance of material, but missed with the less sensitive single-cell approach. Nevertheless, merging the single-cell and the top 200 bulk RNA-seq Areg-specific hits provided a list that was further narrowed-down to 20 common candidates (Suppl. Fig. 3.5a, Table 3.3). The generated overlaps lead to determining a set of candidates for further downstream functional validation. Amongst these candidates, seven corresponded to genes coding for secreted proteins. These included F3, the highest ranked common secreted protein coding candidate gene (C1, 10x and bulk RNA-seq overlap), followed by the next top three secreted protein coding genes Mgp, Clec11a and Gdf10 (Table 3.3). In addition, we also found Cpe, Ban and Cxcl12, given their previously observed involvement in adipogenesis, all together within the top 35 most relevant Aregs-markers within the two single-cell datasets. Two candidates for validation were transcription factor coding genes namely Meox2, the highest ranked common TF-coding candidate (Table 3.3), as well as Pknox2, not present in the "overlap list" but identified in the bulk RNA-seg and reported recently as novel potential requlator of browning in the context of lowly differentiating progenitor clones (Li et al., 2019). Finally, we also identified via bulk transcriptomics only the retinal dehydrogenase (RALDH2) coding gene Aldh1a2, implicated in the catalysis of retinoic acid, an established and potent adipogenesis inhibitor (Takeda et al., 2016).

The expression plots of the candidate genes, ordered by their overall relevance for Aregs, namely *F3*, *Meox2*, *Mgp*, *Clec11a*, *Gdf10*, *Cpe*, *Bgn* and *Cxlc12*, allow appreciating the degree of expression of the selected markers in the context of the considered clusters. The C1 dataset shows the expression of genes within the three identified clusters from P1 to P3, with the blue P3 population corresponding to Aregs (Fig. 3.5a and b) and the 10x dataset depicts the expression within the four clusters from G1 to G4, with the blue G3 group representing Aregs (Fig. 3.5c and d). The specificity of expression of the candidates across all the interrogated cells is illustrated by the tSNE plots representing the presence of genes in the context of the global relative expression relationships amongst all the considered cells (Suppl. Fig. 3.5b and c).

The high specificity of the chosen genes was also confirmed by the bulk RNA sequencing data and represented by their relative expression within Aregs, total ASPCs as well as the molecular counterparts of Aregs, CD142– ASPCs (Fig. 3.5e).





b, Corresponding t-SNE 2D cell map showing the C1 dataset clustering. c, Box plots showing the distribution of log(normalized expression) values of indicated Areg-specific markers across the four 10x populations (G1 - light green, G2 - red, G3 - blue, G4 - dark green). d, Corresponding t-SNE 2D cell map showing the 10x dataset clustering. e, Box plots showing the distribution of log(normalized expression) values of indicated Areg-specific markers in the bulk RNA sequencing of ASPCs, CD142– ASPCs and Aregs. For staa, Box plots showing the distribution of log(normalized expression) values of the indicated Areg-specific markers across the three C1 populations (P1 – green, P2 – red, P3 – blue). tistical details see Methods.

3.2.6 Proteomic analysis of Aregs

Recently, we complemented our genomic dataset collection with an initial mass spectrometry analysis assessing Aregs and CD142– ASPCs. Apart from discovering novel putative candidates, we were eager to interrogate the proteomic dataset with the intention of detecting the common Areg-specific candidates that we identified using the transcriptomic datasets. Using the lists of top 200 Areg-specific





a to **i**, Correlations of LFQs of quantified proteins. **a**, **b** and **c**, Correlation of the LFQ intensities between freshly sorted CD142– ASPCs and freshly sorted Aregs, with highlighted points corresponding to 80 proteins in the top 200 Areg-specific marker genes from the C1 scRNA-seq dataset, 76 proteins in the top 200 Areg-specific genes from the bulk RNA sequencing dataset, represented in orange, in green and in dark blue respectively; **d** and **e**, correlations of the LFQ intensities between CD142–ASPCs and Aregs, in freshly sorted (d) and expanded (e) cells. Purple points correspond to selected Areg-specific marker candidates; **f** and **g**, Represented are statistically differentially expressed proteins with a Z-score sliding window with a FDR <= 0.01, FDR <= 0.05 and FDR < 0.1 represented in red, orange and light blue respectively. Grey points correspond to proteins not differentially expressed (FDR > 0.1); h and i, Correlation of the LFQ intensities between CD142–ASPCs and Aregs secretomes (h) and the corresponding cells (i); The solid black line corresponds to the 1:1 line.

genes we could identify 80 proteins corresponding to C1 single-cell dataset markers, 76 proteins to the 10x list and 35 proteins overlapping with the bulk transcriptomics-derived candidates, many of which showed significantly higher abundance in Aregs (Fig. 3.6a-c, Tables 3.4, 3.5 and 3.6). Further comparisons allowed us to identify 31 proteins corresponding to the candidates common for the C1 and 10x datasets (Suppl. Fig. 3.6a) and 9 proteins overlapping with the markers common for all three genomics datasets (Suppl. Fig. 3.6b). A number of candidates chosen for the experimental follow-up, were validated by the proteomic approach, with the significantly higher abundance of CD142, MEOX2, BGN as well as RALDH2 (encoded by Aldh1a2) in freshly sorted Aregs, all of which (except for MEOX2) were retained after a few days of culture (Fig. 3.6d and e). Interestingly, discovery of novel Areg-specific proteins yielded many candidates corresponding to enzymatic markers identified previously in our genomic assays, with RALDH1 being the top common hit for sorted and expanded Aregs, followed by Indolethylamine N-methyltransferase (encoded by Inmt), Ectonucleoside triphosphate diphosphohydrolase 2 (Entpd2), Dimethylaniline monooxygen-ase [N-oxide-forming] 2 (Fmo2) and Rho GDP-dissociation inhibitor 2 (Arhgdib) (Fig. 3.6a and g, Suppl. Fig. 3.6c and d). Completely novel candidates included a transcription factor LIM and cysteine-rich domains protein 1 (Lmcd1) involved in restricting GATA6 function by inhibiting DNA-binding (Rath et al., 2005).

In addition, we identified Endoglin (*Eng*) a vascular endothelium glycoprotein known to play an important role in the regulation of angiogenesis (Arthur, 2000), evoking the previously discussed link of Aregs to blood vessels (Fig. 2.4a-d, Suppl. Fig. 2.4l and n). Finally, we also found Myosin-11 (*Myh11*), myosin heavy chain, the smooth muscle isoform (Liu et al., 2015b), again suggesting a blood vessel association.

We also interrogated the secretome of Aregs in a, so far, preliminary experiment, which, interestingly, identified CD142 as one of Areg-specific differentially secreted proteins. We could also detect BGN, CPE and CXLC12, but as much as they were specific for Areg cells from which the secretome was harvested, their secreted forms were detected at a higher degree in CD142– ASPCs rather than Areg conditioned medium (Fig. 3.6h and i). Within the secreting Aregs, we could also detect RALDH2 and GDF10, presenting a high abundance fold change compared to CD142– ASPCs (Fig. 3.6i).

Although preliminary, the proteomic analysis of freshly sorted and expanded Aregs, as well as the Areg secretome showed a notable congruence with our genomic data, highlighting the robustness of Aregs as a novel ASPC population also in the context of proteomics, while at the same time yielding some new candidates to be considered if confirmed by the ongoing replicates of the assay.

3.2.7 Recombinant CD142 and GDF10 inhibit *in vitro* adipogenesis

Given the demonstrated secretory nature of the Areg-mediated inhibitory power, candidate genes coding for secreted factors were of our great interest. We therefore decided to perform assays in which CD142– ASPCs, as a progenitor population devoid of Aregs and presenting a considerable adipogenic



DMSO

0.01

1 100 100

 $RA \mu M$

Figure 3.7 | See next page for caption

 $RA \mu M$

0.01

10

DMSO

potential, were treated with various concentrations of recombinant proteins in order to mimic the physiological presence of Aregs.

Bovine serum albumin (BSA) was used as a negative control for the exogenous presence of recombinant proteins in the medium, as it was shown not to affect in vitro adipogenesis at concentrations lower than 2.4 μM (160 μg/ml) (Schlesinger et al., 2006). Epidermal growth factor (EGF), known to be a potent inhibitor of adipogenesis at concentrations higher than 1 nM (6.4 ng/ml), was used as a positive control (Harrington et al., 2007). The CD142- ASPCs were induced to adipogenic differentiation by the adipogenic cue containing half of the usual amount of insulin, IBMX and dexamethasone delivered in DMEM medium (1/2 Complete in DMEM, Methods), in order to diminish the strength of the stimulus, allowing the cells to be more responsive to the introduced recombinant protein. The proteins were administered at the moment of induction as well as during maintenance in order to act simultaneously with the adipogenic cue. We could show that treatment of CD142- ASPCs with 1/2 complete cocktail supplemented with BSA 100 ng/ml, in spite of notable variation, did not overall affect the adipogenic propensity of the tested cells, while administration of 100 ng/ml EGF completely inhibited adipogenesis (Fig. 3.7a and b). Interestingly, complementing the differentiation medium with the recombinant proteins corresponding to the seven selected candidates, namely CD142 (F3), MGP, CLE-C11A, GDF10, CPE and BGN at 100 ng/ml, showed a significant inhibitory effect for CD142 and GDF10, with the former having a stronger effect (Fig. 3.7d and e, Methods). Testing the proteins at various concentrations ranging from 10 to 1000 ng/ml additionally showed the inhibitory activity for MGP at 1000 ng/ml, with no change in the adipogenic propensity of CD142– ASPCs treated with other proteins at the tested concentrations (Suppl. Fig. 3.7a). We also verified if the observed decrease in lipid accumulation was not due to proteins affecting cell proliferation. According to the published reports, EGF treatment dramatically induced cell proliferation, but no other protein influenced cell number in a substantial manner, with the exception of CLEC11A and GDF10 causing a slight though significant cell number increase (Fig. 3.7). While GDF10 is known to inhibit adipogenesis (Camps et al., 2020; Hino et al., 2012), the inhibitory action of CD142 and MGP have not been demonstrated in the context

Figure 3.7 CD142 and GDF10 recombinant proteins inhibit in vitro adipogenesis

a, Representative florescence microscopy images of CD142– ASPCs after in vitro adipogenic differentiation with the differentiation cocktail supplemented with control recombinant proteins BSA (negative control) and EGF (positive control) of inhibition of adipogenesis at 100 ng/ml, n=4 biological replicates, with n=3 technical replicates in each. b, Bar plots showing lipid accumulation (Adipogenic score - arbitrary unit) shown in a; represented are n=4 biological replicates, with n=3 technical replicates in each. c, Representative florescence microscopy images of CD142– ASPCs after in vitro adipogenic differentiation with the differentiation cocktail supplemented with recombinant proteins corresponding to the selected Areg-specific candidates: CD142, MGP, CLEC11A, GDF10, CPE, BGN and CXLC12 at 100 ng/ml. d, Bar plots showing lipid accumulation (Adipogenic score - arbitrary unit) shown in c; represented are n=2-6 biological replicates, with n=3 technical replicates in each. e, Bar plots showing normalised nuclei number of cellular fractions shown in c; represented are n=2-6 biological replicates, with n=3 technical replicates in each. f, Representative florescence microscopy images of CD142– ASPCs after in vitro adipo-genic differentiation with the differentiation cocktail supplemented with DMSO (mock treatment) and retinoic acid (RA, diluted in DMSO) at the indicated concentrations. g, Bar plots showing lipid accumulation (Adipogenic score - arbitrary unit) shown in f; represented are n=3 biological replicates, with n=3 technical replicates in each. h, Bar plots showing normalised nuclei number of cell fractions shown in f; rep-resented are n=3 biological replicates, with n=3 technical replicates in each. In all images, nuclei are stained with Hoechst (blue) and lipids are stained with Bodipy (yellow). All presented images correspond to 25 tiled and thresholded 20x images containing 7-8 z-stacks in order to capture the majority of the well surface (Methods). In d, e, g and h, all values within each independent experiment are normalised to BSA 100 ng/ml, represented by a grey bar. In b, d, e, g and h the height of the bar indicates the mean of the measurements averaged across all replicates; for statistical details see Methods.

of *in vitro* adipogenesis. Interestingly, MGP (Matrix gla protein), a member of a family of vitamin-K2 dependent, Gla-containing proteins, has been reported as an inhibitor of calcification in cartilage and vasculature (Bäck et al., 2019), implying its possible specificity to mesenchymal cells with multi-lineage potential with its inhibitory role. CD142 however, a key actor in the coagulation cascade as Tissue factor/Coagulation factor III, interacting with Factor VII and activating it into Factor VIIa, has never been linked to any adipogenic or differentiation-associated processes and, as much as used as Areg-specific marker, its inhibitory activity towards adipogenesis came as a surprise.

The sensitivity of our assays involving recombinant protein treatments is illustrated by the fact that a new batch of CD142 recombinant protein failed to induce an inhibitory effect (Suppl. Fig. 3.7b). After a close investigation of the two protein batches, we discovered that the fabrication involved completely different parameters for the two lots, while sold under the same reference number. In the first case, the protein was lyophilised from PBS at pH 7.4, while the other batch was lyophilised from sodium-acetate at pH 4.5. This was corroborated by the observation of the DMEM medium turning yellowish after the addition of the second lot of the protein. We requested the concerned company to produce the protein according to the first protocol and are now in the process of validating the new batch.

Another example of the sensitivity of protein treatments is the observation that one GDF10 protein yielded a more pronounced inhibitory effect than the other (prepared by a different company, Suppl. Fig. 3.7c). This sensitivity and a high variation observed for the recombinant protein treatments revealed the value of implementing many replicates to establish the effect in a reproducible fashion. It also indicates that lack of an observable difference might be determined by technical factors and not necessarily by the lack of biological relevance. The recombinant protein treated cells have therefore been collected and transcriptomic assays are ongoing in order to investigate molecular changes upon the corresponding treatments.

Consistent with the secretory nature of the Areg-mediated inhibitory signal, retinal dehydrogenase (RALDH2) encoded by the *Aldh1a2* gene was tested indirectly by treating CD142– ASPCs with retinoic acid (RA, Methods). RA was previously shown to exhibit an inhibitory action on 3T3-L1 cells (Kim et al., 2013), and the same result was observed when treating differentiating CD142– ASPCs, with significantly lowered adipogenic propensity at a concentration of 0.1 μ M (Fig. 3.7g and h). We could observe that cell proliferation was not affected except for the highest concentration of RA namely 100 μ M (Fig. 3.7i). It remains to be seen whether this potent effect occurs in the context of Areg-mediated adipogenic inhibition. Retinoic acid-treated CD142– ASPCs will therefore undergo in-depth transcriptional analysis in the near future.

3.2.8 siRNA-mediated knockdown of specific genes alters the phenotype and activity of Aregs

The validated secreted protein-coding candidates, *F3*, *Mgp* and *Gdf10*, two transcription factor-coding genes, *Meox2* and *Pknox2* as well as *Aldh1a2*, coding for retinal dehydrogenase, were next tested by siRNA-mediated knockdowns in Aregs (Methods). The corresponding knockdowns were performed in total ASPCs as a control, with the rationale that they should only occur in Aregs given that they targeted Areg-specific genes. In parallel, CD142– ASPCs were also subjected to the knockdowns. Adipogenesis was induced in all assayed cell populations two days after the knockdowns, when the siR-NA effect is expected to be the most pronounced.

While scrambled control siRNA impaired adipogenesis of tested cells, the implemented knockdowns led to very consistent results across all assayed ASPCs, with *Pknox2*, *F3* and *Meox2* knockdowns showing the most pronounced effects in terms of increasing adipogenic potential (Fig. 3.8a and b). We thereby found that siRNA-mediated changes in lipid accumulation were very small, pointing to the inherent inability of Aregs to give rise to adipocytes. Interestingly, inactivation of the two TF-coding genes, *Pknox2* and *Meox2* in Aregs resulted in the most pronounced increase in lipid accumulation that could be quantified, followed by the knockdown of *F3* and *Gdf10*. These effects were reflected by the two other populations of ASPCs subjected to siRNA treatment, with total ASPCs showing the most marked relative increase in adipogenic propensity, even though CD142– ASPCs showed the highest absolute changes (normalising all cell populations to their corresponding scrambled conditions *versus* normalising to ASPCs scrambled only, Fig. 3.8b and Suppl. Fig. 3.8a). This agreed with the targeted genes being specific to Aregs, as their knockdown did not induce substantial effects in CD142– ASPCs which are not supposed to express these genes or express them at a much lower level.

A surprising observation was made while considering cell numbers of assayed populations. The inactivation of genes having the highest effect on adipogenic propensity, presented negative effect on the corresponding cell numbers, the effect not determined by the efficiency of the knockdowns (Suppl. Fig. 3.8b). Indeed, Aregs with *F3* and *Pknox2* knocked-down to as little as around 10% of the initial amount, showed the most substantial increase in lipid accumulation while at the same time the most marked decrease in cell number (Fig. 3.8c and Suppl. Fig. 3.8b). The same relative changes can be observed for the two other cell populations, with CD142– ASPCs alterations being less pronounced, again highlighting the Areg-specificity of the knocked-down genes.

The observation of total ASPCs exhibiting the most pronounced relative changes upon inactivation of Areg-specific genes, raises an interesting hypothesis of the knockdowns modulating not so much the molecular identity of Aregs as their function. If Aregs are indeed inherently incapable of differentiating into adipocytes, then inactivating their marker genes would not have an effect on their phenotypic behaviour. Moreover, the small changes that we did observe might originate from the still existing heterogeneity within the sorted population of Aregs and the presence of non-Areg cells that are capable of giving rise to *in vitro* adipocytes. If, however, Aregs are cultured amongst cells with high adipogenic propensity, allowing them to exert their para/endocrine inhibitory effect, then the inactivation of Areg-specific functional genes would alleviate this activity, allowing the potent progenitors to realise their adipogenic potential to the fullest.

In order to test this hypothesis, we performed a transwell assay in which CD142– ASPCs were cultured in the presence of Aregs but without cell to cell contact (Methods). Intriguingly, we observed that CD142– ASPCs that were co-cultured with Aregs whose activity was modified by the inactivation of specific genes, displayed increased adipogenic differentiation. The extent of this effect



Figure 3.8 | See next page for caption

corroborated with the previously observed changes for the genes being knocked-down in total ASPCs, with *Pknox2* and *F3* showing the most significant impact, together with *Mgp* inactivation. The inhibitory activity of Aregs, assessed by their ability of preventing lipid accumulation of the co-cultured CD142– ASPCs, was dramatically decreased and led to an increase of CD142– ASPC adipogenesis by an average of 27, 18 and 16 times for Aregs when respectively *Pknox2*, *Mgp* and *F3* genes were knocked-down (Fig. 3.8d and e). Included controls show the extent of Areg-mediated inhibition of adipogenesis of co-cultured CD142– ASPCs with wild-type Aregs and Aregs transfected with a scrambled siRNA completely preventing lipid accumulation of CD142– APSCs compared to the same cells cultured in the absence of Aregs (Suppl. Fig. 3.8c). We can also appreciate the extent of the effect of *Pknox2* inactivation on Aregs' activity, with the co-cultured CD142– ASPCs showing a degree of differentiation comparable to that of CD142– ASPCs cultured in the absence of Aregs (Suppl. Fig. 3.8c).

Another intriguing observation was the effect of modified Areg activity on the cell number of co-cultured CD142– ASPCs. Strikingly, we could again observe the same dependence as we did for knockdowns in ASPCs, with the CD142– ASPC cell numbers being somewhat inversely proportional to the degree of adipogenesis. Indeed, we could observe a decrease in the number of nuclei to 76%, 78% and 79% when respectively *Pknox2*, *Mgp* and *F3* genes were knocked-down, compared to Aregs treated with scrambled siRNA (Fig. 3.8f).

We are currently performing transcriptomic profiling of 1) Aregs in which the most promising candidate genes are inactivated, and 2) CD142– ASPCs co-cultured with the corresponding impaired Aregs, with the aim of dissecting the molecular mechanisms underlying the Areg-mediated inhibitory effect.

Figure 3.8 siRNA-mediated knockdown of specific genes alters the phenotype and activity of Aregs

a, Representative florescence microscopy images of Aregs (top), ASPCs (middle) and CD142– ASPCs (bottom) after *in vitro* adipogenic differentiation with control (scr) and siRNA-mediated knockdowns of selected Areg-specific candidate genes: *F3, Meox2, Mgp, Gdf10, Aldh1a2* and *Pknox2*; the experiment was repeated n=2-3 with similar results, represented is one biological replicate. **b**, Bar plots showing lipid accumulation (Adipogenic score - arbitrary unit) of cellular fractions shown in a; all values within each independent experiment are normalised to the scrambled control, represented are n=2-3 biological replicates. **c**, Bar plots showing nuclei number of cell fractions shown in a, represented are n=2-3 biological replicates. **d**, Representative florescence microscopy images of CD142– ASPCs, co-cultured with Aregs carrying control (scr) and siRNA-mediated knockdowns of selected Areg-specific candidate genes: *F3, Meox2, Mgp, Gdf10, Aldh1a2* and *Pknox2*, after adipogenic differentiation; the experiment was repeated n=4, with similar results, represented is one biological replicate. **e**, Boxplots showing the distribution of the lipid accumulation (Adipogenic score - arbitrary unit) shown in d, all values within each independent experiment are normalised to the scrambled control, represented are n=4 biological replicates, with n=2-3 technical replicates in each. **f**, Boxplots showing the distribution of the normalised nuclei number for cellular populations shown in d, all values within each independent experiment are normalised to the scrambled control, represented are n=4 biological replicates, with n=2-3 technical replicates in each. In all images, nuclei are stained with Hoechst (blue) and lipids are stained with Bodipy (yellow). For statistics details see Methods.

3.3 Conclusion

In a series of assays employing various strategies of isolating murine subcutaneous Aregs and subjecting them to different adipogenic cues, we demonstrated the phenotypic robustness and stability of this novel adipose stem and precursor cell sub-type. Indeed, Aregs proved to be refractory to adipogenic differentiation even when sorted as a considerably higher percentage of CD142+ ASPCs (20% versus the usual 5-7%). We demonstrated that they remained non-adipogenic even when stimulated with more "aggressive" adipogenic cues. We also showed that they did not present sex-based differences neither in terms of their phenotype nor functionality. Interestingly, we observed a high adipogenic propensity of Aregs isolated from the burgeoning subcutaneous fat pad of new-born mice, revealing potential development-specific properties of Aregs. We next resolved to investigate the mechanisms behind the previously established inhibitory nature of Aregs. Integration of transcriptomic and proteomic datasets resulted in identification of a comprehensive set of Areg-specific candidates, which were further experimentally interrogated in the context of Aregs phenotype and activity. We could show that CD142, one of the most recurrent Areg-specific markers, had inhibitory capacity towards adipogenic differentiation of Areg-depleted ASPCs, with GDF10 and MGP also presenting anti-adipogenic properties, although to a lesser degree. Inactivation of these and two Areg-specific transcription factor-coding genes, Meox2 and Pknox2, again revealed the relevance F3 as well as showed, for the first time, a dramatically reduced inhibitory activity of Aregs devoid of Pknox2 activity. These candidates are now being investigated in the context of transcriptional regulation within functional as well as deactivated Aregs.

3.4 Miscellaneous

Acknowledgements:

We thank the EPFL core facilities: the Animal Facility, the Flow Cytometry Core Facility (especially to M. Garcia, V. Gutz, A. Mozes, L. Tauzin), and the Biolmaging and Optics Platform (especially R. Guiet, O. Burri. T. Laroche, J. Artacho, A. Seitz), for support with our mouse studies, cell sorting, image acquisition and analysis, respectively. We also thank the UNIL Flow Cytometry Facility (especially Stefanie Siegert) for the support with the Image Stream experiments.

Author contributions:

B.D. and M.Z. conceptualised the study. M.Z. performed or prepared all the experiments and related analysis and wrote the manuscript. H.H., J.R. and R.F. contributed to performing the experiments.

P.Y.R. reanalysed the scRNA-seq data. M.L. performed the statistical analysis and produced R-based scripts for plot generation. R.H. performed the proteomics-related experiments. F.A. performed the analysis of the proteomic datasets.

3.5 Tables

Table 3.1 Comparison of experimental strategies between Schwalie et al. and Merrick et al.

| Feature | Schwalie et al. | Merrick et al. |
|-----------------------------------|--|--|
| Mouse genetic background | C57BL/6 | C57BL/6 CD1 129S6/SvEv |
| Sex | Male & female | Male & female Male |
| Age | 8-12 weeks | P0.5 P12 Adult |
| Temp. Conditions | 22°C | Thermoneutrality (30°C) |
| Tissue digestion | Collagenase Type II Clostridium histolyticum | Collagenase D Dispase II |
| RBC lysis | Home-made buffer | BioLegend lysis buffer |
| Antibody | Anti-CD142-PE clone 001 SinoBiological 50413-R001-PE | Anti-CD142- clone 001 SinoBiological 50413-R001 Coupled to Biotin CF647 |
| Selection | CD31- CD45- SCA1+ | CD31- CD45- |
| Gating for CD142+ | 3-5% | 15-20% |
| Plating | 4k cells/96wp 4-5 days till confluence | Plated at near confluence |
| Culture medium | DMEM 10% FBS 10% FBS 1% P/S | DMEM/F12 10% FBS 10% FBS 50 ng/ml Primocin |
| Complete differentiation cocktail | Insulin 1μg/ml (170 nM) IBMX 0.5 μΜ Dex 1 μΜ | Insulin 20 nM IBMX 0.5 μM Dex 1 μM T3 1 nM Indomethacin 125 nM |
| Minimal differentiation cocktail | _ | Insulin 20 nM |
| Maintenance cocktail | Insulin 1µg/ml (170 nM) | Insulin 20 nM T3 1 nM |
| End of experiment | 6-8 days post-differentiation | 4-6 days post-differentiation |

| Rank | Gene | P3 Rank | G3 Rank | Rank | Gene | P3 Rank | G3 Rank |
|------|---------|---------|---------|------|---------------|---------|---------|
| 1 | Gdf10 | 9 | 5 | 34 | Spock2 | 27 | 88 |
| 2 | Bgn | 8 | 8 | 35 | Сре | 67 | 57 |
| 3 | lgfbp7 | 18 | 2 | 36 | lgf1 | 59 | 72 |
| 4 | Ndrg2 | 14 | 14 | 37 | Tspan11 | 42 | 92 |
| 5 | С2 | 10 | 22 | 38 | Olfml3 | 44 | 93 |
| 6 | Entpd2 | 17 | 17 | 39 | Deptor | 90 | 52 |
| 7 | Meox2 | 7 | 27 | 40 | Dcn | 25 | 121 |
| 8 | Cygb | 26 | 9 | 41 | Gpnmb | 54 | 102 |
| 9 | Gas6 | 30 | 7 | 42 | Арое | 154 | 4 |
| 10 | Steap4 | 34 | 3 | 43 | Cryab | 107 | 54 |
| 11 | Fmo2 | 4 | 34 | 44 | Arhgdib | 45 | 118 |
| 12 | Gsn | 22 | 16 | 45 | Atp1a2 | 68 | 106 |
| 13 | F3 | 5 | 38 | 46 | Podn | 15 | 169 |
| 14 | Vit | 37 | 10 | 47 | Ecm2 | 117 | 86 |
| 15 | Clec11a | 40 | 12 | 48 | Oat | 83 | 124 |
| 16 | Nfib | 33 | 23 | 49 | Tnfsf13b | 96 | 111 |
| 17 | Cst3 | 12 | 47 | 50 | Adh1 | 144 | 67 |
| 18 | Cav1 | 48 | 15 | 51 | Cxcl16 | 156 | 55 |
| 19 | Fth1 | 32 | 32 | 52 | ll6st | 115 | 97 |
| 20 | Inmt | 19 | 45 | 53 | Emp2 | 101 | 116 |
| 21 | Tgfbi | 16 | 49 | 54 | Cd302 | 158 | 65 |
| 22 | Cd9 | 55 | 26 | 55 | Gabra3 | 60 | 184 |
| 23 | Sepp1 | 24 | 58 | 56 | 3632451006Rik | 138 | 107 |
| 24 | Мдр | 2 | 81 | 57 | Dock9 | 77 | 175 |
| 25 | Nrp1 | 72 | 11 | 58 | Epha3 | 51 | 201 |
| 26 | Fgf7 | 21 | 63 | 59 | Ср | 200 | 53 |
| 27 | Pid1 | 23 | 62 | 60 | Adcyap1r1 | 147 | 119 |
| 28 | Cxcl12 | 80 | 6 | 61 | lfitm1 | 114 | 159 |
| 29 | Abi3bp | 62 | 28 | 62 | Rspo1 | 165 | 114 |
| 30 | Cfh | 11 | 80 | 63 | Ndufb6 | 128 | 155 |
| 31 | Kitl | 58 | 35 | 64 | Osr1 | 121 | 166 |
| 32 | Nmb | 70 | 29 | 65 | Ghr | 190 | 145 |
| 33 | Slco2b1 | 61 | 44 | | | | |

Table 3.2 Genes overlapping between the top 200 P3 (C1) and the top 200 G3 (10x) hits

| Rank | Gene | P3 Rank | G3 Rank | Aregs Rank |
|------|----------|---------|---------|------------|
| 1 | Fmo2 | 3 | 33 | 3 |
| 2 | F3 | 4 | 37 | 10 |
| 3 | Meox2 | 6 | 26 | 31 |
| 4 | Ndrg2 | 13 | 13 | 61 |
| 5 | Мдр | 1 | 80 | 8 |
| 6 | Clec11a | 39 | 11 | 41 |
| 7 | Vit | 36 | 9 | 47 |
| 8 | Gdf10 | 8 | 4 | 124 |
| 9 | Gas6 | 29 | 6 | 102 |
| 10 | Tspan11 | 41 | 91 | 9 |
| 11 | Tgfbi | 15 | 48 | 111 |
| 12 | Arhgdib | 44 | 117 | 17 |
| 13 | C2 | 9 | 21 | 158 |
| 14 | Spock2 | 26 | 87 | 113 |
| 15 | Pid1 | 22 | 61 | 150 |
| 16 | Сре | 66 | 56 | 178 |
| 17 | lfitm1 | 113 | 158 | 53 |
| 18 | Dock9 | 76 | 174 | 134 |
| 19 | Tnfsf13b | 95 | 110 | 182 |
| 20 | Epha3 | 50 | 200 | 181 |

Table 3.3 Genes overlapping between the top 200 P3 (C1), the top 200 G3 (10x) hits and the top 200 differentially expressed genes in Aregs vs. CD142– ASPCs in bulk RNA-seq

| Overlap with P3 | Overlap with P3 | |
|-----------------|-----------------|--|
| Fmo2 | Cav1 | |
| Bgn | Nfib | |
| Ephb2 | Nucb2 | |
| Dock9 | Lum | |
| Cdh13 | Tmod3 | |
| Susd2 | Alpl | |
| Ср | Kctd12 | |
| Arhgdib | Serping1 | |
| S100b | Dcn | |
| Meox2 | Мрр1 | |
| Epha3 | Gsn | |
| Steap4 | Adh1 | |
| lgfbp7 | Cltb | |
| Lmo7 | Sord | |
| F3 | Serpinf1 | |
| Cd9 | Fnbp1 | |
| Pid1 | ltpr1 | |
| Cygb | Ikbip | |
| Entpd2 | Add3 | |
| Rbp1 | Fuca1 | |
| Cst3 | Ikbip | |
| Inmt | Arap1 | |
| Atp1a2 | Nrp1 | |
| Ndrg2 | Cdc42bpa | |
| Ank2 | Lamb2 | |
| ltgbl1 | Fth1 | |
| C7 | Oat | |
| Sgcb | Zmpste24 | |
| Gm2a | Pmm2 | |
| Gas6 | Gcc1 | |
| Scarf2 | Ecm1 | |
| Arrb1 | Psmd2 | |
| Ndufa4l2 | Ctsl | |
| ll6st | Mfap1b | |
| Gpnmb | Serpina3k | |
| Арое | Svep1 | |
| Rab34 | Arhgap17 | |
| Vps53 | Трр1 | |
| Ahnak | Akap13 | |
| Cd302 | Ampd3 | |

Table 3.4 Aregs' proteomic hits overlap with the top 200 P3 (C1) genes

| Overlap with G3 | Overlap with G3 | |
|-----------------|-----------------|--|
| Fmo2 | Pcolce | |
| Bgn | Cstb | |
| Sec61g | Gstm2 | |
| Dock9 | Al607873 | |
| Ср | Nrp1 | |
| Arhgdib | Prdx1 | |
| Meox2 | Erp44 | |
| Epha3 | Sra1 | |
| Steap4 | Pfdn2 | |
| lgfbp7 | Rpl3 | |
| Cd9 | Ррр3са | |
| Pid1 | SIc29a1 | |
| Cygb | Fth1 | |
| Entpd2 | Oat | |
| Cst3 | Prrx1 | |
| Inmt | Eif1 | |
| Pik3r1 | Ranbp1 | |
| Naalad2 | Zhx3 | |
| Atp1a2 | Dbi | |
| Ndrg2 | Cisd1 | |
| Fbxo6 | Eif4ebp1 | |
| S100a4 | Ddost | |
| Gas6 | Gbp7 | |
| Ppl | Gm10094 | |
| ll6st | Mrpl12 | |
| Gpnmb | Cebpb | |
| lcam1 | Rp12211 | |
| Арое | Cox7a2 | |
| Cd44 | Srsf7 | |
| Cd302 | Rab11b | |
| Cav1 | Dnajb1 | |
| Nfib | Mt1 | |
| Maged2 | Ftl1 | |
| Dcn | Jund | |
| Gsn | Myd88 | |
| Adh1 | Cd81 | |
| Uap1l1 | Tex264 | |
| Trim47 | Map1lc3b | |

Table 3.5 Aregs' proteomic hits overlap with the top 200 G3 (10x) genes

Table 3.6 Aregs' proteomic hits overlap with the top 200 bulk RNA-seq-derived DE Areg-specific genes

| Overlap with Aregs | | |
|--------------------|--|--|
| Fmo2 | | |
| Acsbg1 | | |
| Dock9 | | |
| Susd2 | | |
| Folh1 | | |
| Норх | | |
| Aldh1a2 | | |
| Arhgdib | | |
| Meox2 | | |
| Epha3 | | |
| Lmo7 | | |
| Ces2b | | |
| F3 | | |
| Vill | | |
| Pid1 | | |
| Aldh3a1 | | |
| Naalad2 | | |
| Ndrg2 | | |
| Ces2e | | |
| Sgcd | | |
| ltgbl1 | | |
| C7 | | |
| Sgip1 | | |
| Cda | | |
| Gas6 | | |
| Gpr39 | | |
| Ndufa4l2 | | |
| Cav1 | | |
| Alpl | | |
| Pygb | | |
| Ube2v1 | | |
| Serpina3k | | |
| Pabpc1 | | |
| Serpinb6 | | |
| Fyn | | |

3.6 Supplementary Figures



Supplementary Figure 3.1 Aregs show a bigger size than the total ASPCs and CD142– ASPCs

a, Back-gating analysis showing the emplacement of Aregs (left) and CD142– ASPCs (right) within the ancestry plots; Aregs and CD142– ASPCs are displayed as child populations coloured in red within each parent population, including all parent gates with an overlay showing gate effect, the analysis corresponds to the sample stained with the SB anti-CD142–PE antibody showed in Fig. 3.1a., the experiment was repeated at least four times with similar results, represented is one biological replicate. **b** to **e**, Image Stream profiles of human ASPCs stained with anti-CD142 and anti-CD146 antibodies, CD142+ ASPCs are represented in pink and beige (b); showed are profiles of **c**, circularity, **d**, area and **e**, intensity of the considered populations; the experiment was repeated for three different individuals with similar results, shown is one biological replicate. **f**, Bar plots representing normalised average nuclei area of the total ASPCs, CD142– ASPCs and Aregs, represented are n=2 biological replicates containing n=3 technical replicates each; for statistical details see Methods.



Supplementary Figure 3.2 Differentiation and proliferation of ASPCs with various adipogenic cues and culture media

a, Bar plots showing normalised nuclei number of cellular fractions shown in Fig 3.2b, the height of the bar indicates the mean of the measurements averaged across all replicates; represented are n=4 biological replicates, with at least n=3 technical replicates in each. **b**, Representative florescence microscopy images of CD142– ASPCs, after *in vitro* adipogenic differentiation with the indicated adipogenic differentiation cocktails (for details see Methods); the presented images correspond to 25 tiled and thresholded 20x images containing 7-8 z-stacks in order to capture the majority of the well surface (Methods). **c**, Bar plots showing lipid accumulation (Adipogenic score - arbitrary unit) shown in b; the height of the bar indicating the mean of the measurements averaged across all replicates, represented is one biological replicates, with n=3 technical replicates; for statistical details see Methods. In all images, nuclei are stained with Hoechst (blue) and lipids are stained with Bodipy (yellow).



Supplementary Figure 3.3 Pups- and adult-derived ASPCs present phenotypic differences

a, **b** and **c**, Back-gating analysis showing the emplacement of the indicated populations within the ancestry plots; the back-gated population is displayed as a child population coloured in red within each parent population, including all parent gates with an overlay showing gate effect. **a**, Back-gating analysis of pups- (left) and adult (right)-derived SCA1+ populations. **b**, Back-gating analysis of the pups-specific population. **c**, Back-gating analysis of pups-derived Aregs (left), CD142+ ASPCs (middle) and CD142– ASPCs (right) populations. The experiments were repeated at least n=4 times with similar results, represented is one biological replicate for each back-gating.



Supplementary Figure 3.4 Pups- and adult-derived ASPCs show different proliferation rates

a, Representative florescence microscopy images of pups-derived Aregs, 20% CD142+ ASPCs and CD142– ASPCs, isolated following the sorting strategy shown in Fig. 3.3a and b after *in vitro* adipogenic differentiation with the indicated adipogenic cocktails (Methods); the presented images correspond to 25 tiled and thresholded 20x images containing 7-8 z-stacks in order to capture the majority of the well surface (Methods), the experiments containing Aregs was performed once with n=2 or 3 technical replicates, the corresponding quantification of adipogenic differentiation is shown in Fig. 3.3h. **b**, Bar plots showing nuclei number of cell fractions shown in Fig. 3.3d, represented are n=2-3 biological replicates with n=1-3 technical replicates in each. **c**, **d** and **e**, Bar plots showing nuclei number of pups-(blue) and adult-(green) derived from c, Aregs, d, 20% CD142+ ASPCs and e, CD142– ASPCs, represented are n=2-3 biological replicates with n=1-3 technical replicates in each. In b-e the height of the bar indicates the mean of the measurements averaged across all replicates; for statistical details see Methods. In all images, nuclei are stained with Hoechst (blue) and lipids are stained with Bodipy (yellow).



Supplementary Figure 3.5 Single-cell and bulk RNA-seq-based Aregs-specific candidate genes

a, Venn diagram illustrating overlaps between top 200 Areg-specific genes in C1 dataset (P3-specific genes), 10x dataset (G3-specific genes) and bulk RNA-seq dataset (significantly differentially expressed genes in Aregs *versus* CD142– ASPCs) (Tables 3.2 and 3.3). **b**, t-SNE 2D maps of all analysed cells (C1) highlighting the expression of the indicated Aregs-specific markers. **c**, t-SNE 2D maps of all analysed cells (10x), highlighting the expression of the indicated Aregs-specific markers.



Supplementary Figure 3.6 Mass-spectrometry confirms most Areg-specific candidate genes

a to **d**, Represent correlations of LFQs of quantified proteins. **a** and **b** Correlation of the LFQ intensities between freshly sorted CD142– ASPCs and freshly sorted Aregs, with highlighted points corresponding to 31 proteins common with the C1 and 10x scRNA-seq overlap (a) and 9 proteins common with the C1, 10x and bulk RNA-seq overlap (b). **c** and **d**, Represented are statistically differentially expressed proteins with a Z-score sliding window with a FDR <= 0.01, FDR <= 0.05 and FDR < 0.1 represented in red, orange and light blue respectively. Grey points correspond to proteins not differentially expressed (FDR > 0.1).



Supplementary Figure 3.7 CD142 and GDF10 recombinant proteins inhibit in vitro adipogenesis

a, Bar plots showing lipid accumulation (Adipogenic score - arbitrary unit) of cellular fractions shown in Fig. 3.3c; the individual graphs represent lipid accumulation of CD142– ASPCs after *in vitro* adipogenic differentiation with the differentiation cocktail supplemented with control (BSA) and recombinant proteins corresponding to the selected Areg-specific candidates as indicated: BSA, CD142, MGP, CLEC11A, GDF10, CPE, BGN and CXLC12 at indicated concentrations; represented are n=3-6 biological replicates, with n=3 technical replicates in each. **b**, Bar plots showing lipid accumulation (Adipogenic score - arbitrary unit) of CD142– ASPCs after *in vitro* adipogenic differentiation with the differentiation cocktail supplemented with two different lot of CD142 recombinant protein: SinoBiological Lot 1 (yellow) and SinoBiological Lot 2 (blue); represented are n=2-6 biological replicates, with n=3 technical replicates in each. **c**, Bar plots showing lipid accumulation (Adipogenic score - arbitrary unit) of CD142– ASPCs after *in vitro* adipogenic differentiation with the differentiation cocktail supplemented with two different GDF10 recombinant proteins: LifeSpan BioSciences (yellow) and SinoBiological (blue); represented are n=2-6 biological replicates, with n=3 technical replicates in each. All values within each independent experiment are normalised to BSA 100 ng/ml, represented by a grey bar. The height of the bar indicates the mean of the measurements averaged across all replicates; for statistical details see Methods.


Elipids DNA

Supplementary Figure 3.8 siRNA-mediated knockdown of specific genes alters the phenotype and activity of Aregs

a, Bar plots showing lipid accumulation (Adipogenic score - arbitrary unit) of cellular fractions shown in Fig. 4.4a; all values within each independent experiment are normalised to the ASPCs scrambled control, represented are n=2-3 biological replicates. **b**, Bar plots showing the expression of indicated genes relative to *Hprt1* housekeeping gene, in Aregs with control siRNA and in Aregs with siRNA targeting the specific gene, the expression was tested 48 hours after transfection, represented are n=1-3 biological replicates with n=3 technical replicates in each. **c**, Representative florescence microscopy images of CD142– ASPCs after adipogenic differentiation, co-cultured in a transwell assay with wild-type Aregs (Aregs), Aregs carrying control (scr Aregs) or siRNA-mediated knockdown of *Pknox2* gene or with an empty transwell; the experiment containing wild-type Aregs was repeated n=2, with similar results, represented is one biological replicate. In all images, nuclei are stained with Hoechst (blue) and lipids are stained with Bodipy (yellow). The height of the bar indicates the mean of the measurements averaged across all replicates; for statistical details see Methods.

Chapter 4 Discussion

4.1 The discovery of Aregs, a novel cell sub-type

Using the resolving power of scRNA-seq, with the aim of dissecting the heterogeneity of adipose stem and precursor cells (ASPCs) within the murine subcutaneous fat pad, we identified a subpopulation marked with a high expression of genes including F3 (coding for CD142), Meox2, Map and Gdf10 (Schwalie et al., 2018). FACS-based isolation of CD142+ ASPCs, comprising less than 10% of total ASPCs, yielded a cellular fraction presenting a completely non-adipogenic character upon a differentiation cue. Moreover, we observed that depleting total APSCs of this small population, significantly increased adipogenic differentiation of the remaining cells. The observed increase seemed too important to be caused and explained simply by removal of non-differentiating cells, suggesting their inhibitory activity. A series of assays including titration and transwell-based cell co-culture experiments, proved that the CD142+ population negatively regulates the adipogenic capacity of other ASPCs. As such negative modulatory activity is conceptually evocative of T regulatory cells, Tregs, which exert an inhibitory activity to maintain homeostasis within the immune niche, we decided to term the CD142+ APSCs "Aregs", for "adipogenesis regulators". Subsequently, Aregs were demonstrated to inhibit adipogenesis in vivo, in an assay where subcutaneously injected ASPCs devoid of Aregs underwent a significantly higher adipogenesis than total ASPCs in mice on high fat diet. Interestingly, Aregs, positive for SCA1 and CD142 markers, were shown to localise along blood vessels, in agreement with the concept of a peri-vascular pre-adipogenic stem cell niche (Rodeheffer et al., 2008). We also demonstrated the existence of CD142+ ASPCs in human subcutaneous adipose tissue, where they present the expression of Areg-specific marker genes and a decreased adipogenic potential.

The robustness of murine Aregs was assessed by isolating CD142+ ASPCs with antibodies from various sources and defining Aregs with different percentages of the CD142+ cells within the ASPC pool. Aregs were also challenged with a number of adipogenic cocktails, differing in strength of the adipogenic cue. In addition, sex-based differences were considered regarding Aregs and their adipogenic propensity. None of these conditions lead to an adipogenic character of Aregs. With these findings, we reinforced the phenotypic identity of Aregs and confirmed their status as a novel sub-population of murine ASPCs, especially in light of the conundrum in the field created by a recent study reporting highly adipogenic properties of CD142+ ASPCs (Hwang and Kim, 2019; Merrick et al., 2019). In contrast, integrating single-cell transcriptomics data from our (Schwalie et al., 2018) and Merrick and

colleagues assays yielded a clearly delineated cluster corresponding to Aregs (Merrick et al., 2018; Ferrero et al., in preparation). This was however not the case for FIPs, whose profile overlapped both with Aregs and Population P2/G2, corresponding to committed pre-adipocytes from our dataset (Hepler et al., 2018; Ferrero et al., in preparation) opposing the hypothesis of FIPs being the visceral counterparts of Aregs.

Intriguingly, our in-depth investigation revealed that Aregs derived from the burgeoning subcutaneous fat depots of new-born mice exhibited a highly adipogenic potential, with virtually every cell undergoing adipogenesis *in vitro*, an unprecedented observation in our hands in the context of the Areg phenotype.

The discovery of Aregs sheds a new light on the regulatory dynamics within the adipose stem and precursor cells. We see it as an example of how complex and highly regulated the interplay within these cell subpopulations proves to be. Our results provide valuable insights into the extensively used in vitro adipogenesis model, explaining, for example, why a fraction of ASPCs within the dish does not undergo adipogenic differentiation. Most importantly however, Aregs have intriguing implications in the context of adipose biology. The existence of such an inhibitory cell population has powerful implications regarding the composition and plasticity of the fat depot and subsequently metabolic health. The complex regulation within a fat depot, would therefore not be solely determined by the number of resident adipose progenitors but also by their propensity to give rise to mature adipocytes and by the modulatory signals they secrete. For example, depot- and condition-specific differences in de novo differentiation might be controlled by the number of endogenous Aregs. Our findings reveal a bigger fraction of Aregs within the visceral depot, begging the question whether Aregs are partially responsible for the dramatically decreased adipogenic differentiation potential of the visceral ASPCs (Meissburger et al., 2016). The visceral pre-adipogenic niche has already been proposed to receive and/or generate negatively modulating signals, including decorin and Sparc-like 1 proteins (Meissburger et al., 2016), as well as retinoic acid-mediated activity (Takeda et al., 2016).

Interestingly, we also observed a bigger portion of Aregs within the ASPCs of obese mice. An observation, which at first seemed counterintuitive, could be explained by Aregs striving to counter-balance the excess pro-adipogenic signals. It remains to be answered through what mechanisms Aregs increased in number within the obese fat pad. Another intriguing question concerns the way in which the identity and activity of Aregs is determined. It would be interesting to test, whether the inhibitory signal is constitutive or triggered by excessive adipogenic cues. An indication might come from the fact that new-born mice-derived Aregs present a high adipogenic potential. If additionally, they would be demonstrated not to have the anti-adipogenic activity, this could suggest that the inhibitory signal has not yet manifested in the developing, equilibrated environment, which possibly does not yet receive excessive adipogenic stimuli. It will be interesting to demonstrate at which point during post-natal development Aregs arise with their hallmark identity and function.

Establishing robust and stable Areg-specific marker genes will be required for lineage tracing assays. The latter will provide answers to numerous questions regarding the *in vivo* origin of Aregs as well as their variable depot- and condition-specific numbers. It may also clarify whether Aregs or Areg-like cells reside in distinct organs subjected to adipogenic differentiation, like for example, muscle or bone marrow. Fibro-adipogenic progenitors (FAPs), support muscle regeneration in healthy conditions (Brack and Rando, 2012). Upon aging or muscle disease however, their regenerative potential turns into adipogenic propensity. It would be interesting to speculate that Areg-like cells reside within the muscle stem cell niche and regulate the dual differentiation capacity of FAPs.

Another system in which we could hypothesise the existence of Areg-like cells is bone marrow. A similar to muscle phenomenon occurs during aging, where red, haematopoietic marrow gradually transforms into yellow, non-haematopoietic marrow, filled with adipocytes (Kricun, 1985). This transition is also observed in patients having undergone intensive ablative chemotherapy, or haematopoietic cell transplantation. In the majority of patients after the therapy, the yellow bone marrow initially colonising the bones reverts back to the functional red marrow (Naveiras et al., 2009). In a small group of patients however, the fatty marrow persists, compromising haematopoietic function, which often becomes life-threatening. It would be interesting to explore whether Areg-like cells contribute to regulating the adiposity of the bone marrow, and if yes, what is their origin.

An interesting observation was our histological analysis, which revealed that Aregs localise peri-vascularily within murine subcutaneous depots (Fig. 2.4b-d, Suppl. Fig. 2.7e-g). This corroborated with a the certain level of vascular association of Aregs, which showed a significant enrichment of blood vessel GTEx tissue terms as well as the higher expression of several endothelial genes including *Vcam1*, *Thbd* and *Cdh5* compared to CD142– ASPCs. Upon closer analysis of our histological data, we found Aregs residing around bigger rather than smaller vessels, which were round in shape. Aggregates of Aregs were identified within a certain distance from the endothelial, CD31+ cells, suggesting that they might be located to what corresponds to the adventitia of arteries (Majesky et al., 2011). Recent studies propose, that adventitia should no longer be perceived as a loose, disorganised collection of fibroblasts embedded in the collagen-rich extracellular matrix. It has been shown that this most outer layer of the arterial wall supports a dynamic composition of interacting cell types, including fibroblasts, macrophages but also CD34+ SCA1+ progenitor cells and an outer layer of adipocytes (Majesky et al., 2011). This collection of cells was proposed to be in the domain of the restricted to adventitia Sonic hedgehog signalling (Passman et al., 2008).

It would be interesting to interrogate Aregs in the context of Sonic Hedgehog signalling, by targeting, for example *Gli1* gene (Hedgehog signalling effector), which Aregs express, although not at a highly specific level. Another interesting hypothesis based on these assumptions, would be that Aregs are present systemically and exert their anti-adipogenic activity towards the layer of peri-vascular adipocytes, mechanically protecting the arteries. A histological approach, identifying Aregs and artery-associated adipocytes across all major organs could address such a hypothesis.

4.2 In search of Aregs' mechanism

Given the considerable implications of Aregs in adipose biology, it is extremely important to understand the mechanism through which they exert their inhibitory action towards other adipose stem and precursor cells. Integration of transcriptomic and proteomic datasets allowed us to identify a comprehensive set of highly Areg-specific candidates, which we validated in the context of Aregs identity and function. Amongst the selected candidates, seven corresponded to genes coding for secreted proteins, namely CD142 (coded by F3), MGP, CLEC11A, GDF10, CPE, BGN and CXCL12 (ordered according to their overall relevance to Aregs). These candidates were used in assays where ASPCs devoid of Aregs (CD142- ASPCs) were treated with the corresponding recombinant proteins, with the intention of mimicking the physiological presence of active Aregs. CD142 and GDF10 decreased the capacity of CD142– APSCs to undergo adipogenesis, complemented by MGP, having an inhibitory effect at a higher concentration. In the frame of addressing the secretory nature of Aregs, we assayed the effect of retinoic acid (RA) on CD142– ASPCs to indirectly address the potential involvement of RALDH2 (coded by Aldh1a2), the enzyme catalysing the formation of RA (Chambers et al., 2007) and an Areg-specific candidate. We confirmed the previously reported strongly inhibitory effect of RA in the context of CD142- ASPCs. These promising candidates as well as the two relevant transcription factors coding genes, Meox2 and Pknox2, were next tested in siRNA-mediated knockdown assays. Our results showed that inactivating the specific genes in Aregs, caused an increase in the lipid accumulation compared to control, although to a very small extent. Interestingly, this effect was most noticeable for the inactivation of the two transcription factors, followed by F3 and Map. The most pronounced effects however, were observed within the total ASPCs, where inactivating Pknox2, F3 and Meox2 genes caused a substantial increase in adipogenic differentiation. We also observed that knockdowns of the genes causing the highest increase in adipogenesis, resulted in a corresponding decrease in cell numbers (which was not correlated with the knockdown efficiency). We next addressed the functionality of Aregs by testing the effect of inactivating the specific genes in Aregs co-cultured with CD142- ASPCs within the transwell set-up. Intriguingly, we observed that Aregs devoid of the activity of Pknox2, were no longer capable of inhibiting adipogenesis of co-cultured ASPCs compared to a scrambled control and to wild-type Aregs. This was also the case for the inactivation of F3 and Mgp, although to a lesser degree, with Meox2 showing an even smaller effect. We were surprised to notice that the cell numbers of ASPCs co-cultured with the most inactivated Aregs were accordingly lowered.

F3 gene, coding for CD142, has proved to be one of the most specific markers for Aregs throughout all our datasets. We subsequently demonstrated that CD142 is secreted by cultured Aregs and that supplementing CD142– ASPCs with recombinant CD142, inhibits adipogenesis (at 100 ng/ml). Finally, our results confirmed its importance through *in vitro* functional assays, where we showed that inactivating F3 gene in Aregs alleviates their anti-adipogenic nature towards differentiating cells. Its highly consistent relevance in the context of Aregs' activity is surprising given the acclaimed physiological role of CD142, being a coagulation factor (Chu, 2011). CD142, also known as Tissue factor, is the primary initiator in the extrinsic coagulation pathway (Petersen et al., 1995). It is a single pass type I transmembrane

and shed protein, which initiates blood coagulation by forming one-to-one stoichiometric complex with factor VII, enhancing the proteolytic activity of an activated factor VIIa towards its further substrates (Petersen et al., 1995). Its association with adipose biology was demonstrated by elevated circulating CD142 levels in childhood and adult obesity, evidencing of a pro-coagulant state (Faber et al., 2009; Singh et al., 2012). Another link is the reported F3 overexpression in obesity, accompanied by upregulated adipose inflammation and fibrosis (Chu, 2011; Faber et al., 2009). Indeed, in human studies, CD142 was shown to set up inflammation, in part accounting for increased levels of IL-6, IL-8 and TNF α in obese individuals (Popko et al., 2010). The expression of F3 was also shown to be upregulated by leptin and downregulated by adiponectin, presenting high and low levels in obesity respectively (Chu, 2011). This was suggested to imply a mechanistic role of CD142 in developing inflammatory obesity. This hypothesis was supported by F3 being downregulated by PPARy agonists including fenofibric acid, WY14643 and GW2331 (Chu, 2011). Another line of interest could be Tissue factor's involvement in angiogenesis (Carneiro-Lobo et al., 2014). CD142 activity was suggested to be linked to a G protein-coupled receptor, protease-activated receptors (PARs), whose activation leads to the expression of IL-8 and VEGF (a potent angiogenic factor), with VEGF and CD142 expressions mutually enhancing each other (Carneiro-Lobo et al., 2014).

Interestingly, CD142 as a factor VIIa-dependent activator of protease activated receptor-2 (PAR2) was interrogated in the context of obesity in mice and was shown to be implicated in weight gain regulation as well as in the process of inflammation (Badeanlou et al., 2011). The involved signalling occurs via PAR2 activation and phosphorylation-dependent cross-talk of the cytoplasmic domain of CD142 and integrins (Dorfeurtner et al., 2004). It was shown that CD142-PAR2 signalling in hematopoietic cells reduces adipose tissue macrophage inflammation and pharmacological inhibition of CD142 signalling improves glucose resistance. In non-hematopoietic cells, CD142-VIIa-PAR2 was demonstrated to specifically promote obesity (Bedeanlou et al., 2011), observation opposing our findings of CD142 showing anti-adipogenic activity. It was further shown that the CD142 cytoplasmic domain-dependent signalling suppresses Akt phosphorylation with subsequent adverse changes in transcriptional regulation of key factors involved in obesity and metabolism including Adiponectin and TNFa (Bedeanlou et al., 2011). Pharmacological inhibition of adipocyte-specific CD142 in vivo was shown to reverse the effects of CD142-VIIa signalling and improve energy expenditure. It remains to be seen whether this conceptual discrepancy could be explained by differences between the nature of the cytoplasmic versus extracellular CD142-signalling. The use of antibodies differentially blocking the two CD142-dependent signalling pathways (i.e. 10H10 specifically inhibiting human CD142-VIIa-PAR2 signalling, Bedeanlou et al., 2011) could help reveal this potential dual, "opposing" activity of CD142.

These rather vast associations remain to be addressed in detail in the context of our discoveries of CD142 being one of the most promising candidates for elucidating Aregs' mechanism of action. A number of above-discussed molecular factors will be examined in a transcriptional analysis of Aregs carrying *F3* knockdown as well as in CD142– ASPCs co-cultured with Aregs lacking *F3* activity.

Meox2, coding for Homeobox protein MOX-2, is another highly specific marker of Aregs, and the highest ranked Areg-specific transcriptional factor. This mesodermal transcription factor plays a key role in somitogenesis and is required for sclerotome development (Candia et al.; Mankoo et al., 2003).

MEOX2 induces expression of cyclin dependent kinase inhibitor genes *Cdkn1a* and *Cdkn2a* in endothelial cells, where it acts as a regulator of vascular cell proliferation (Douville et al., 2011). Interestingly, in a study investigating vascular remodelling in obesity, MEOX2 was shown to suppress proliferation and differentiation of peri-vascular adipocytes (PVACs) (Liu et al., 2015a). In this system, MEXO2 activity was counteracting the activity of insulin-like growth factor 1 (IGF-1), which, surprisingly, we find specific to Aregs in our single-cell transcriptomic datasets. The study also reported that MEOX2 knockdown resulted in a increased PVAC lipid content (Liu et al., 2015a).

Intriguingly, the physiological role of MEOX2 reveals a vascular link, a recurring feature while dissecting the identity of Aregs. Moreover, its activity to suppress proliferation and differentiation of peri-vascular adipocytes corroborates the hypothesis of Aregs systemically protecting vasculature against peri-vascular adipocytes overgrowth. As much as *Meox2* inactivation in Aregs showed to negatively affect Aregs' activity, this effect was nevertheless not as pronounced as for other candidates. Overexpression of *Meox2* in differentiating ASPCs as well as examining the genomic regions under MEOX2 control by performing ChiP-seq and ATAC-seq assays will provide further insights into the relevance of *Meox2* in Aregs' mechanism.

Another tested candidate, Matrix gla protein (MGP), showed to inhibit adipogenesis at a high concentration (1 µg/ml) and its inactivation impaired the anti-adipogenic properties of Aregs, although to a lesser degree than some other candidates. MGP is an established calcification inhibitor (Canfield et al. 2000). It has been proposed to deactivate BMP2 and BMP4 activity, but also suggested to exist in activator-inhibitor relationship with BMP2/4 and VEGF in the context of pattern formation of mesenchymal cells *in vitro* as well as in branching on the pulmonary vasculature *in vivo* (Yao et al., 2006, 2007). It was also reported that *Mgp* knockout lead to retardation of 3T3-L1 differentiation (Li et al., 2020).

Interestingly, *Mgp* was shown to be highly expressed in visceral as opposed to subcutaneous fat and ASPCs (Li et al., 2020; Meissburger et al., 2016; Takeda et al., 2016). A study examining the phenotypic differences between the visceral and subcutaneous stromal vascular fraction reported its potential involvement in the inhibitory interplay within the visceral compartment (Meissburger et al., 2016). In the related context of human visceral ASPCs being non-adipogenic *in vitro*, MGP was shown to be specific to visceral fat and APSCs and proposed to be important for non-adipogenic character of ASPCs through WT1-mediated upregulation of the retinoic signalling pathway (Takeda et al., 2016). Recently, these findings were supported by the demonstration of an increase in circulating MGP in visceral obesity and the proposal of it as a novel clinical marker (Li et al., 2020).

Growth-differentiation factor-10 (GDF10, also known as BMP3b) was another candidate identified as one of the most Areg-specific secreted factors and showed to negatively affect adipogenesis of ASPCs devoid of Aregs (at 100 ng/ml). GDF10 is a member of the transforming growth factor-beta (TGF β) family (Hino et al., 2012). It was demonstrated to be primarily present in murine uterus, adipose tissue and brain as well as neonatal and adult bones, where it has been proposed to have a critical role in regulation of osteogenesis by inhibiting osteoblast differentiation (Matsumoto et al., 2012). In the context of adipose tissue, it was shown to be expressed at higher levels in murine preadipocytes than in mature adipocytes (Hino et al., 2012) with high expression observed also in 3T3-L1 murine preadipocyte cell line. Subsequent tests have shown that the siRNA-mediated knockdown of *Gdf10* in 3T3-L1 cells have led to an enhanced *in vitro* adipogenic propensity, while treating differentiating 3T3-L1 cells with recombinant GDF10 protein inhibited adipogenesis (Hino et al., 2012), corroborating our findings.

In addition, *Gdf10* was recently identified in a scRNA-seq-based study as one of the highest markers of a novel population of muscle interstitial cells, which also exhibited a specific expression of *F3* (Camps et al., 2020). GDF10 was shown to be secreted by the novel interstitial population, which presented inhibitory activity towards CD142–interstitial cells. Inactivation of *Gdf10* in the interstitial cells, caused their impaired anti-adipogenic potential towards the adipogenic capacity of differentiating cells. Validating these finding, overexpressing *Gdf10* in CD142–interstitial cells decreased their ability to give rise to *in vitro* adipocytes (Camps et al., 2020). These corroborating findings prompt us to retain *Gdf10* as an interesting candidate to follow in the context of Aregs, in spite of its thus far apparent lack of involvement in the actual Areg mechanism.

Clec11a, Cpe, Bgn and *Cxcl12*, further Areg-specific candidates, all coding for secreted factors, were tested in the context of the inhibitory activity of Aregs, where differentiating CD142– ASPCs were treated with the corresponding recombinant proteins at various concentrations. However, none of them revealed inhibitory activity towards CD142– ASPCs and they will not be further discussed within the scope of this study.

It is nevertheless important to keep in mind, that differentiation assays involving recombinant proteins are extremely sensitive. An example of that was the lack of inhibitory activity of the recombinant CD142, prepared in a pH 4.5 buffer as opposed to the one lyophilized from pH 7.5 PBS. Such a change might have affected the structure of the protein by compromising the formation of disulphide bridges (of which two are present within CD142 (Chu, 2011), as well as the stability of salt bridges and hydrogen bonds (Monahan et al., 1995; O'Brien et al., 2012; Tetsch et al., 2011). A modification of a protein structure strongly implies modification of its activity. Numerous other reasons might affect successfully demonstrating a protein's physiological role in assays involving recombinant proteins. As a function of the organism in which they were produced, they may lack essential post-translational modifications such as glycosylation or phosphorylation, or simply present insufficient stability. If produced in bacteria, they also might be contaminated by residual endotoxins such as lipopolysaccharide (LPS) (Schwarz et al., 2014). In exploratory assays, tested proteins might not have revealed their anticipated activity due to lack of a necessary co-factor or conditions to, for example, homodimerize. In the context of our assays, it is possible that even though spanning two orders of magnitude, the necessary concentration might have been outside of the used range. Finally, in our experiments the proteins were administered to CD142- ASPCs at the moment of adipogenic induction with the aim of mimicking the physiological presence of Aregs. It is possible however that Aregs might control differentiation at different mechanistic points, for example, before the adipogenic cue, or once the cells are already committed. Absence of an effect in the presented assays should therefore not definitively eliminate the potential relevance of the tested candidates.

The approach for testing the potential involvement of Retinal dehydrogenase 2 (coded by *Aldh1a2*) in Aregs' activity was two-fold and consisted of 1) an indirect way, by treating CD142– ASPCs with retinoic acid (RA) as a product of RALDH2 activity and 2) inactivating *Aldh1a2* in Aregs and examining

its effect on their activity. As much as RA exerted a dramatic inhibitory effect on adipogenesis (as previously reported, (Berry et al., 2012), *Aldh1a2* knockdown did not impair the anti-adipogenic function of Aregs arguing against the involvement of RA signalling in the activity of Aregs.

Interestingly though, *ALDH1A2* gene was shown to be significantly higher expressed in visceral as opposed to subcutaneous adipose tissue both in humans and in mice (Reichert et al., 2011; Takeda et al., 2016) and this in the context of the non-adipogenic character of visceral *versus* subcutaneous ASPCs.

In the same human study, *PKNOX2* was reported to also be expressed specifically in the visceral compartment (Takeda et al., 2016).

Curiously, the last tested candidate, *Pknox2*, encoding a poorly characterized transcription factor Homeobox protein PKNOX2, revealed to be the most relevant in the context of Aregs activity. Indeed, inactivating *Pknox2* in Aregs caused a dramatic effect on their activity rendering them incapable of inhibiting adipogenic differentiation of co-cultured Areg-depleted ASPCs. We also observed a corresponding significant increase of differentiation within total ASPCs transfected with a *Pknox2*-targeting siRNA compared to control, accounting for a high specificity of *Pknox2* to Aregs and suggesting its involvement in the secreted inhibitory signal.

Interestingly, PKNOX2 was proposed as one of the candidates in a study investigating a variant of histone H2A, MacroH2A1 in the context of its role in promoting metabolic health and leanness through inhibiting adipogenesis (Pazienza et al., 2016). In addition, it was identified in the bone marrow-derived mesenchymal stem cells in the context of determining cellular identity (Cagnan et al., 2019). *PKNOX2* mRNA was shown to be significantly downregulated in Fanconi anaemia (FA) compared to healthy donors, which however was not the case at the protein level. It was also demonstrated that the addition of TGFβ1 recombinant protein to the cultured cells increased the expression of *PKNOX2* in both FA- and healthy donor-derived cells (Cagnan et al., 2019).

The integration of transcriptomic and proteomic datasets, which we generated in the context of Aregs revealed other candidates eligible for experimental validation. This is the case for, i.e. highly Areg-specific *Fmo2* gene coding for Dimethylaniline monooxygenase [N-oxide-forming] 2, which hasbeen shown to be relevant in the oxidative metabolism of a variety of xenobiotics (Krueger and Williams, 2005). Another candidate, interesting given its secreted nature, is *Gas6*, coding for Growth arrest-specific protein 6, whose signalling was shown to be implicated in cell growth and survival, cell adhesion and endothelial cell survival (Angelillo-Scherrer et al., 2001).

4.3 Aregs' role in biology

Having experimentally validated the discussed set of Areg-specific candidates, it is, at this moment, still difficult to come to a clear conclusion about the mechanistic regulation or a signalling pathway involved in the inhibitory activity of Aregs, with numerous leads begging for further investigation.

One line of inquiry may be the association of Aregs to vasculature, a feature evoked already by their transcriptional signature, with the increased expression of endothelial genes such as Vcam, Thbd and Mcam, especially pronounced after a few hours of culture (Suppl. Fig. 2.4n). The recurring pertinence of CD142 in the context of Aregs' activity supports this hypothesis, given the acclaimed physiological role of CD142 in coagulation and angiogenesis. In agreement with this, our results showed the peri-vascular localization of the CD142 positive cells within the murine subcutaneous fat pad (Fig. 2.4.a-d and Suppl. Fig. 2.7c-q). We also suggest that Aregs reside within the arterial adventitia, where CD34+ SCA1+ progenitor cells were shown to act under Sonic hedgehog signalling (Majesky et al., 2011). Intriguingly, one of the other most Areg-specific candidates, MEOX2, was shown to suppress proliferation and differentiation of peri-vascular adipocytes (PVAC) derived from the thoracic aortic ring (Liu et al., 2015a). Adding to this speculation, MGP was shown to act, by indirectly increasing VEGF, in the context of pattern formation of mesenchymal cells in vitro as well as in branching of the pulmonary vasculature in vivo (Garfinkel et al., 2004; Yao et al., 2006, 2007). It would be interesting to interrogate Aregs in the context of their angiogenic potential in in vitro assays or inquire whether they could be systemically responsible for modulating peri-vascular adipogenesis. Further still, another line of investigation could be to study their involvement in obesity-specific fibrosis and inflammation.

Another surprising pattern emerging from the obtained results is the similarity of the tested candidates to the visceral adipose compartment, and more precisely, to the non-adipogenic properties of the visceral adipose stem and precursor cells. A number of Areg-specific genes have been shown to be significantly higher expressed in visceral *versus* subcutaneous ASPCs in human and mice, including *Aldh1a2*, *F3*, *Mgp*, *Bgn* and *Pknox2* (Li et al., 2020; Meissburger et al., 2016; Reichert et al., 2011; Takeda et al., 2016). Moreover, *F3* and *MGP* genes in human, have been shown to be regulated by retinoic acid, which itself has been demonstrated to be endogenously higher in visceral compared to subcutaneous ASPCs and directly associated with their impaired capacity to give rise to adipocytes (Takeda et al., 2016). Further, Wilms tumor protein (coded by *Wt1*) has been proposed to control the expression of the visceral-specific *ALDH1A2* by binding to its promoter region. This was corroborated by the *WT1* knockdown in visceral ASPCs leading to a reduced expression of *ALDH1A2* and improved adipogenesis (Takeda et al., 2016). It will be interesting to further investigate the relevance of RA-mediated signalling in Aregs, through, for example, transcriptionally interrogating RA-treated CD142– ASPCs in the context of Areg-specific genes.

In addition, *Bgn* and *Mgp* have been shown to be significantly higher expressed in visceral as opposed to subcutaneous stromal vascular fraction (Meissburger et al., 2016). These, together with other factors including Sparc-like 1 (*Sparc1*) and Decorin (*Dcn*, also found specific for Aregs), have been

identified in the context of the inhibitory power of visceral SVF-derived secretome (Meissburger et al., 2016).

The hypothesis of Aregs sharing phenotypic and functional properties with visceral ASPCs will have to be further researched. A starting point could be an in-depth molecular and functional characterization of visceral Aregs in the context of the degree of their non-adipogenic phenotype as well as the power of their inhibitory activity compared to subcutaneous Aregs. It is tempting to speculate that Aregs, through their number and/or the potency of their signal may contribute to the impaired capacity of visceral adipose stem and precursor cells to undergo adipogenesis. This would, in turn, contribute to the hypertrophic expansion of the visceral tissue. As visceral hypertrophy in obesity causes insulin resistance, adipose tissue fibrosis and inflammation, all together responsible for the decline of overall metabolic health (Ghaben and Scherer, 2019), Aregs involvement would be extremely interesting from the potential therapeutic point of view.

Chapter 5 Methods

No statistical methods were used to predetermine sample size. The experiments were not randomized and investigators were not blinded to allocation during experiments and outcome assessment, except for the quantification of *in vivo* implantation experiments.

Bioethics

All mouse experiments were conducted in strict accordance with the Swiss law and all experiments were approved by the ethics commission of the state veterinary office (VD2984/2015, 60/2012, 43/2011, 80/2014). The work on human ASPC cultures derived from human lipoaspirate samples was approved by the ethical commission of Canton Ticino (CE 2961 from 22.10.2015) and conforms to the guide-lines of the 2000 Helsinki declaration. The anonymized samples were collected under signed informed consent.

Generation of the DLK1 (also known as PREF1)-RFP mouse

The new mouse strain Tg(Pref1-CreER)426 Biat (Pref1-CreER) was generated using the bacterial artificial chromosome (BAC) RP24-334E14 (BACPAC Resources Center) according to previously published methods (Johansson et al., 2010, Rosenwald et al., 2013).

Isolation of the adult mouse SVF

Subcutaneous and visceral adipose tissue depots were dissected from male and female 8–12-weekold wild-type, new-born wild-type, DLK1–RFP or male ob/ob C57BL/6J mice into ice-cold PBS. The tissue was finely minced using scissors, transferred into collagenase (Sigma-Aldrich #C6885-1G, 2 mg/ml of collagenase buffer (25 mM NaHCO3, 12 mM KH2PO4, 1.2 m MgSO4, 4.8 mM KCl, 120 mM NaCl, 1.4 M CaCl2, 5 mM Glucose, 2.5% BSA, pH = 7.4)) and incubated for 1 h at 37 °C under agitation, resuspended after 45 min of digestion. The cell suspension was then fil-tered through a 100 µm, then 40 µm cell strainer to ensure a single cell preparation. Next, the cells were pelleted by a 5 min centrifugation at 400 g at room temperature and a red blood cell lysis was performed by incubating the pelleted cells in the red blood cell lysis buffer (154 mM NH4Cl, 10 mM KHCO3, 0.1 mM EDTA) for 5 min, followed by two washes (a 5 min centrifugation at 400 g, room temperature) with FACS buffer (PBS with 3% foetal bovine serum (FBS) (Gibco #10270-106), 1 mM EDTA, 1% penicillin–streptomycin (Gibco #15140122)).

Isolation of the new-born mouse SVF cells

Burgeoning depots of subcutaneous adipose tissue were dissected from new-born (P0.5) wild-type C57BL/6J mice into ice-cold PBS. The tissue was transferred into collagenase (Sigma-Aldrich #C6885-1G, 2 mg/ml of collagenase buffer (25 mM NaHCO3, 12 mM KH2PO4, 1.2 m MgSO4, 4.8 mM KCl, 120 mM NaCl, 1.4 mM CaCl2, 5 mM Glucose, 2.5% BSA, pH = 7.4)) and incubated for 1 h at 37 °C under agitation, resuspended after 45 min of digestion. The cell suspension was then filtered through a 100- μ m, then 40- μ m cell strainer to ensure a single cell preparation. Next, the cells were pelleted by a 5-min centrifugation at 400 g at room temperature and a red blood cell lysis was performed by incubating the pelleted cells in the red blood cell lysis buffer (154 mM NH4Cl, 10 mM KHCO3, 0.1 mM EDTA) for 5 min, followed by two washes (a 5-min centrifugation at 400 g, room temperature) with FACS buffer (PBS with 3% foe-tal bovine serum (FBS) (Gibco #10270-106), 1 mM EDTA, 1% penicillin–streptomycin (Gibco #15140122)).

Isolation of human SVF

Fresh lipoaspirates were washed twice with DPBS with calcium and magnesium (Gibco #14040091) in 100-ml syringes (VWR International #720-2528) (50 ml lipoaspirate per 40 ml DPBS with calcium and magnesium) and incubated with 0.28 U/ml of liberase TM (Roche #05401119001) for 45 min at 37 °C under agitation. The digested tissue was mixed with 1% human albumin (CSL Behring) in DPBS –/– (Gibco #14190094) (50 ml lipoaspirate per 40 ml 1% human albumin in DPBS –/–) and shaken vigorously to liberate the stromal cells (procedure performed twice). The aqueous phase was recovered and centrifuged (all centrifugations performed at 400 g for 5 min at room temperature). The cell pellet was resuspended in 15 ml remaining buffer and filtered through a 100- μ m and then a 40- μ m cell strainer to ensure a single-cell preparation, centrifuged and resuspended in 5 ml of 5% human albumin (CSL Behring). The viability and the number of nucleated cells in the cell suspension obtained was determined using a Nucleostainer, after which a red blood cell lysis was per-formed using VersaLyse solution (Beckman Coulter #A09777) according to the manufacturer's recommendations.

scRNA-seq

For the initial (Fluidigm C1) scRNA-seq experiments, we used the DLK1–RFP mouse strain, as recent results have suggested that adipogenic pro- genitors may be marked by the expression of *Dlk1*. Specifically, in this strain, RFP is induced in *Dlk1*-expressing cells upon feeding with ta-moxifen. Thus, all cells in which the *Dlk1* promoter has been active become RFP+ and can be easi-ly isolated by FACS. We investigated both RFP+ and RFP– cells to explore the differences (if any) in cell composition and molecular characteristics between the two cell populations. However, as no differences were observed, we treated and discussed the data irrespective of RFP status. Cells (550 RFP+ and 550 RFP–, 1,100 in total) at a concentration of 200 cells/µl were sorted by FACS directly into the C1 Single-Cell Auto Prep Array IFC 10–17 µm chip (Fluidigm #100-5760, C1) for 96 cells with the efficiency of ~10%. The presence of the cells was validated by microscopy visualiza-tion (Olympus Cell Auto Prep system (Fluidigm) according to the manufacturer's specifications. Single-cell libraries were multiplexed and sequenced across 3 lanes of HiSeq 2000 (Illumina) using 100-bp single-end sequencing. All results relating to the

Fluidigm C1 single-cell experiment (Figs 2.1a–c, 2a and Supplementary Figs 2.1, 2a and 8) are based on 3 biological replicates performed on 3 different days, each stemming from 3–6 male and female 10–11-week-old mice.

A second scRNA-seq experiment was performed using the Chromium Single Cell Gene Expression Solution (10x Genomics), following the manufacturer's protocol. The SVF was isolated from six 8-week-old mice (3 males and 3 females) as described above. Cells were stained with the anti-mouse antibodies CD31–AF488, CD45–AF488 and TER119 AF488 (Biolegend #303110, #304017 and #116215, respectively), and 280,000 Lin– cells were isolated using a Becton Dickinson FACS Aria III sorter. Cells were washed and resuspended in 250 µl FACS buffer (PBS, 2% FBS, 1 mM EDTA), targeting the required 1,000 cells/µl concentration, accounting fora10–20%loss.We pipetted 9.7 µl cell suspension (concentration of 913 cells/µl, ~8,800 cells), targeting the recovery of ~5,000 cells. Single-cell RNA-seq libraries were obtained following the 10x Genomics recommended protocol, using the reagents included in the Chromium Single Cell 3' v2 Reagent Kit. Libraries were sequenced on the NextSeq 500 v2 (Illumina) instrument using 150 cycles (18 bp barcode + UMI, and 132-bp transcript 3' end), obtaining ~5 × 108 raw reads.

scRNA-seq data analysis

For the C1 scRNA-seq experiments, FASTQ files con- taining 100-bp-long single-end sequenced tags (reads) from 3 replicates of 96 cells each were trimmed and filtered using prinseq 0.20.328 with the parameters '-custom_params 'A 70%;T 70%;G 70%;C 70%' -trim_tail_left 36 -trim_tail_right 36 -lc_ method dust -lc_threshold 45 -min_gc 1 -out_format 3' and cutadapt 1.529 with the parame-ters '-m 36 -q 20' and the Nextera adaptor sequence. The retained tags were evaluated using FastQC v.0.11.2 and aligned to the Ensembl 8430 gene annotation of the NCBI38/mm10 mouse genome using STAR 2.4.0g31 with the parameters '-runThreadN 4 -runMode alignReads -outFilterType BySJout -out-FilterMultimapNmax 20 – alignSJoverhangMin 8 – alignSJDB over-hangMin 1 – out- FilterMismatchNmax 999 –outFilterMismatchNoverLmax 0.04 –alignIntronMin 20 –alignIntronMax 1000000 –alignMates-GapMax 1000000 g -outSAMtype BAM SortedByCoordinate'. The number of tags per gene was calculated using htseq-count 0.6.032 with the parameters 'htseq-count -m intersection-nonempty -s no -a 10 -t exon -i gene_id'. Cells that appeared as doublets in the microscopy images (33) and those that had <40% or <400,000 aligned reads were excluded, which resulted in a total of 208 cells, of which 63 were RFP- according to the microscopy images. Genomic alignment rates, number of detected genes per cell, RNA spike-in recovery as well as correlations between replicate experiments and those with bulk population (control) samples suggested that our data were of high quality (Supplementary Fig. 2.1a-d). RFP+ cells showed significantly higher expression of RFP than RFP- cells (P < 0.01, Wilcoxon rank-sum test, data not shown). For each gene, expression estimates per gene were expressed as log-transformed counts per million (cpm) (log_cpm/log(norm_expr) in Fig. 2.1c and Supplementary Fig. 2.1), by dividing total tags per gene by the total number of gene-aligned reads per cell and taking the log(x + 1) of this value. Using the marker genes listed in Supplementary Table 4, we calculated 'scores'—a single numeric value representative of the expression of multiple marker genes-as the sum of log_cpms across all markers in a category. We further filtered and normalized the data using

the package M3Drop 1.0.133 and the function M3DropCleanData() with the parameters 'is.counts=T, min_detected_genes=3000', obtaining 17,287 genes and 208 cells. We used the function M3Drop-DropoutModels from the M3Drop package to fit the modified Michaelis–Menten equation, a logistic regression (logistic) or a double exponential (ZIFA) function to the relationship between mean expression and dropout-rate (proportion of zero values). After visual inspection of the three fits and examination of the sum of squared residuals and sum of absolute residuals, we determined that the Michaelis-Menten method produced the best fit to our data. We therefore used it to estimate genes that had a significantly higher number of dropouts than expected by chance (referred to as differentially expressed genes above, and informative genes below) using the M3Drop function M3DropDifferential-Expression() at a false discovery rate (FDR) of 0.05, which resulted in 527 genes (listed in Supplementary Table 1). We used these genes to obtain a 2D representation of the cells, while maintaining the similarity relationships between them using t-SNE34 as implemented in the package Rt-SNE_0.1134. Cluster analysis, including silhouette analysis, was performed using the SC3_1.3.6 package35 on the 527 differentially expressed genes, using k = 3 or 4 (Fig. 2.1b and Supplementary Fig. 2.8a). P1 and P3 were stable across different cluster number choices, whereas P2 could be further subdivided. The cell grouping we obtained did not correspond to biological repli-cates, which suggests that the main signal captured by the selected genes is biological and not technical (Supplementary Fig. 2.8b, c). Silhouette analysis supported a three-cluster partitioning (Supplementary Fig. 2.8d), which also corresponded closely to the distribution of Fabp4 expression. Marker genes per cluster were obtained using M3DropGet-Markers() function, with the top 200 included in Supplementary Table 2 and used for functional enrichment analysis (Supplementary Table 3). To test the robustness of our results to methodological choices, we alternatively used a set of 1,827 biologically highly variable (>0.5, FDR 0.05) genes calculated using the scran_1.2.2 pack-age36 instead of the drop-out-based gene selection. We found that 520 of these genes were among the 527 significantly differentially expressed genes described above (Supplementary Fig. 2.8e). The clustering was highly similar to that previously described, with P3 being largely unchanged and ~20% of P1 cells attributed as P2 cells (Supplementary Fig. 2.8f). Over 60% of the top 100 markers identified for each of the three populations were identical (Supplementary Fig. 2.8g) and the t-SNE projection placed P3 outside the P1–P2 space (Supplementary Fig. 2.8h). In Fig. 2.1c, we displayed in colour the top 10 markers per cluster, additional transcription-factor coding genes that were among the top 100 markers per cluster and highlighted genes that code for transcription factors and cell surface proteins. Heat maps were generated using gplots_3.0.1 and the function heatmap.2() with the parameters 'scale="row", Colv = F, Rowv = as.dendrogram (cluster)' (per row z-score transformed log(normalized expression), blue-to-red). Row means per gene are displayed on the left, in white-todark-red. All correlations were calculated based on log_cpm values, with the function cor() and the parameters 'method="Spearman'. Multiple testing correction using the function 'p.adjust and the parameters 'method = Bonferroni') was applied for Fabp4 and Cd34 expression correlations, respectively.

The 10x Genomics scRNA-seq data was processed using cellranger-2.1.0, default parameters and the mouse NCBI38/mm10 genome. Molecular counts were obtained for 2,919 cells (filtered matrix), with 174,362 mean reads/cell, an average of 60.5% reads mapping to the transcriptome and 3,404 median genes detected per cell. We also filtered outlier cells using the median absolute deviation from the

median total library size (logarithmic scale) as well as total gene numbers (logarithmic scale), as implemented in scran36, using a cutoff of 3 (isOutlier, nmads = 3). log(normalized ex-pression) values were obtained using size factors per cell, estimated with scran. The majority of the cells expressed high levels of the housekeeping gene Actb, as well as the ASPC marker genes Cd34, Ly6a and Itqb1 (Supplementary Fig. 2.8i). An initial analysis using all cells revealed that one of the major sources of variation was related to the expression of the gene Xist, as exemplified by the strong clustering of cells expressing Xist in the 2D t-SNE projection (Supplementary Fig. 2.8j). As Xist was expressed (>2 reads/cell) in 93% of the C1 cells, for comparative consistency we included only cells that expressed Xist (≥2 molecular counts per cell) in the reported results. We also noted a strong cell division signal in a small number of cells, as quantified by Cyclone, and exemplified by *Mki67* expression (Supplementary Fig. 2.8). As the C1 data contained cells that were predicted only to be in G1 phase (with a single exception), we also excluded the cells predicted to be in S or G2-M phase. Finally, as we noticed high levels of expression of epithelial-specific genes in a small subset of cells (again, dissimilar to the C1 experiment), we excluded all cells showing any (>0) Epcam and Krt19 or Krt18 expression. The final reported dataset consists of 1,804 cells (Fig. 2.1d). Genomic alignment rates and number of detected genes/cell suggested that our data were of high quality (Supplementary Fig. 2.2a, b). We further used both methods detailed above (M3Drop and highly variable genes) to determine a set of informative genes, and obtained a set of 550 genes with a significantly higher number of drop-outs than expected by chance (FDR = 0.0001, a stringent cutoff designed to exclude the selection of many genes expressed at very low levels, given that the 10x Genomics data showed a different drop-out distribution as compared to the C1 data) and 223 highly variable genes (FDR 0.1 and biological variability >0.3). Combining these two sets of genes gave 631 unique informative genes (Supplementary Table 6), which we used to perform consensus clustering using SC3 1.3.6 and to display the cells in 2D using t-SNE, similar to as described above. Although the sum of the squared differences decreased rapidly from using two to using three or four clusters (Supplementary Fig. 2.8k), silhouette analysis suggested a local maximum at four clusters (Supplementary Fig. 2.8l). We therefore used k = 4 in the manuscript. We note that a larger choice of k could provide further insight into additional subgroupings of the reported populations. Marker genes per cluster were again obtained using M3DropGetMarkers() function; the top 200 are included in Supplementary Table 7, and were used for functional enrichment analysis. The top 100 marker genes were used to compare the clusters (groups, G) with the previous ones (populations, P) (Fig. 2.1f and Supplementary Figs 2.2e, 2.8m), revealing >30% overlap for each population with its respective group.

FACS-based cell isolation of mouse cells

The isolated single-cell suspension was diluted to 0.75 or 1 \times 107 cells/ml with FACS buffer (PBS with 3% FBS, 1 mM EDTA, 1% penicillin–streptomycin) and the following fluorophore-conjugated antibodies were added (in titration-determined quantities, Supplementary Table 17): anti-mouse CD31–AF488, anti-mouse CD45–AF488, anti-mouse TER119–AF488 (BioLegend #303110, #304017 and #116215, respectively) for selecting the Lin– population; anti-mouse SCA1-PE-Cy7 (BioLegend #122513), anti-mouse CD34-BV421 (BioLegend #119321) and anti-mouse CD29-PerCP-efluor710 (eBiosciences

#46-0291) to enrich the Lin– population with ASPCs; anti-mouse CD55-APC (Bio-Legend #131802), anti-mouse VAP1 (Abcam #ab81673) conjugated with allophycocyanin (APC) (Lightning-Link Allophycocyanin (APC) Conjugation Kit (Innova Biosciences #705-0030), anti-mouse CD142–PE (SinoBiological #50413-R001) and anti-ABCG1 antibody (Invitrogen #PA5-13462) conjugated with APC for separating populations negative and positive for the given marker. The cells were incubated with the cocktail of antibodies on ice for 20 min protected from light, after which they were washed and stained with DAPI (Sigma #D9542) or propidium iodide (Molecular Probes #P3566) for assessing viability, and subjected to FACS using a Becton Dickinson FACSAria II sorter. Compensation measurements were performed for single stains using compensation beads (eBiosciences #01-2222-42).

The following gating strategy was applied while sorting the cells: first, the cells were selected based on their size and granularity or complexity (side and forward scatter), and then any events that could represent more than one cell were eliminated. Next, the Lin–(CD31–CD45–TER119–) popula-tion was selected, followed by Lin–SCA1+CD34+CD29+ (C1), Lin–SCA1+CD34+, Lin–SCA1+, or Lin– (10x) selection, which were used as controls for the further analysed populations that were negative or positive for a given marker: Lin–SCA1+CD55– (CD55–) and Lin–SCA1+CD55+ (CD55+), Lin–SCA1+VAP1– (VAP1–) and Lin–SCA1+VAP1+ (VAP1+), Lin–SCA1+ABCG1– (ABCG1–) and Lin–SCA1+AB-CG1+ (ABCG1+), Lin–SCA1+CD34+CD142– (CD142–) and Lin–SCA1+CD142+ (CD142+), and Lin–SCA1+CD142–ABCG1– (CD142–ABCG1–) and Lin–SCA1+CD142+ABCG1+ (CD142+ABCG1+). The measurements were acquired using Diva software supplemented on the Becton Dickinson FACS Aria II sorter and analysed using Kaluza analysis software. The mouse sorting experiments were performed two to four times all stemming from male mice. Subsequent data analysis was performed using Beckman Coulter Kaluza Analysis software or Becton Dickinson FlowJo 10.6.2 version.

FACS-based cell isolation of mouse cells using different anti-CD142 antibodies

SVF cells from adult mice were stained with the above-described antibodies in order to select for ASPCs: Lin–(CD31–CD45–TER119–) followed by selection for SCA1+ cells. Next, four different anti-CD142 antibodies were used in order to isolate CD142– ASPCs and Aregs, namely: 1) SinoBiological, monoclonal rabbit IgG clone #001, PE-conjugated, #50413-R001-PE, 2) SinoBiological, monoclonal rabbit IgG clone #001, #50413-R001, in-house conjugated to Lightning-Link PE with Conjugation Kit (Innova Biosciences #703-0010), 3) BiOrbyt, monoclonal mouse Igg clone HTF-1, PE-conjugated, # ORB507485, 4) R&D Systems, polyclonal goat IgG, PE-conjugated, #FAB3178P.

FACS-based cell isolation of human cells

The isolated single cell suspension was diluted to 1 × 107 cells/ml with FACS buffer (DPBS –/–) with 1% human platelet serum (Cook Medical #G34936 PL-NH-500) and the following fluorophore-conjugated antibodies (Supplementary Table 17) were added: anti-human CD31–AF488, anti-human CD45– AF488 for selecting the Lin– population, and anti-human CD142–PE (BioLegend #303110, #304017 and #365204, respectively) for separating the CD142+ and CD142– populations. 7-Aminoactinomycin D (7-AAD) (Beckman Coulter #A07704) was used for assessing viability and Syto40 (Molecular Probes #S11351) was used for discerning nucleated cells. The cells were then processed as detailed above. The human *ex vivo* experiments were replicated for three different individuals (individuals 1 and 5, individual 4 not shown) (CD142+ ASPCs = $3.10\% \pm 0.85\%$). The human *in vitro* experiments were performed for four different individuals (individuals 1 to 4) (ASPCs = $3.38\% \pm 0.64\%$). The qPCR gene expression assay was performed on *ex vivo* and differentiated human CD142+ ASPCs and CD142– ASPCs from three different individuals (individual 1, individual 4 and individual 5) (Supplementary Fig. 2.6b). The 3' RNA-seq gene expression assay (see below) was performed on *in vitro* and differentiated human ASPCs, CD142+ and CD142– ASPCs from four distinct individuals (individuals 1 to 4).

Imaging Flow Cytometry

Samples were run in a 2-camera, 12-channel ImageStreamX multispectral imaging flow cytometer (Luminex Corporation) at low speed and 40X magnification. Instrument setup and performance tracking was performed daily using the Amnis SpeedBead Kit (Luminex Corporation) for verifying optimal instrument performance. Cells were excited using a 405 nm laser (120 mW), 488 nm laser (200mW), 561nm yellow laser (200mW) and a 642 nm red laser (150 mW). Classifiers were set to eliminate collection of debris based on low area in the brightfield channel (as described in Henery et al., 2008). Data were acquired for \approx 50,000 focused events per sample. Experimental samples contained images and data for Brightfield (Channels 1 and 9), CD31/45-AF488 (Channel 2), CD142–PE (Channel 3), CD34-Pacific Blue (Channel 7), CD146-APC (Channel 11) and Side Scatter (SSC, Channel 12). Single colour controls for each fluorochrome were acquired to generate the compensation matrix that was applied to all the experimental files prior to analysis using IDEAs (Image Data Exploration and Analysis Software) software (Amnis Corporation).

Ex vivo adipogenic differentiation of mouse cells

The same number of cells was sorted directly into flat-bottom microscopy-adapted cell culture plates (Corning #353219), cultured to confluence in high glucose DMEM medium (Gibco #61965026) supplemented with 10% FBS and 1% penicillin–streptomycin, and treated with a single dose of white adipocyte differentiation induction cocktail (0.5 μ M 3-isobutyl-1-methylxanthine (IBMX, Sigma #15879), 1 μ M dexamethasone (Sigma #D2915), 170 nM (1 μ g/ml) insulin (Sigma #19278)), followed by maintenance treatment (170 nM insulin) every 48 h. The culture was carried out until day 8, 9 or 10 after induction, at which point cells were stained for imaging or collected for RNA extraction.

Ex vivo adipogenic differentiation of mouse cells using different differentiation cocktails

The same number of cells was sorted and cultured as described above. At confluence, the cells were treated with a single dose of one of four different versions of white adipocyte differentiation induction cocktails: 1) Complete cocktail in DMDM medium: 0.5 μ M IBMX, 1 μ M dexamethasone, 170 nM (1 μ g/ml) insulin; 2) Min in DMEM medium: 170 nM insulin; 3) Complete + Indomethacin + T3 in DMEM/ F12 (1:1 ratio) medium: 0.5 μ M IBMX, 1 μ M dexamethasone and 20 nM, 125 nM indomethacin, 1 nM Triiodothyronine (T3); 4) Min in DMEM/F12 medium: 20 nM insulin. After 2-3 days of induction, maintenance cocktail was applied every 2-3 days with 1) 170 nM insulin in DMEM for cocktail 1); 2) DMEM

for cocktail 2); 3) 20 nM insulin in DMEM/F12 for cocktail 3); 4) DMEM/F12 for cocktail 4). DMEM for all the corresponding treatments was supplemented with 10% FBS and 1% penicillin–streptomycin. DMEM/F12 for all the corresponding treatments was supplemented with 10% FBS and 50 ng/ml Primocin. The culture was carried till day 6-8 post induction.

Ex vivo adipogenic differentiation of mouse cells with the use of recombinant proteins and retinoic acid

The same number of cells was sorted and cultured as described above. At confluence, the cells were treated with a single dose of white adipocyte differentiation induction cocktail at half of its usual concentration: 0.25 μ M IBMX, 0.5 μ M dexamethasone, 85 nM (0.5 μ g/ml) insulin (in DMEM supplemented with 10% FBS and 1% penicillin–streptomycin), supplemented with the following recombinant proteins: BSA (Sigma, # A3059, A9418), EGF (ThermoFisher Scientific, # PMG8043), CD142 (LifeSpan BioSciences, # LS-G12283), MGP (LifeSpan BioSciences, #LS-G13865), CLEC11A (R&D Systems, #3729-SC), GDF10 (SinoBiological, #50165-M01H), CPE (ArcoBioSystems, #CAE-M5222), BGN (R&D Systems, #8128-CM), CXCL12 (R&D Systems, #460-SD-CF) at various concentrations: 10, 50, 100, 500, 750 and 1000 ng/ml. For retinoic acid treatments, the above described differentiation and maintenance cocktails were supplemented with retinoic acid (Sigma, # R2625) diluted in Dimethyl sulfoxide (DMSO, AppliChem, #A3672,0250). The corresponding doses of the recombinant proteins and the retinoic acid, were applied after 2-3 days of induction with the maintenance medium at half of its usual concentration, 85 nM (0.5 μ g/ml) insulin in DMEM supplemented with 10% FBS and 1% penicillin–streptomycin.

Ex vivo adipogenic differentiation of human cells

The same number of cells was sorted directly into flat-bottom microscopy-adapted cell culture plates in high glucose MEMa medium (Gibco #32561037) supplemented with 5% human platelet serum and 50 µg/ml Primocin (InvivoGen #ant-pm-1). The cells at confluence were treated with induction cocktail (high glucose DMEM, 10% FBS, 50 µg/ml Primocin, 0.5 µM IBMX, 1 µM dexamethasone, 1.7 µM insulin, 0.2 mM indomethacin) for 7 days, followed by maintenance cocktail treatment (high glucose DMEM, 10% FBS, 50 µg/ml Primocin, 1.7 µM insulin) for another 7 days. For expanded human ASPCs, TrypLE Select reagent (Gibco #12563011) was used to collect the cells from the cell culture plates.

Imaging and quantification of *in vitro* differentiation (mouse and human)

For experiments shown in Fig. 2.2c–f, h, i and Spplementary Figs. 2.3d, e, 2.5d, e, h, differentiated cells were fixed with 4% formaldehyde before staining with Hoechst (nuclei) and LD540 (lipid droplets). Images were taken per well with an automatic imaging system (Operetta, Perkin Elmer) and analysed for lipid droplet content using the Harmony software. The differentiation quantification experiments were repeated at least 3 times, with multiple independent wells per experiment. At least 15 images per well (96-well plate) were acquired. One well is represented (Fig. 2.2c and Supplementary Fig. 2.3d), and quantification of 4 or 5 independent wells is shown (Fig. 2.2d and Supplementary Fig. 2.3e).

For experiments shown in Fig. 2.3a–d and Supplementary Figs 2.5a, b, 2.6j, k, once the cells differentiated fully, they were stained with live fluorescence dyes: Bodipy (boron-dipyrromethene, Invitrogen

#D3922) for lipids and Hoechst for nuclei. Cells were incubated with the dyes in FluoroBrite phenol red-free DMEM medium (Gibco #A1896701) supplemented with 10% FBS and 1% penicillin-streptomycin for 30 min at 37 °C in the dark, washed twice with warm PBS and imaged in FluoroBrite medium using a Leica DMI4000 wide filed microscope (objectives: PL-S-APO 5×/0.15, PL-S-APO 10×/0.30, HC-PL-APO 20×/0.70) or a Zeiss LSM700 confocal inverted microscope (objectives: EC Plan-Neofluar 10×/0.30, Plan-Apochromat 20×/0.80). To accurately estimate and represent differences in adipose differentiation, a quantification algorithm for image treatment was developed in collaboration with the EPFL BIOP imaging facility. In brief, image analysis was performed in ImageJ/Fiji, lipid droplets (yellow) and nuclei (blue) images were filtered using a Gaussian blur (sigma equal to 2 and 3, respectively) before an automatic thresholding. The automatic thresholding algorithm selections were chosen on the basis of visual inspection of output images. At least 7 images per well (96-well plate) were acquired. Images of one technical replicate (field of view) are represented in Fig. 3a, and quantification for 2 individuals is represented in Fig. 3b for ex vivo experiments. Images of one technical replicate are represented in Fig. 3c, and quantification for 4 individuals is represented in Fig. 3d for in vitro experiments. Data points shown in Fig. 3b, d correspond to technical replicates (fields of view). The experiments were performed for at least three individuals.

For experiments shown in Chapter 3, once the cells differentiated fully, they were stained with live fluorescence dyes: Bodipy (boron-dipyrromethene, Invitrogen #D3922) for lipids and Hoechst for nuclei. Cells were incubated with the dyes in FluoroBrite, phenol red-free DMEM medium (Gibco #A1896701), supplemented with 10% FBS and 1% penicillin–streptomycin for 30 min at 37 °C in the dark, washed twice with PBS and imaged in FluoroBrite medium. The automated platform Operetta (Per-kin Elmer) was used for imaging. 25 images per well were acquired using a Plan Apochromat 20x Air, NA 0.8 objective, with 10% overlap for further/downstream tiling. The Hoechst, blue, channel was acquired with 40 ms exposure time and 50% of the illumination power. 7-8 z-stacks were acquired for every field of view. The images, supported by Harmony software, were exported as tifs. They were subsequently tiled using the acquired 10% image overlap and Z-projection was performed with the maximum intensity of acquired z-stacks using pipelines develop developed in collaboration with the EPFL BIOP imaging facility.

To accurately estimate and represent differences in adipose differentiation, a quantification algorithm for image treatment was developed in collaboration with the EPFL BIOP imaging facility. In brief, image analysis was performed in ImageJ/Fiji, lipid droplets (yellow) and nuclei (blue) images were filtered using a Gaussian blur (sigma equal to 2 and 3, respectively) before an automatic thresholding. The automatic thresholding algorithm selections were chosen on the basis of visual inspection of output images. The areas corresponding to the thresholded lipid and nuclei signals were next used to calculate the normalised lipid accumulation and finally score the assessed individuals based on the degree of adipose differentiation potential *in vitro*.

Titration experiments

An equal number of Lin–SCA1+CD142–ABCG1– and Lin–SCA1+CD142+ABCG1+ cells (see 'FACSbased cell isolation of mouse cells') were plated in 10 cm collagen I (Corning #354249)-coated cell culture plates and cultured for expansion in high glucose DMEM medium supplemented with 10% foetal bovine serum and 1% penicillin–streptomycin, refreshed every 48 h. At 90% confluence, the CD142–ABCG1– and CD142+ABCG1+ populations were mixed at different ratios (ranging from 0 to 100%) in a 96-well plate at a density of 25,000 cells per well. Cells were differentiated as described in *'Ex vivo* adipogenic differentiation of mouse cells'. The experiment was replicated twice, with n = 4 independent wells each. At least 15 images per well (96-well plate) were acquired. One independent well is represented in Fig. 2.2e, and quantification of four independent wells is rep-resented in Fig. 2.2f.

siRNA-mediated knockdown experiments

For each gene, a pool of 2–4 siRNA probes (Supplementary Table 18) was reverse-transfected to total SVF (Supplementary Fig. 2.5c–f) or CD142+ ASPCs (Aregs) (Fig. 2.2h, i). Seventy-five thousand cells/cm2 were plated with 100 nM of a given siRNA dissolved in 1.5% Lipofectamine RNAiMAX (Invitrogen #13778150) in Opti-MEM I reduced serum medium (Invitrogen #31985062), and high glucose DMEM medium supplemented with 2.5% FBS (w/o penicillin–streptomycin). After 24 h, the medium was changed to high glucose DMEM medium supplemented with 10% FBS and 1% penicillin–streptomycin and after 48 h the cells were collected for determining knockdown efficiency. The experiment was replicated twice, with n = 6–8, 2 biological replicates and 3–4 independent wells each. At least 15 images per well (96-well plate) were acquired. One independent well is represented in Supplementary Fig. 2.5d, and quantification of n = 6–8 independent wells from two biological replicates and two independent experiments (S1 and S2) is represented in Supplementary Fig. 2.5e, f.

Transwell experiments

The corresponding genes were knocked down in Aregs on the transwell inserts (Corning #3381) as described in 'siRNA-mediated knockdown experiments'. 24 h after transfection, the inserts were washed with PBS and changed to high glucose DMEM medium supplemented with 10% FBS and 1% penicillin-streptomycin. Afterwards, the inserts were co-cultured with CD142- ASPCs and at 48 h, both cell populations were treated with white adipocyte differentiation cocktail (see 'Ex vivo adipogenic differentiation of mouse cells') and imaged at day 6 after induction (see 'Quantification of in vitro differentiation'). For Chapter 2, the experiment was replicated twice, with n = 6, 2 biological replicates and 3 independent wells each. At least 15 images per well (96-well plate) were acquired. One independent well is represented in Fig. 2.2h, and quantification of n = 6-8 independent wells from two biological replicates and two independent experiments (S1 and S2) is represented in Fig. 2.2i. The experiment shown in Supplementary Fig. 2.5a, b was performed three times, a technical replicate (two fields of views of one well) is shown in Supplementary Fig. 2.5a and the quantification of two biological replicates is represented in Supplementary Fig. 2.5b; data points correspond to technical replicates (fields of view of one well). The experiments were imaged at day 8, 9 or 10. For Chapter 4, the experiments were replicated four times with n=2-3 technical replicates within each experiment, shown is one biological replicate per experiment.

In vivo differentiation experiments

In Fig. 2.4f–h, 106 SVF cells and 106 CD142–ABCG1– SVF cells were resuspended in 150 µl of Matrigel (Corning #356234) and injected subcutaneously in each flank of the same 5–6-week-old mouse. This experimental set-up minimizes inter-individual variation, and enables a direct comparison of the effect of CD142+ABCG1+ cell removal on adipocyte differentiation capacity *in vivo*. In Supplementary Fig. 2.7i, j, 106 Lin–SCA1+ cells and 106 Lin–SCA1+CD142– cells were injected as described for Fig. 4f–h. After 3 weeks of high fat diet, all Matrigel plugs were excised and fixed in 4% paraformaldehyde (PFA) overnight, dehydrated and embedded in paraffin. Sections of 4 µm were stained with haematoxylin and eosin. From each plug, images of at least 3 full sections were taken and adipocyte numbers, as well as the number of nuclei, were determined with the software CellProfiler, as previously described. Five (Fig. 2.4f–h and Supplementary Fig. 2.7h) and seven (Supplementary Fig. 2.7i, j) biological replicates are represented. The experiments were performed once each.

Vascularisation experiments

The Matrigel plugs were obtained as described in '*In vivo* differentiation'. After excision, the Matrigel plugs were frozen in Neg-50 Frozen Section Medium (ThermoFisher #6502). Sections of 100 μ m were prepared using a Reichert cryostat microtome. Primary antibody anti-isolectin GS-IB4 Alexa Fluor-488 (Invitrogen #121411) was applied in 5% goat serum and incubated 12 h at 4 °C. The sections were then washed with PBS 3 times for 10 min, at room temperature. Finally, the sections were incubated with Hoechst (5 μ g/ml), washed twice for 10 min with PBS and mounted with Pro-Long Diamond Antifade Mountant (Thermo Fisher #P36965). The slides were then imaged with a Leica TCS SP8 microscope. One replicate is represented in Supplementary Fig. 2.7k. The experiment was performed once, and included 6 biological replicates (Supplementary Fig. 2.7l).

RNA isolation and quantitative PCR

RNA isolation of sorted cells. Live cells were collected in RLT+ lysis buffer (Qiagen #1053393) and flash-frozen on dry ice. Cell lysates were homogenized with QIAshredders before RNA isolation using the RNeasy Plus Micro Kit (Qiagen #74034). Reverse transcription was performed using the QuantiTect whole transcriptome kit (Qiagen #207045), following the manufacturer's recommendations for a standard-yield reaction (2 h of amplification time), or SuperScript VILO cDNA Synthesis Kit (Invitrogen #11754050). mRNA expression was normalized to *36b4* (also known as *Rplp0*) (for all mouse experiments, if not otherwise specified) or to *Hprt1* (for the human experiments and the experiments represented in Fig. 2.2b and Supplementary Fig. 2.3c (CD142+ versus CD142–)).

RNA isolation of differentiated cells. mRNA was isolated and transcribed into cDNA using the Multi-MACS cDNA Synthesis Kit (Miltenyi #130-094-410) or collected into Tri-Reagent (Molecular Research Center #TR118). Direct-zol RNA kit (Zymo Research #R2052) was used to extract RNA, followed by reverse transcription using the SuperScript VILO cDNA Synthesis Kit (Invitrogen). Expression levels of mRNA were assessed by real-time PCR using the PowerUp SYBR Green Master Mix (Thermo Fisher Scientific #A25743). mRNA expression was normalized to *36b4* (for mouse experiments) or to *Hprt1* or *HPRT1* (for mouse or human experiments presented in Fig. 2.2b (third panel), Supplementary Fig. 2.3c (third panel) and Supplementary Fig. 4.4b).

Gene expression assays in Supplementary Fig. 2.3c show mean values over n = 4 or 5 biological replicates; the experiment was performed once. Figure 2.2b and Supplementary Fig. 2.3c (CD142+ *versus* CD142–) show n = 3 technical qPCR replicates, from 1 sorting tube. The experiment was performed twice. Supplementary Fig. 2.3f shows n = 4 or 5 independent wells, the experiment was replicated four times. Supplementary Fig. 2.5c, g represents 2–3 independent wells from 2 biological replicates each (n = 4–6 independent wells). The experiment was performed once. Supplementary Fig. 2.5j represents 3 independent wells from 2 biological replicates each (n = 6). The experiment was replicated two times. Supplementary Fig. 2.6b rep- resents three biological replicates, from three individuals (individuals 1, 4 and 5).

Bulk mRNA-seq

Bulk mRNA sequencing (Figs 2.2, 2.3 and Supplementary Figs 2.4, 2.6) was performed as previously described. In brief, 50 ng of total RNA from each sample was reverse transcribed in a 96-well plate using Maxima H Minus Reverse Transcriptase (Thermo Fisher Scientific #EP0753) with individual oligo-dT primers, featuring a 6-nt-long multiplexing barcode, and template switch oligo (Microsynth Custom made). Specifically, each oligo-dT primer is biotinylated and has the following structure: 5'- ACACTCT-TTCCCTACACGACGCTCTTCCGATCT[BC6] [N15] [T30] VN-3', in which [BC6] represents a 6-nt barcode that is specific to each well and N15 represents a stretch of random nucleotides forming a unique molecular identifier (UMI). Thus, for each well, we have generated a unique combination of barcodes and UMIs to identify each well (sample) and transcript. Next, all the samples were pooled together, purified using the DNA Clean and Concentrator kit (Zymo Research #D4014), and treated with exonuclease I (NEB or New England BioLabs #M0293S). The full-length cDNA library was amplified using a single primer and purified with Agencourt AMPure XP beads (Beckman Coulter A63881). The sequencing library was prepared by tagmentation of 10 ng full-length cDNA with a Tn5 transposase made in house, at 55 °C for 9 min41. Tagmented DNA was purified with the DNA Clean and Concentrator kit and PCR amplified using NEBNext High-Fidelity 2X PCR Master Mix (NEB or New England BioLabs #M0541S) with an i7 adaptor identical to Illumina Nextera and custom i5 (Microsynth custom made). The PCR reaction was then purified twice with AMPure beads or Agencourt AMPure XP beads and the average fragment size of the library was evaluated using a Fragment Analyzer (Advanced Analytical) before paired-end se-quencing with NextSeq 500 (Illumina).

Analysis of bulk mRNA-seq data

For conventional bulk mRNA sequencing analysis, FASTQ files containing 100-bp-long single-end sequenced tags (reads) from four replicates of Lin–CD29+CD34+SCA1+RFP+ and Lin–CD29+CD34+S-CA1+RPF– each were analysed. Reads from each sample were trimmed and filtered using prinseq 0.20.328 with the parameters '-custom_params 'A 70%;T 70%;G 70%;C 70%' -trim_tail_left 36 -trim_tail_right 36 -lc_method dust -lc_threshold 45 -min_gc 1 -out_format 3' and cutadapt 1.529 with the parameters '-m 36 -q 20' and the Nextera adaptor sequence. The retained tags were evaluated using FastQC v.0.11.2 and aligned to the Ensembl 8430 gene annotation of the NCBI38/mm10 mouse genome using STAR 2.4.0g31 with the parameters '-runThreadN 4–runMode alignReads–outFilterType

BySJout–outFilterMul- timapNmax 20–alignSJoverhangMin 8 –alignSJDB overhangMin 1–outFil- ter-MismatchNmax 999–outFilterMismatchNoverLmax 0.04–alignIntronMin 20–alignIntronMax 1000000– alignMatesGapMax 1000000 g–outSAMtype BAM Sort-edByCoordinate'. The number of tags per gene was calculated using htseq- count 0.6.032 with the parameters 'htseq-count -m intersection-nonempty -s no -a 10 -t exon -i gene_id'. For each gene, expression estimates per gene were expressed as log-transformed counts per million (log_cpm), by dividing total tags per gene by the total number

of gene-aligned reads per cell and taking the log(x + 1) of this value.

To analyse the data from barcoded mRNA sequencing (Areg-related experiments in mouse and human, Figs 2.2, 2.3 and Supplementary Figs 2.4, 2.6)40, the following workflow was used. Reads from barcoded mRNA-seq experiments have two barcodes, corresponding to the two levels of multiplexing. The first one is common to standard protocols and is used to separate the libraries. The second is specific to the barcoded mRNA-seq protocol and is used to separate the multiplexed samples from the bulk data. The first demultiplexing step was performed with the Illumina BaseSpace platform, and the second was performed using custom scripts. FASTQ files containing 62-bp-long single-end sequenced tags (reads) from at least four biological replicates (4 biological replicates, each with 1–3 independent wells) per ASPC population and sample were analysed: 6 ASPC replicates D0, 4 ASPC samples D12, 8 CD142- ASPC replicates D0, 7 CD142- ASPC 5 h, 7 CD142- ASPC 24 h, 4 CD142- ASPC D12, and 4 replicates each for CD142+ ASPC D0, 5 h, 24 h and D12 (Fig. 2.2g and Supplementary Fig 2.4). Reads from each sample were trimmed and filtered using prinseq 0.20.328 with the parameters '-custom_params 'A 70%;T 70%;G 70%;C 70%' -trim_tail_left 36 -trim_tail_right 36 -lc_method dust -lc_threshold 45 -min_gc 1 -out_format 3' and cutadapt 1.529 with the parameters '-m 36 -g 20' and the Nextera adaptor sequence. The retained tags were evaluated using FastQC v.0.11.2 and aligned to the Ensembl 8430 gene annotation of the NCBI38/mm10 mouse genome using STAR 2.4.0g31 with the parameters '-runThreadN 4-runMode alignReads-outFilterType BySJout-out- FilterMultimapNmax 20-alignSJoverhangMin 8 –alignSJDB overhangMin 1–out- FilterMismatchNmax 999–outFilterMismatchNoverLmax 0.04-alignIntronMin 20-alignIntronMax 1000000-alignMatesGapMax 1000000 g-outSAMtype BAM SortedByCoordi-nate'. The number of tags per gene was calculated using htseq- count 0.6.032 with the parameters 'htseq-count -m intersection-nonempty -s no -a 10 -t exon -i gene_id'.

Genes with a count per million greater than 2 in at least 4 samples were retained, providing a filtered dataset of 26,159 expressed genes across 52 samples. The samples were of high quality, as assessed by the number of aligned reads and detected genes per cell and by correlations with the P3 single-cell data (Supplementary Fig. 2.4c, d). Raw counts were normalized using mean-variance modelling at the observational level, as implemented in the voom() function in limma_3.30.442 and further using combat() in sva_3.22.043 to adjust for batch effects. Differential expression was computed on the normalized values using the limma_3.30.4 pipe- line at an FDR of 0.05 and fold-change cutoff of 2. We detected a large number of changes that were induced upon adipogenic differentiation in all three cell fractions (CD142+, CD142– and ASPCs) (Supplementary Fig. 2.4e, Supplemen-tary Table 11), each of which had over 3,500 significantly differentially expressed genes (FDR 0.05, fold-change 2). Across time points, the highest number of differences was observed between Aregs and CD142– cells or ASPCs (Supplementary Fig. 2.4e). The high transcriptional similarity between CD142– cells and ASPCs (Supplementary Fig. 2.4e, f) was expected based on the low fraction of Aregs (generally <10%) contained in ASPCs. Most differences arose early upon plating (after 5 h and 24 h), but dampened after adipogenic differentiation (Supplementary Fig. 2.4e). Heat maps dis-playing row-normalized (z-score transformation) expression (log_cpm) values were generated using pheatmap_1.0.8 and the parameters 'clustering_distance_ rows = "correlation", cluster_cols = F, clustering_method = "average", scale = "row". Supplementary Fig. 2.4g contains all genes that were expressed at significantly lower levels after adipogenic differentiation (day 12, D12) in CD142+ ASPCs compared to both CD142– ASPCs and all ASPCs; Fig. 2.2g contains all genes that were expressed at significantly higher levels in CD142+ ASPCs compared to CD142– ASPCs after sorting (day 0, D0) and after culturing (5 h and day 1, D1); Supplementary Fig. 2.4k contains all genes that were expressed at significantly higher levels in CD142+ ASPCs compared to CD142- ASPCs after sorting (day 0, D0); Supplementary Fig. 2.4n shows the expression of selected endothelial marker genes. All correlations were calculated based on log cpm values, with the function cor() and the parameters 'method="Spearman". The analysis of the human barcoded mRNA-seq data (displayed in Fig. 2.3 and Supplementary Fig. 2.6) was performed in a manner analogous to that described above for mouse data, but reads were aligned to the Ensembl 8430 gene annotation of the GRCh38/hg20 human genome instead. Figure 2.3e contains only the top 30 genes that were expressed at significantly higher levels (fold-change = 2, FDR = 0.1) in CD142- ASPCs compared to CD142+ ASPCs, and Supplementary Fig. 2.6g contains all genes that were expressed at significantly higher levels (fold-change = 2, FDR = 0.1) in CD142+ ASPCs compared to CD142– ASPCs.

Immunofluorescence

Mice were anaesthetized with isoflurane and perfused with PBS (5 min) followed by 4% PFA (paraformaldehyde, electron microscopy grade (VWR #100504-858) (5 min). The subcutaneous fat pads were dissected and post-fixed in 4% PFA for 2 h at 4 °C upon gentle shaking. Next, the tissue was washed with PBS and incubated with 30% sucrose for 24 h at 4 °C upon gentle shaking. Cryoblocks were prepared using a Cryomatrix (Thermo Fisher Scientific #6769006) and 30-µm sections were generated using a Leica CM3050S cryostat at -30 °C. The sections were deposited onto glass slides and incubated in -20 °C acetone for 10 min, after which they were dried at room temperature for 10 min, rehydrated in PBS and blocked with 10% goat serum (Invitrogen #31872) supplemented with 0.3% TritonX100 (Sigma #T9284) for 1 h. Primary antibodies (Supplementary Table 17, hamster anti-mouse CD31, BioRad #MCA1370A, rabbit anti-mouse CD142, SinoBiological #50413-R001, rat anti-mouse SCA1, Bio-Legend #122501) were applied in 1% goat serum with 0.3% TritonX100 and incubated for 24h at 4 °C. The sections were then washed with PBS three times for 10 min at room temperature. Secondary antibodies (Supplementary Table 17, goat anti-hamster AF-546, Molecular Probes #A21111, donkey anti-rabbit AF-488, Molecular Probes #A21247, goat anti-rat AF-647, Molecular Probes #A21206) were applied in PBS with 0.3% TritonX100 and incubated in the dark for 1 h at room temperature, followed by three 10-min washes with PBS at room temperature in the dark. Finally, the sections were incubated with Hoechst (5 μg/ml), washed two times for 10 min with PBS and mounted with Fluoromount G (Southern Biotech #0100-01). The slides were then imaged with a Zeiss confocal LSM700 microscope (objectives: EC Plan-Neofluar 10×/0.30, Plan-Apochromat 20×/0.80, Plan-Apochromat 40×/1.30). The

results presented in Fig. 2.4a–d were replicated in at least three independent experiments. We note that we also verified that the signal we detected is not the result of autofluorescence of the adipose tissue (Supplementary Fig. 2.7a) or from unspecific binding of secondary antibodies (Supplementary Fig. 2.7b). We also showed that the peri-vascular staining is not an artefact caused by perfusing the animals with 4% PFA (Supple-mentary Fig. 2.7c).

Mass spectrometry sample preparation

Each sample was digested by Filter Aided Sample Preparation (FASP) (Wiśniewski et al., 2009) with minor modifications. Dithiothreitol (DTT) was replaced by Tris (2-carboxyethylphosphine (TCEP) as reducing agent and lodoacetamide by Chloracetamide as alkylating agent. A combined proteolytic digestion was performed using Endoproteinase Lys-C and Trypsin. Peptides were desalted on stagetips (Rappsilber et al., 2007) and dried under a vacuum concentrator. For LC-MS/MS analysis, resuspended peptides were separated by reversed phase chromatography on a Dionex Ultimate 3000 RSLC nano UPLC system connected in-line with an Orbitrap QExactive HF (Thermo Fisher Scientific, Waltham, USA). Protein identification and quantification were performed with the search engine MaxQuant 1.5.1.2 (Cox and Mann, 2008). The Mouse Uniprot database (Last Modified: 2017-05-16, 51434 canonical and isoform sequences) was used. Carbamidomethylation was set as fixed modification, whereas oxidation (M), phosphorylation (S,T,Y) and acetylation (Protein N-term) were considered as variable modifications. A maximum of two missed cleavages were allowed. "Match between runs" was enabled. A minimum of 2 peptides was allowed for protein identification and the false discovery rate (FDR) cutoff was set to 0.01 for both peptides and proteins. Label-free quantification and normalisation was performed by Maxquant using the MaxLFQ algorithm, with the standard settings (Cox and Mann, 2008).

Mass spectrometry data analysis

For preliminary experiment with only one replicate, a sliding window Z-score transformation is used as a simple statistical testing method (Wilmarth, Z-score_GUI). This method removes abundance-dependent dispersion. For the calculation of individual Z-score, the chosen sliding window width is 351 proteins and the percentage of data that is trimmed, meaning the removal of large Z-scores, corresponds to 10%. To calculate P-values, a gaussian was fit to the distribution of Z-scores. Multi-ple testing correction is addressed with the Benjamini-Hochberg method. Statistical analysis and further graphical displays were generated using homemade programs written in R (version 3.6.1).

Statistical methods

In Fig. 3.1c-d, Fig. 3.5 and Fig 3.8e and f: the line in the middle of the box indicated the median. The box itself represents the middle 50% of the data. The box edges are the 25th and 75th percentiles. Statistical significance in difference in adipogenic score and normalized nuclei count was accessed through ANOVA complemented with Tukey HSD test. In order to control for the performed multiple tests, Tukey test was performed if and only if p-value of ANOVA < 0.05. The difference between groups was considered significant is p-value of the Tukey test < 0.05.

In Suppl. Fig. 3.1f, Fig. 3.2c, Suppl. Fig. 3.2, Fig. 3.3d, Suppl. Fig. 3.4, Fig 3.4, Fig. 3.7, Suppl. Fig. 3.7, Fig. 3.8 and Supple. Fig. 3.8: the height of the bar in the plot indicates the mean of the measurements averaged across all replicates. Error bars indicate standard deviation and various shapes of the points denotes biological replicates. Pairwise t-tests between all groups were conducted and p-values were adjusted for multiple testing by FDR. Significance level was set on 0.05.

Annotation and functional enrichment analyses

Gene annotations were obtained from Ensembl 84 (mar2016.archive.ensembl.org), either by di-rect download or through biomaRt_2.30.044. Enrichment analysis was performed using the web-server Enrichr with default parameters and annotations provided by KEGG, Wikipathway (Kelder et al., 2012), or gene expression data provided by the GTEx project (The GTEx Consortium et al., 2015). For Supplementary Fig. 2.1g, the top 10 enriched terms were considered. Only high- lights are displayed in the figures, and full results are available in Supplementary Tables 3, 8, 12 and 16.

Other computational analyses and data processing remarks concerning Chapter 2

All computational analyses were performed using R version 3.3.2 and Bioconductor version 3.4. All t-tests and Wilcoxon rank-sum tests were unpaired and two-sided, if not otherwise specified. All box plots were generated and displayed in R, using the boxplot() function with default parameters. The median value is indicated with a black line, and a coloured box (hinges) is drawn between the 1st and 3rd quartiles (interquartile range, IQR). The whiskers correspond to approximately $1.5\times$ inter-quartile range (±1.58 interquartile range divided by the square root of n) and outliers are drawn as individual points. All bean plots are generated and displayed in R, using the beanplot() (package beanplot) function with default parameters ('bw = "SJ-dpi", kernel = "gaussian", cut = 3, cutmin = -Inf, cutmax = Inf '). The mean value is indicated with the widest black line, and individual values are indicated with narrower lines. Kernel density estimates are displayed in colours. All bar plots display mean values as centres and the standard deviation as error bars. All included microscopy images and macroscopic images are representative. Exact P values are provided in the Supplementary Table 19; only the P value ranges are provided in the figure legends.

Reporting summary concerning Chapter 2

Further information on experimental design is available in the Nature Research Reporting Summary linked to this paper. Code availability. Sample scripts used to process the data are available at https://github.com/DeplanckeLab/Areg.

Data availability concerning Chapter 2

All raw and processed RNA-seq data have been uploaded in the ArrayExpress database (www. ebi.ac.uk/arrayexpress) with the accession numbers E-MTAB-5785, E-MTAB-5818, E-MTAB-5802, E-MTAB-5787 and E-MTAB-6677. Microscopy images are available from the corre-sponding authors upon reasonable request.

Bibliography

- Ahima, R.S., Prabakaran, D., Mantzoros, C., Qu, D., Lowell, B., Maratos-Flier, E., and Flier, J.S. (1996). Role of leptin in the neuroendocrine response to fasting. Nature 382, 250–252.
- Ambrosi, T.H., and Schulz, T.J. (2017). The emerging role of bone marrow adipose tissue in bone health and dysfunction. J. Mol. Med. 95, 1291–1301.
- Angelillo-Scherrer, A., de Frutos, P.G., Aparicio, C., Melis, E., Savi, P., Lupu, F., Arnout, J., Dewerchin, M., Hoylaerts, M.F., Herbert, J.-M., et al. (2001). Deficiency or inhibition of Gas6 causes platelet dysfunction and protects mice against thrombosis. Nat. Med. 7, 215–221.
- Arthur, H.M. (2000). Endoglin, an Ancillary TGFβ Receptor, Is Required for Extraem bryonic Angiogenesis and Plays a Key Role in Heart Developm ent. 12.
- Bäck, M., Aranyi, T., Cancela, M.L., Carracedo, M., Conceição, N., Leftheriotis, G., Macrae, V., Martin, L., Nitschke, Y., Pasch, A., et al. (2019). Endogenous Calcification Inhibitors in the Prevention of Vascular Calcification: A Consensus Statement From the COST Action EuroSoftCalcNet. Front. Cardiovasc. Med. 5, 196.
- Badeanlou, L., Furlan-Freguia, C., Yang, G., Ruf, W., and Samad, F. (2011). Tissue factor–protease-activated receptor 2 signaling promotes diet-induced obesity and adipose inflammation. Nat Med 17, 1490–1497.
- Barak, Y., Nelson, M.C., Ong, E.S., Jones, Y.Z., Ruiz-Lozano, P., Chien, K.R., Koder, A., and Evans, R.M. PPARg Is Required for Placental, Cardiac, and Adipose Tissue Development. Mol. Cell 11.
- Bartelt, A., and Heeren, J. (2014). Adipose tissue browning and metabolic health. Nat. Rev. Endocrinol. 10, 24–36.
- Bartness, T.J., Vaughan, C.H., and Song, C.K. (2010). Sympathetic and sensory innervation of brown adipose tissue. Int. J. Obes. 34, S36–S42.
- Berry, R., and Rodeheffer, M.S. (2013). Characterization of the adipocyte cellular lineage in vivo. Nat. Cell Biol. 15, 302–308.
- Berry, D.C., DeSantis, D., Soltanian, H., Croniger, C.M., and Noy, N. (2012). Retinoic Acid Upregulates Preadipocyte Genes to Block Adipogenesis and Suppress Diet-Induced Obesity. Diabetes 61, 1112–1121.
- Berry, R., Jeffery, E., and Rodeheffer, M.S. (2014). Weighing in on Adipocyte Precursors. Cell Metab. 19, 8–20.
- Betz, M.J., and Enerbäck, S. (2015). Human Brown Adipose Tissue: What We Have Learned So Far. Diabetes 64, 2352–2360.

- Billon, N., Iannarelli, P., Monteiro, M.C., Glavieux-Pardanaud, C., Richardson, W.D., Kessaris, N., Dani, C., and Dupin, E. (2007). The generation of adipocytes by the neural crest. Development 134, 2283–2292.
- Birsoy, K., Berry, R., Wang, T., Ceyhan, O., Tavazoie, S., Friedman, J.M., and Rodeheffer, M.S. Analysis of gene networks in white adipose tissue development reveals a role for ETS2 in adipogenesis. 12.
- Bloomgarden, Z.T. (2003). Fat Metabolism and Diabetes: 2003 American Diabetes Association Postgraduate Course. Diabetes Care 26, 2198–2203.
- Bowers, R.R., Kim, J.W., Otto, T.C., and Lane, M.D. (2006). Stable stem cell commitment to the adipocyte lineage by inhibition of DNA methylation: Role of the BMP-4 gene. Proc. Natl. Acad. Sci. 103, 13022–13027.
- Brack, A.S., and Rando, T.A. (2012). Tissue-Specific Stem Cells: Lessons from the Skeletal Muscle Satellite Cell. Cell Stem Cell 10, 504–514.
- Bremer, A.A., and Jialal, I. (2013). Adipose Tissue Dysfunction in Nascent Metabolic Syndrome. J. Obes. 2013, 1–8.
- Bunnell, B., Flaat, M., Gagliardi, C., Patel, B., and Ripoll, C. (2008). Adipose-derived stem cells: Isolation, expansion and differentiation. Methods 45, 115–120.
- Burl, R.B., Ramseyer, V.D., Rondini, E.A., Pique-Regi, R., Lee, Y.-H., and Granneman, J.G. (2018). Deconstructing Adipogenesis Induced by β3-Adrenergic Receptor Activation with Single-Cell Expression Profiling. Cell Metab. 28, 300-309.e4.
- Cagnan, I., Cosgun, E., Konu, O., Uckan, D., and Gunel-Ozcan, A. (2019). PKNOX2 expression and regulation in the bone marrow mesenchymal stem cells of Fanconi anemia patients and healthy donors. Mol. Biol. Rep. 46, 669–678.
- Candia, A.F., Hu, J., Crosby, J., Lalley, P.A., Noden, D., and Wright, C.V.E. Mox-1 and Mox-2 define a novel homeobox gene subfamily and are differentially expressed during early mesodermal patterning in mouse embryos. 14.
- Cannon, B., and Nedergaard, J. (2004). Brown Adipose Tissue: Function and Physiological Significance. Physiol. Rev. 84, 277–359.
- Carbone, F., La Rocca, C., and Matarese, G. (2012). Immunological functions of leptin and adiponectin. Biochimie 94, 2082–2088.
- Carneiro-Lobo, T.C., Lima, M.T., Mariano-Oliveira, A., Dutra-Oliveira, A., Oba-Shinjo, S.M., Marie, S.K.N., Sogayar, M.C., and Mon-teiro, R.Q. (2014). Expression of tissue factor signaling pathway elements correlates with the production of vascular endothelial growth factor and interleukin-8 in human astrocytoma patients. Oncol. Rep. 31, 679–686.
- Cawthorn, W.P., Scheller, E.L., and MacDougald, O.A. (2012). Adipose tissue stem cells meet preadipocyte commitment: going back to the future. J. Lipid Res. 53, 227–246.
- Cederberg, A., Grønning, L.M., Ahrén, B., Taskén, K., Carlsson, P., and Enerbäck, S. (2001). FOXC2 Is a Winged Helix Gene that Counteracts Obesity, Hypertriglyceridemia, and Diet-Induced Insulin Resistance. Cell 106, 563–573.
- Celi, F.S., Le, T.N., and Ni, B. (2015). Physiology and relevance of human adaptive thermogenesis response. Trends Endocrinol. Metab. 26, 238–247.
- Chambers, D., Wilson, L., Maden, M., and Lumsden, A. (2007). RALDH-independent generation of retinoic acid during vertebrate embryogenesis by CYP1B1. Development 134, 1369–1383.
- Chen, G., Ning, B., and Shi, T. (2019). Single-Cell RNA-Seq Technologies and Related Computational Data Analysis. Front. Genet. 10, 317.

- Chen, H., Charlat, O., Tartaglia, L.A., Woolf, E.A., Weng, X., Ellis, S.J., Lakey, N.D., Culpepper, J., More, K.J., Breitbart, R.E., et al. (1996). Evidence That the Diabetes Gene Encodes the Leptin Receptor: Identification of a Mutation in the Leptin Receptor Gene in db/db Mice. Cell 84, 491–495.
- Chondronikola, M., Volpi, E., Borsheim, E., Porter, C., Annamalai, P., Enerback, S., Lidell, M.E., Saraf, M.K., Labbe, S.M., Hurren, N.M., et al. (2014). Brown Adipose Tissue Improves Whole-Body Glucose Homeostasis and Insulin Sensitivity in Humans. Diabetes 63, 4089–4099.
- Chu, A.J. (2011). Tissue Factor, Blood Coagulation, and Beyond: An Overview. Int. J. Inflamm. 2011, 1–30.
- Cohen, P., and Spiegelman, B.M. (2015). Brown and Beige Fat: Molecular Parts of a Thermogenic Machine. Diabetes 64, 2346–2351.
- Considine, R.V., Kriauciunas, A., Ohannesian, J.P., and Bauer, T.L. (1996). Serum Immunoreactive-Leptin Concentrations in Normal-Weight and Obese Humans. N. Engl. J. Med. 334, 4.
- Cox, J., and Mann, M. (2008). MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. Nat. Biotechnol. 26, 1367–1372.
- Crisan, M., Yap, S., Casteilla, L., Chen, C.-W., Corselli, M., Park, T.S., Andriolo, G., Sun, B., Zheng, B., Zhang, L., et al. (2008). A Peri-vascular Origin for Mesenchymal Stem Cells in Multiple Human Organs. Cell Stem Cell 3, 301–313.
- Cristancho, A.G., and Lazar, M.A. (2011). Forming functional fat: a growing understanding of adipocyte differentiation. Nat. Rev. Mol. Cell Biol. 12, 722–734.
- Cypess, A.M., Lehman, S., Williams, G., Tal, I., Rodman, D., Goldfine, A.B., Kuo, F.C., Palmer, E.L., Tseng, Y.-H., Doria, A., et al. (2009). Identification and Importance of Brown Adipose Tissue in Adult Humans. N. Engl. J. Med. 360, 1509–1517.
- Dorfleutner, A., Hintermann, E., Tarui, T., Takada, Y., and Ruf, W. (2004). Cross-talk of Integrin α3β1 and Tissue Factor in Cell Migration. Molecular Biology of the Cell 15, 10.
- Douville, J.M., Cheung, D.Y.C., Herbert, K.L., Moffatt, T., and Wigle, J.T. (2011). Mechanisms of MEOX1 and MEOX2 Regulation of the Cyclin Dependent Kinase Inhibitors p21CIP1/WAF1 and p16INK4a in Vascular Endothelial Cells. PLoS ONE 6, e29099.
- Dulloo, A.G. (2008). Thrifty energy metabolism in catch-up growth trajectories to insulin and leptin resistance. Best Pract. Res. Clin. Endocrinol. Metab. 22, 155–171.
- Dulloo, A.G., Jacquet, J., Solinas, G., Montani, J.-P., and Schutz, Y. (2010). Body composition phenotypes in pathways to obesity and the metabolic syndrome. Int. J. Obes. 34, S4–S17.
- Enerbäck, S., Jacobsson, A., Simpson, E.M., Guerra, C., Yamashita, H., Harper, M.-E., and Kozak, L.P. (1997). Mice lacking mitochondrial uncoupling protein are cold-sensitive but not obese. Nature 387, 90–94.
- Faber, D.R., de Groot, Ph.G., and Visseren, F.L.J. (2009). Role of adipose tissue in haemostasis, coagulation and fibrinolysis. Obes. Rev. 10, 554–563.
- Frederich, R.C., Hamann, A., Anderson, S., Löllmann, B., Lowell, B.B., and Flier, J.S. (1995). Leptin levels reflect body lipid content in mice: Evidence for diet-induced resistance to leptin action. Nat. Med. 1, 1311–1314.
- Garfinkel, A., Tintut, Y., Petrasek, D., Bostrom, K., and Demer, L.L. (2004). Pattern formation by vascular mesenchymal cells. Proc. Natl. Acad. Sci. 101, 9247–9250.
- Ghaben, A.L., and Scherer, P.E. (2019). Adipogenesis and metabolic health. Nat. Rev. Mol. Cell Biol. 20, 242–258.

- Goldstein, B.J., Scalia, R.G., and Ma, X.L. (2009). Protective vascular and myocardial effects of adiponectin. Nat. Clin. Pract. Cardio-vasc. Med. 6, 27–35.
- Greenwood MR, Hirsch J. (1974). Postnatal development of adipocyte cellularity in the normal rat. J Lipid Res 15(5), 474-483.
- Gupta, R.K., Arany, Z., Seale, P., Mepani, R.J., Ye, L., Conroe, H.M., Roby, Y.A., Kulaga, H., Reed, R.R., and Spiegelman, B.M. (2010). Transcriptional control of preadipocyte determination by Zfp423. Nature 464, 619–623.
- Gupta, R.K., Mepani, R.J., Kleiner, S., Lo, J.C., Khandekar, M.J., Cohen, P., Frontini, A., Bhowmick, D.C., Ye, L., Cinti, S., et al. (2012). Zfp423 Expression Identifies Committed Preadipocytes and Localizes to Adipose Endothelial and Perivascular Cells. Cell Metab. 15, 230–239.
- Halberg, N., Khan, T., Trujillo, M.E., Wernstedt-Asterholm, I., Attie, A.D., Sherwani, S., Wang, Z.V., Landskroner-Eiger, S., Dineen, S., Magalang, U.J., et al. (2009). Hypoxia-Inducible Factor 1α Induces Fibrosis and Insulin Resistance in White Adipose Tissue. Mol. Cell. Biol. 29, 4467–4483.
- Halpern, B., Mancini, M.C., and Halpern, A. (2014). Brown adipose tissue: what have we learned since its recent identification in human adults. Arq. Bras. Endocrinol. Metabol. 58, 889–899.
- Han, J., Lee, J.-E., Jin, J., Lim, J.S., Oh, N., Kim, K., Chang, S.-I., Shibuya, M., Kim, H., and Koh, G.Y. (2011). The spatiotemporal devel-opment of adipose tissue. Development 138, 5027–5037.
- Harms, M., and Seale, P. (2013). Brown and beige fat: development, function and therapeutic potential. Nat. Med. 19, 1252–1263.
- Harrington, M., Pond-Tor, S., and Boney, C.M. (2007). Role of Epidermal Growth Factor and ErbB2 Receptors in 3T3-L1 Adipogenesis*. Obesity 15, 563–571.
- Hepler, C., Shan, B., Zhang, Q., Henry, G.H., Shao, M., Vishvanath, L., Ghaben, A.L., Mobley, A.B., Strand, D., Hon, G.C., et al. (2018). Identification of functionally distinct fibro-inflammatory and adipogenic stromal subpopulations in visceral adipose tissue of adult mice. ELife 7, e39636.
- Hepler, C., Vishvanath, L., and Gupta, R.K. (2017). Sorting out adipocyte precursors and their role in physiology and disease. Genes Dev. 31, 127–140.
- Hino, J., Miyazawa, T., Miyazato, M., and Kangawa, K. (2012). Bone morphogenetic protein-3b (BMP-3b) is expressed in adipocytes and inhibits adipogenesis as a unique complex. Int. J. Obes. 36, 725–734.
- Hocking, S.L., Stewart, R.L., Brandon, A.E., Suryana, E., Stuart, E., Baldwin, E.M., Kolumam, G.A., Modrusan, Z., Junutula, J.R., Gunton, J.E., et al. (2015). Subcutaneous fat transplantation alleviates diet-induced glucose intolerance and inflammation in mice. Diabetologia 58, 1587–1600.
- Holland, W.L., Adams, A.C., Brozinick, J.T., Bui, H.H., Miyauchi, Y., Kusminski, C.M., Bauer, S.M., Wade, M., Singhal, E., Cheng, C.C., et al. (2013). An FGF21-Adiponectin-Ceramide Axis Controls Energy Expenditure and Insulin Action in Mice. Cell Metab. 17, 790–797.
- Huang, H., Song, T.-J., Li, X., Hu, L., He, Q., Liu, M., Lane, M.D., and Tang, Q.-Q. (2009). BMP signaling pathway is required for commitment of C3H10T1/2 pluripotent stem cells to the adipocyte lineage. Proc. Natl. Acad. Sci. 106, 12670–12675.
- Hudak, C.S., Gulyaeva, O., Wang, Y., Park, S., Lee, L., Kang, C., and Sul, H.S. (2014). Pref-1 Marks Very Early Mesenchymal Precursors Required for Adipose Tissue Development and Expansion. Cell Rep. 8, 678–687.
- Hwang, I., and Kim, J.B. (2019). Two Faces of White Adipose Tissue with Heterogeneous Adipogenic Progenitors. Diabetes Metab. J. 43, 752.
- Hwang, B., Lee, J.H., and Bang, D. (2018). Single-cell RNA sequencing technologies and bioinformatics pipelines. Exp. Mol. Med. 50, 96.
- Iglesias, P., Selgas, R., Romero, S., and Díez, J.J. (2012). MECHANISMS IN ENDOCRINOLOGY: Biological role, clinical significance, and therapeutic possibilities of the recently discovered metabolic hormone fibroblastic growth factor 21. Eur. J. Endocrinol. 167, 301–309.
- Jeffery, E., Church, C.D., Holtrup, B., Colman, L., and Rodeheffer, M.S. (2015). Rapid depot-specific activation of adipocyte precursor cells at the onset of obesity. Nat. Cell Biol. 17, 376–385.
- Jiang, Y., Berry, D.C., Tang, W., and Graff, J.M. (2014). Independent Stem Cell Lineages Regulate Adipose Organogenesis and Adipose Homeostasis. Cell Rep. 9, 1007–1022.
- Johansson, T., Broll, I., Frenz, T., Hemmers, S., Becher, B., Zeilhofer, H.U., and Buch, T. (2010). Building a zoo of mice for genetic analyses: A comprehensive protocol for the rapid generation of BAC transgenic mice. Genesis NA-NA.
- Kajimura, S., and Saito, M. (2014). A New Era in Brown Adipose Tissue Biology: Molecular Control of Brown Fat Development and Energy Homeostasis. Annu. Rev. Physiol. 76, 225–249.
- Kajimura, S., Spiegelman, B.M., and Seale, P. (2015). Brown and Beige Fat: Physiological Roles beyond Heat Generation. Cell Metab. 22, 546–559.
- Kelder, T., van Iersel, M.P., Hanspers, K., Kutmon, M., Conklin, B.R., Evelo, C.T., and Pico, A.R. (2012). WikiPathways: building research communities on biological pathways. Nucleic Acids Res. 40, D1301–D1307.
- Kim, D.M., Choi, H.-R., Park, A., Shin, S.-M., Bae, K.-H., Lee, S.C., Kim, I.-C., and Kim, W.K. (2013). Retinoic acid inhibits adipogenesis via activation of Wnt signaling pathway in 3T3-L1 preadipocytes. Biochem. Biophys. Res. Commun. 434, 455–459.
- Kim, J.I., Huh, J.Y., Sohn, J.H., Choe, S.S., Lee, Y.S., Lim, C.Y., Jo, A., Park, S.B., Han, W., and Kim, J.B. (2015). Lipid-Overloaded Enlarged Adipocytes Provoke Insulin Resistance Independent of Inflammation. Mol. Cell. Biol. 35, 1686–1699.
- Knittle, J.L., Timmers, K., Ginsberg-Fellner, F., Brown, R.E., and Katz, D.P. (1979). The growth of adipose tissue in children and adolescents. Cross-sectional and longitudinal studies of adipose cell number and size. J. Clin. Invest. 63, 239–246.
- Kolodziejczyk, A.A., Kim, J.K., Svensson, V., Marioni, J.C., and Teichmann, S.A. (2015). The Technology and Biology of Single-Cell RNA Sequencing. Mol. Cell 58, 610–620.
- Kricun, M.E. (1985). Red-yellow marrow conversion: Its effect on the location of some solitary bone lesions. Skeletal Radiol. 14, 10–19.
- Krotkiewski, M., Björntorp, P., Sjöström, L., and Smith, U. (1983). Impact of obesity on metabolism in men and women. Importance of regional adipose tissue distribution. J. Clin. Invest. 72, 1150–1162.
- Krueger, S.K., and Williams, D.E. (2005). Mammalian flavin-containing monooxygenases: structure/function, genetic polymorphisms and role in drug metabolism. Pharmacol. Ther. 106, 357–387.
- Lee, G.-H., Proenca, R., Montez, J.M., Carroll, K.M., Darvishzadeh, J.G., Lee, J.I., and Friedman, J.M. (1996). Abnormal splicing of the leptin receptor in diabetic mice. Nature 379, 632–635.
- Lee, K.Y., Luong, Q., Sharma, R., Dreyfuss, J.M., Ussar, S., and Kahn, C.R. (2019). Developmental and functional heterogeneity of white adipocytes within a single fat depot. EMBO J. 38.

- Leinninger, G.M., Jo, Y.-H., Leshan, R.L., Louis, G.W., Yang, H., Barrera, J.G., Wilson, H., Opland, D.M., Faouzi, M.A., Gong, Y., et al. (2009). Leptin Acts via Leptin Receptor-Expressing Lateral Hypothalamic Neurons to Modulate the Mesolimbic Dopamine Sys-tem and Suppress Feeding. Cell Metab. 10, 89–98.
- Li, C., Li, J., He, F., Li, K., Li, X., and Zhang, Y. (2020). Matrix Gla protein regulates adipogenesis and is serum marker of visceral adiposity. Adipocyte 9, 68–76.
- Li, Y., Schwalie, P.C., Bast-Habersbrunner, A., Mocek, S., Russeil, J., Fromme, T., Deplancke, B., and Klingenspor, M. (2019). Systems-Genetics-Based Inference of a Core Regulatory Network Underlying White Fat Browning. Cell Rep. 29, 4099-4113.e5.
- Lidell, M.E. (2018). Brown Adipose Tissue in Human Infants. In Brown Adipose Tissue, A. Pfeifer, M. Klingenspor, and S. Herzig, eds. (Cham: Springer International Publishing), pp. 107–123.
- Liu, P., Kong, F., Wang, J., Lu, Q., Xu, H., Qi, T., and Meng, J. (2015a). Involvement of IGF-1 and MEOX2 in PI3K/Akt1/2 and ERK1/2 pathways mediated proliferation and differentiation of perivascular adipocytes. Exp. Cell Res. 331, 82–96.
- Liu, R., Leslie, K.L., and Martin, K.A. (2015b). Epigenetic regulation of smooth muscle cell plasticity. Biochim. Biophys. Acta BBA - Gene Regul. Mech. 1849, 448–453.
- Lundgren, M., Svensson, M., Lindmark, S., Renström, F., Ruge, T., and Eriksson, J.W. (2007). Fat cell enlargement is an independent marker of insulin resistance and 'hyperleptinaemia.' Diabetologia 50, 625–633.
- Macaulay, I.C., and Voet, T. (2014). Single Cell Genomics: Advances and Future Perspectives. PLoS Genet. 10, e1004126.
- Macosko, E.Z., Basu, A., Satija, R., Nemesh, J., Shekhar, K., Goldman, M., Tirosh, I., Bialas, A.R., Kamitaki, N., Martersteck, E.M., et al. (2015). Highly Parallel Genome-wide Expression Profiling of Individual Cells Using Nanoliter Droplets. Cell 161, 1202–1214.
- Maffei, M., Halaas, J., Ravussin, E., Pratley, R.E., Lee, G.H., Zhang, Y., Fei, H., Kim, S., Lallone, R., Ranganathan, S., et al. (1995). Leptin levels in human and rodent: Measurement of plasma leptin and ob RNA in obese and weight-reduced subjects. Nat. Med. 1, 1155–1161.
- Majesky, M.W., Dong, X.R., Hoglund, V., Mahoney, W.M., and Daum, G. (2011). The Adventitia: A Dynamic Interface Containing Resident Progenitor Cells. Arterioscler. Thromb. Vasc. Biol. 31, 1530–1539.
- Majka, S.M., Fox, K.E., Psilas, J.C., Helm, K.M., Childs, C.R., Acosta, A.S., Janssen, R.C., Friedman, J.E., Woessner, B.T., Shade, T.R., et al. (2010). De novo generation of white adipocytes from the myeloid lineage via mesenchymal intermediates is age, adipose depot, and gender specific. Proc. Natl. Acad. Sci. 107, 14781–14786.
- Mankoo, B.S., Skuntz, S., Harrigan, I., Grigorieva, E., Candia, A., Wright, C.V.E., Arnheiter, H., and Pachnis, V. (2003). The concerted action of Meox homeobox genes is required upstream of genetic pathways essential for the formation, patterning and differentiation of somites. Development 130, 4655–4664.
- Matsumoto, Y., Otsuka, F., Hino, J., Miyoshi, T., Takano, M., Miyazato, M., Makino, H., and Kangawa, K. (2012). Bone morphogenet-ic protein-3b (BMP-3b) inhibits osteoblast differentiation via Smad2/3 pathway by counteract-ing Smad1/5/8 signaling. Mol. Cell. Endocrinol. 350, 78–86.
- Meissburger, B., Perdikari, A., Moest, H., Müller, S., Geiger, M., and Wolfrum, C. (2016). Regulation of adipogenesis by paracrine factors from adipose stromal-vascular fraction a link to fat depot-specific differences. Biochim. Biophys. Acta BBA Mol. Cell Biol. Lipids 1861, 1121–1131.
- Merrick, D., Sakers, A., Irgebay, Z., Okada, C., Calvert, C., Morley, M.P., Percec, I., and Seale, P. (2019). Identification of a mesenchymal progenitor cell hierarchy in adipose tissue. Science 364, eaav2501.

- Michalakis, K., Mintziori, G., Kaprara, A., Tarlatzis, B.C., and Goulis, D.G. (2013). The complex interaction between obesity, metabolic syndrome and reproductive axis: A narrative review. Metabolism 62, 457–478.
- Miyazaki, Y., Mahankali, A., Matsuda, M., Mahankali, S., Hardies, J., Cusi, K., Mandarino, L.J., and Defronzo, R.A. Effect of Pioglita-zone on Abdominal Fat Distribution and Insulin Sensitivity in Type 2 Diabetic Patients. 8.
- Monahan, F.J., German, J.B., and Kinsella, J.E. (1995). Effect of pH and temperature on protein unfolding and thiol/disulfide interchange reactions during heat-induced gelation of whey proteins. J. Agric. Food Chem. 43, 46–52.
- Moseti, D., Regassa, A., and Kim, W.-K. (2016). Molecular Regulation of Adipogenesis and Potential Anti-Adipogenic Bioactive Molecules. Int. J. Mol. Sci. 17, 124.
- Naveiras, O., Nardi, V., Wenzel, P.L., Hauschka, P.V., Fahey, F., and Daley, G.Q. (2009). Bone-marrow adipocytes as negative regulators of the haematopoietic microenvironment. Nature 460, 259–263.
- Nedergaard, J., Golozoubova, V., Matthias, A., Asadi, A., Jacobsson, A., and Cannon, B. UCP1: the only protein able to mediate adaptive non-shivering thermogenesis and metabolic ine¤ciency. Biochim. Biophys. Acta 25.
- O'Brien, E.P., Brooks, B.R., and Thirumalai, D. (2012). Effects of pH on Proteins: Predictions for Ensemble and Single-Molecule Pulling Experiments. J. Am. Chem. Soc. 134, 979–987.
- Ottaviani, E., Malagoli, D., and Franceschi, C. (2011). The evolution of the adipose tissue: A neglected enigma. Gen. Comp. Endocrinol. 174, 1–4.
- Passman, J.N., Dong, X.R., Wu, S.-P., Maguire, C.T., Hogan, K.A., Bautch, V.L., and Majesky, M.W. (2008). A sonic hedgehog signaling domain in the arterial adventitia supports resident Sca1+ smooth muscle progenitor cells. Proc. Natl. Acad. Sci. 105, 9349–9354.
- Pazienza, V., Panebianco, C., Rappa, F., Memoli, D., Borghesan, M., Cannito, S., Oji, A., Mazza, G., Tamburrino, D., Fusai, G., et al. (2016). Histone macroH2A1.2 promotes metabolic health and leanness by inhibiting adipogenesis. Epigenetics Chromatin 9, 45.
- Petersen, L.C., Valentin, S., and Hedner, U. (1995). Regulation of the extrinsic pathway system in health and disease: the role of factor Vha and tissue factor pathway inhibitor. 79, 47.
- Popa, S., Moţa, M., Popa, A., Moţa, E., Serafinceanu, C., Guja, C., Catrinoiu, D., Hâncu, N., Lichiardopol, R., Bala, C., et al. (2016). Prevalence of overweight/obesity, abdominal obesity and metabolic syndrome and atypical cardiometabolic phenotypes in the adult Romanian population: PREDATORR study. J. Endocrinol. Invest. 39, 1045–1053.
- Popko, K., Gorska, E., Stelmaszczyk-Emmel, A., Plywaczewski, R., Stoklosa, A., Gorecka, D., Pyrzak, B., and Demkow, U. (2010). Pro-inflammatory cytokines IL-6 and TNF-α and the development of inflammation in obese subjects. Eur. J. Med. Res. 15, 120.
- Pradhan, R.N., Zachara, M., and Deplancke, B. (2017). A systems perspective on brown adipogenesis and metabolic activation: Systems perspective on brown adipogenesis. Obes. Rev. 18, 65–81.
- Rappsilber, J., Mann, M., and Ishihama, Y. (2007). Protocol for micro-purification, enrichment, pre-fractionation and storage of peptides for proteomics using StageTips. Nat. Protoc. 2, 1896–1906.
- Rath, N., Wang, Z., Lu, M.M., and Morrisey, E.E. (2005). LMCD1/Dyxin Is a Novel Transcriptional Cofactor That Restricts GATA6 Function by Inhibiting DNA Binding. Mol. Cell. Biol. 25, 8864–8873.
- Ravussin, Y., Leibel, R.L., and Ferrante, A.W. (2014). A Missing Link in Body Weight Homeostasis: The Catabolic Signal of the Overfed State. Cell Metabolism 20, 565–572.

- Reichert, B., Yasmeen, R., Jeyakumar, S.M., Yang, F., Thomou, T., Alder, H., Duester, G., Maiseyeu, A., Mihai, G., Harrison, E.H., et al. (2011). Concerted Action of Aldehyde Dehydrogenases Influences Depot-Specific Fat Formation. Mol. Endocrinol. 25, 799–809.
- Rennert, R.C., Januszyk, M., Sorkin, M., Rodrigues, M., Maan, Z.N., Duscher, D., Whittam, A.J., Kosaraju, R., Chung, M.T., Paik, K., et al. (2016). Microfluidic single-cell transcriptional analysis rationally identifies novel surface marker profiles to enhance cell-based therapies. Nat. Commun. 7, 11945.
- Rodeheffer, M.S., Birsoy, K., and Friedman, J.M. (2008). Identification of White Adipocyte Progenitor Cells In Vivo. Cell 135, 240–249.
- Rosen, E.D., and Spiegelman, B.M. (2000). Molecular Regulation of Adipogenesis. Annu. Rev. Cell Dev. Biol. 16, 145–171.
- Rosen, E.D., and Spiegelman, B.M. (2014). What We Talk About When We Talk About Fat. Cell 156, 20-44.
- Rosenwald, M., Perdikari, A., Rülicke, T., and Wolfrum, C. (2013). Bi-directional interconversion of brite and white adipocytes. Nat Cell Biol 15, 659–667.
- Salans, L.B., Knittle, J.L., and Hirsch, J. (1968). The role of adipose cell size and adipose tissue insulin sensitivity in the carbohydrate intolerance of human obesity. J. Clin. Invest. 47, 153–165.
- Sanchez-Gurmaches, J., and Guertin, D.A. (2014). Adipocyte lineages: Tracing back the origins of fat. Biochim. Biophys. Acta BBA Mol. Basis Dis. 1842, 340–351.
- Schlesinger, J.B., van Harmelen, V., Alberti-Huber, C.E., and Hauner, H. (2006). Albumin inhibits adipogenesis and stimulates cytokine release from human adipocytes. Am. J. Physiol.-Cell Physiol. 291, C27–C33.
- Schulz, T.J., Huang, P., Huang, T.L., Xue, R., McDougall, L.E., Townsend, K.L., Cypess, A.M., Mishina, Y., Gussoni, E., and Tseng, Y.-H. (2013). Brown-fat paucity due to impaired BMP signalling induces compensatory browning of white fat. Nature 495, 379–383.
- Schupp, M., and Lazar, M.A. (2010). Endogenous Ligands for Nuclear Receptors: Digging Deeper. J. Biol. Chem. 285, 40409–40415.
- Schwalie, P.C., Dong, H., Zachara, M., Russeil, J., Alpern, D., Akchiche, N., Caprara, C., Sun, W., Schlaudraff, K.-U., Soldati, G., et al. (2018). A stromal cell population that inhibits adipogenesis in mammalian fat depots. Nature 559, 103–108.
- Schwarz, E.J., Reginato, M.J., Shao, D., Krakow, S.L., and Lazar, M.A. (1997). Retinoic Acid Blocks Adipogenesis by Inhibiting C/EBPβ-Mediated Transcription. MOL CELL BIOL 17, 10.
- Schwarz, H., Schmittner, M., Duschl, A., and Horejs-Hoeck, J. (2014). Residual Endotoxin Contaminations in Recombinant Proteins Are Sufficient to Activate Human CD1c+ Dendritic Cells. PLoS ONE 9, e113840.
- Seale, P., Bjork, B., Yang, W., Kajimura, S., Chin, S., Kuang, S., Scimè, A., Devarakonda, S., Conroe, H.M., Erdjument-Bromage, H., et al. (2008). PRDM16 controls a brown fat/skeletal muscle switch. Nature 454, 961–967.
- Shook, B., Rivera Gonzalez, G., Ebmeier, S., Grisotti, G., Zwick, R., and Horsley, V. (2016). The Role of Adipocytes in Tissue Regen-eration and Stem Cell Niches. Annu. Rev. Cell Dev. Biol. 32, 609–631.
- Sidossis, L., and Kajimura, S. (2015). Brown and beige fat in humans: thermogenic adipocytes that control energy and glucose homeostasis. J. Clin. Invest. 125, 478–486.
- Singh, A., Foster, G.D., Gunawardana, J., McCoy, T.A., Nguyen, T., Vander Veur, S., Komaroff, E., and Rao, A.K. (2012). Elevated circulating tissue factor procoagulant activity, factor VII, and plasminogen activator inhibitor-1 in childhood obesity: evidence of a procoagulant state. Br. J. Haematol. 158, 523–527.

- Smas, C.M., and Sul, H.S. (1993). Pref-1, a protein containing EGF-like repeats, inhibits adipocyte differentiation. Cell 73, 725–734.
- Spiegelman, B.M. (1998). PPAR- : Adipogenic Regulator and Thiazolidinedione Receptor. 47, 9.
- Spiegelman, B.M., and Flier, J.S. (1996). Adipogenesis and Obesity: Rounding Out the Big Picture. Cell 87, 377–389.
- Svensson, V., Vento-Tormo, R., and Teichmann, S.A. (2018). Exponential scaling of single-cell RNA-seq in the past decade. Nat. Protoc. 13, 599–604.
- Sul H. S. (2009). Minireview: Pref-1: role in adipogenesis and mesenchymal cell fate. Molecular endocrinology (Baltimore, Md.), 23(11), 1717–1725.
- Takeda, K., Sriram, S., Chan, X.H.D., Ong, W.K., Yeo, C.R., Tan, B., Lee, S.-A., Kong, K.V., Hoon, S., Jiang, H., et al. (2016). Retinoic Acid Mediates Visceral-Specific Adipogenic Defects of Human Adipose-Derived Stem Cells. Diabetes 65, 1164–1178.
- Tang, F., Barbacioru, C., Wang, Y., Nordman, E., Lee, C., Xu, N., Wang, X., Bodeau, J., Tuch, B.B., Siddiqui, A., et al. (2009). mRNA-Seq whole-transcriptome analysis of a single cell. Nat. Methods 6, 377–382.
- Tang, W., Zeve, D., Suh, J.M., Bosnakovski, D., Kyba, M., Hammer, R.E., Tallquist, M.D., and Graff, J.M. (2008). White Fat Progenitor Cells Reside in the Adipose Vasculature. Science 322, 583–586.
- Tchernof, A., Belanger, C., Morisset, A.-S., Richard, C., Mailloux, J., Laberge, P., and Dupont, P. (2006). Regional Differences in Adipose Tissue Metabolism in Women: Minor Effect of Obesity and Body Fat Distribution. Diabetes 55, 1353–1360.
- Tchkonia, T., Thomou, T., Zhu, Y., Karagiannides, I., Pothoulakis, C., Jensen, M.D., and Kirkland, J.L. (2013). Mechanisms and Meta-bolic Implications of Regional Differences among Fat Depots. Cell Metab. 17, 644–656.
- Tetsch, L., Koller, C., Dönhöfer, A., and Jung, K. (2011). Detection and function of an intramolecular disulfide bond in the pH-responsive CadC of Escherichia coli. BMC Microbiol. 11, 74.
- The GTEx Consortium, Ardlie, K.G., Deluca, D.S., Segre, A.V., Sullivan, T.J., Young, T.R., Gelfand, E.T., Trowbridge, C.A., Maller, J.B., Tukiainen, T., et al. (2015). The Genotype-Tissue Expression (GTEx) pilot analysis: Multitissue gene regulation in humans. Science 348, 648–660.
- Tran, K.-V., Gealekman, O., Frontini, A., Zingaretti, M.C., Morroni, M., Giordano, A., Smorlesi, A., Perugini, J., De Matteis, R., Sbarbati, A., et al. (2012). The Vascular Endothelium of the Adipose Tissue Gives Rise to Both White and Brown Fat Cells. Cell Metab. 15, 222–229.
- Turer, A.T., Khera, A., Ayers, C.R., Turer, C.B., Grundy, S.M., Vega, G.L., and Scherer, P.E. (2011). Adipose tissue mass and location affect circulating adiponectin levels. Diabetologia 54, 2515–2524.
- Turer, A.T., and Scherer, P.E. (2012). Adiponectin: mechanistic insights and clinical implications. Diabetologia 55, 2319–2326.
- Uezumi, A., Fukada, S., Yamamoto, N., Takeda, S., and Tsuchida, K. (2010). Mesenchymal progenitors distinct from satellite cells contribute to ectopic fat cell formation in skeletal muscle. Nat. Cell Biol. 12, 143–152.
- Vergara, R.C., Jaramillo-Riveri, S., Luarte, A., Moënne-Loccoz, C., Fuentes, R., Couve, A., and Maldonado, P.E. (2019). The Energy Homeostasis Principle: Neuronal Energy Regulation Drives Local Network Dynamics Generating Behavior. Front. Comput. Neuro-sci. 13, 49.
- Vignali, D.A.A., Collison, L.W., and Workman, C.J. (2008). How regulatory T cells work. Nat. Rev. Immunol. 8, 523–532.

- Vishvanath, L., MacPherson, K.A., Hepler, C., Wang, Q.A., Shao, M., Spurgin, S.B., Wang, M.Y., Kusminski, C.M., Morley, T.S., and Gupta, R.K. (2016). Pdgfrβ+ Mural Preadipocytes Contribute to Adipocyte Hyperplasia Induced by High-Fat-Diet Feeding and Prolonged Cold Exposure in Adult Mice. Cell Metab. 23, 350–359.
- Wang, F., Mullican, S.E., DiSpirito, J.R., Peed, L.C., and Lazar, M.A. (2013a). Lipoatrophy and severe metabolic disturbance in mice with fat-specific deletion of PPAR. Proc. Natl. Acad. Sci. 110, 18656–18661.
- Wang, G.-X., Zhao, X.-Y., and Lin, J.D. (2015). The brown fat secretome: metabolic functions beyond thermogenesis. Trends Endocrinol. Metab. 26, 231–237.
- Wang, Q.A., Tao, C., Gupta, R.K., and Scherer, P.E. (2013b). Tracking adipogenesis during white adipose tissue development, expansion and regeneration. Nat. Med. 19, 1338–1344.
- Wiśniewski, J.R., Zougman, A., Nagaraj, N., and Mann, M. (2009). Universal sample preparation method for proteome analysis. Nat. Methods 6, 359–362.
- Wrann, C.D., Laue, T., Hübner, L., Kuhlmann, S., Jacobs, R., Goudeva, L., and Nave, H. (2012). Short-term and longterm leptin exposure differentially affect human natural killer cell immune functions. Am. J. Physiol.-Endocrinol. Metab. 302, E108–E116.
- Wu, J., Boström, P., Sparks, L.M., Ye, L., Choi, J.H., Giang, A.-H., Khandekar, M., Virtanen, K.A., Nuutila, P., Schaart, G., et al. (2012). Beige Adipocytes Are a Distinct Type of Thermogenic Fat Cell in Mouse and Human. Cell 150, 366–376.
- Xue, R., Lynes, M.D., Dreyfuss, J.M., Shamsi, F., Schulz, T.J., Zhang, H., Huang, T.L., Townsend, K.L., Li, Y., Takahashi, H., et al. (2015). Clonal analyses and gene profiling identify genetic biomarkers of the thermogenic potential of human brown and white preadi-pocytes. Nat. Med. 21, 760–768.
- Yamauchi, T., and Kadowaki, T. (2013). Adiponectin Receptor as a Key Player in Healthy Longevity and Obesity-Related Diseases. Cell Metab. 17, 185–196.
- Yao, Y., Zebboudj, A.F., Shao, E., Perez, M., and Boström, K. (2006). Regulation of Bone Morphogenetic Protein-4 by Matrix GLA Protein in Vascular Endothelial Cells Involves Activin-like Kinase Receptor 1. J. Biol. Chem. 281, 33921–33930.
- Yao, Y., Nowak, S., Yochelis, A., Garfinkel, A., and Boström, K.I. (2007). Matrix GLA Protein, an Inhibitory Morphogen in Pulmonary Vascular Development. J. Biol. Chem. 282, 30131–30142.
- Zannettino, A.C.W., Paton, S., Arthur, A., Khor, F., Itescu, S., Gimble, J.M., and Gronthos, S. (2008). Multipotential human adipose-derived stromal stem cells exhibit a perivascular phenotype in vitro and in vivo. J. Cell. Physiol. 214, 413–421.
- Zhang, Y., Proenca, R., Maffei, M., Barone, M., and Leopold, L. Positional cloning of the mouse obese gene and its human homologue. 8.

Magda Zachara

Address: IBI-LSBG, SV-3823, Station 19, EPFL, Lausanne, Switzerland E-mail: magda.zachara@epfl.ch Citizenship: Polish Languages: English, French, Polish

Education

| École Polytechnique Fédérale de Lausanne (EPFL), Faculty of Life Sciences | 2015-2020 |
|---|-----------|
| PhD in Life Molecular Life Sciences PhD Adviser: Prof. Bart Deplancke | |
| Doctoral thesis title: "A single-cell-based identification and characterisation of Aregs, an inhibitory subpopulation of adipose stem and precursor cells" | |
| Geneva University, Faculty of Science | 2012-2014 |
| Master in Biochemistry Master Adviser: Prof. Robbie Loewith Master thesis title: "Identification and characterization of potential upstream effectors of TOBC2 in S. cerevisiae" | |
| | 2010 2012 |
| Geneva University, Faculty of Science Pachalor in Piachamietry | 2010-2012 |
| Bachelor Adviser: Prof. Jean-Marc Matter Bachelor bibliography title: "Sonic Hedgehog's strategy in neural identity assignment" | |
| <i>Claude Bernard University, Lyon, France, Faculty of Science</i> Biochemistry (Classified: 8th/152, sem.1; 23rd/101, sem.2) | 2009-2010 |
| <i>Claude Bernard University, Lyon, France</i> Medicine (Classified: 260th/700) | 2007-2009 |
| PEG French School, Geneva; IFAGE School, Geneva Diplôme Alliance Française | 2005-2006 |
| <i>Collegium Medicum, Jagiellonian University, Kraków, Poland</i> First year (departure due to family reasons) | 2003-2004 |

Professional Experience

| <i>École Polytechnique Fédérale de Lausanne (EPFL)</i> PhD in Life Molecular Life Sciences in the Laboratory of Systems Biology and Genetics | 2015-2020 |
|--|-----------|
| <i>Geneva University</i> Master Internship in Molecular Biology in Prof. Robbie Loewith laboratory | 2012-2014 |
| <i>Geneva University</i> Collaboration with Prof. Dominique Belin in the creation of the MOOC course: Les Classiques de la Génétique Moléculaire | 2014 |

Publications

Pradhan, R. N.*, Zachara, M.*, and Deplancke, B. A systems perspective on brown adipogenesis and metabolic activation. Obesity Reviews, 18: 65–81 (2017). doi: 10.1111/obr.12512.

* shared first author

Schwalie, P.C.*, Dong, H.*, Zachara, M.* et al. A stromal cell population that inhibits adipogenesis in mammalian fat depots. Nature 559, 103–108 (2018). https://doi.org/10.1038/s41586-018-0226-8

* shared first author

Chen, W., Guillaume-Gentil, O., Dainese, R., Rainer, P. Y., Zachara, M., Gäbelein, C. G., Vorholt, J. A., Deplancke, B. Live cell transcriptome profiling using Live-seq. Science (in revision), (2020)

Selected Talks

Wihuri Research Institute Symposium – "Translational Insights to Organ-Specific Vasculature" – June 2019, Biomedicum Helsinki, Finland

23rd EASD-(European Association for the Study of Diabetes)-Hagedorn Oxford Workshop – "Big Data, Omics and Bioinformatics in Diabetes" – August 2018, Keble College, Oxford, United Kingdom

Stem Cell Retreat - May 2018, Freudenstadt, Germany

SV in Extenso – Multidisciplinary symposium organised by EPFL students and postdocs – March 2018, EPFL, Lausanne, Switzerland

Selected Poster Presentations

Sciences Keystone Symposia – "Obesity and Adipose Tissue Biology" – January 2017, Keystone, Colorado, USA

Single Cell Genomics 2016 – September 2016, Welcome Genome Campus, Hinxton, Cambridge, United Kingdom

Thematic LIMNA (Lausanne Integrative Metabolism & Nutrition Alliance) symposium – "Muscle stem cells, metabolism and aging" – September 2016, Lausanne, Switzerland

Prizes and Grants

First Prize in the category "Scientific Insight" at the microscopy contest "Seeing Life Science" – March 2018, EPFL, Lausanne, Switzerland

Travel Grant to an overseas conference from Lausanne Integrative Metabolism & Nutrition Alliance (LIMNA) – January 2017, Lausanne, Switzerland

Best Poster Prize at the Thematic LIMNA (Lausanne Integrative Metabolism & Nutrition Alliance) symposium – "Muscle stem cells, metabolism and aging" – September 2016, Lausanne, Switzerland