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RELEVANCE OF THE CLUSTER miR-424(322)/503 IN THE DEVELOPMENT OF HYPERPLASTIC ADIPOSE TISSUE

Francisco José Ortega Delgado
Hospital Dr. Josep Trueta - IDIBGi Institut d'Investigació Biomèdica de Girona
David Llobet Navas
IDIBELL Institut d'Investigació Biomèdica de Bellvitge

1. Project summary

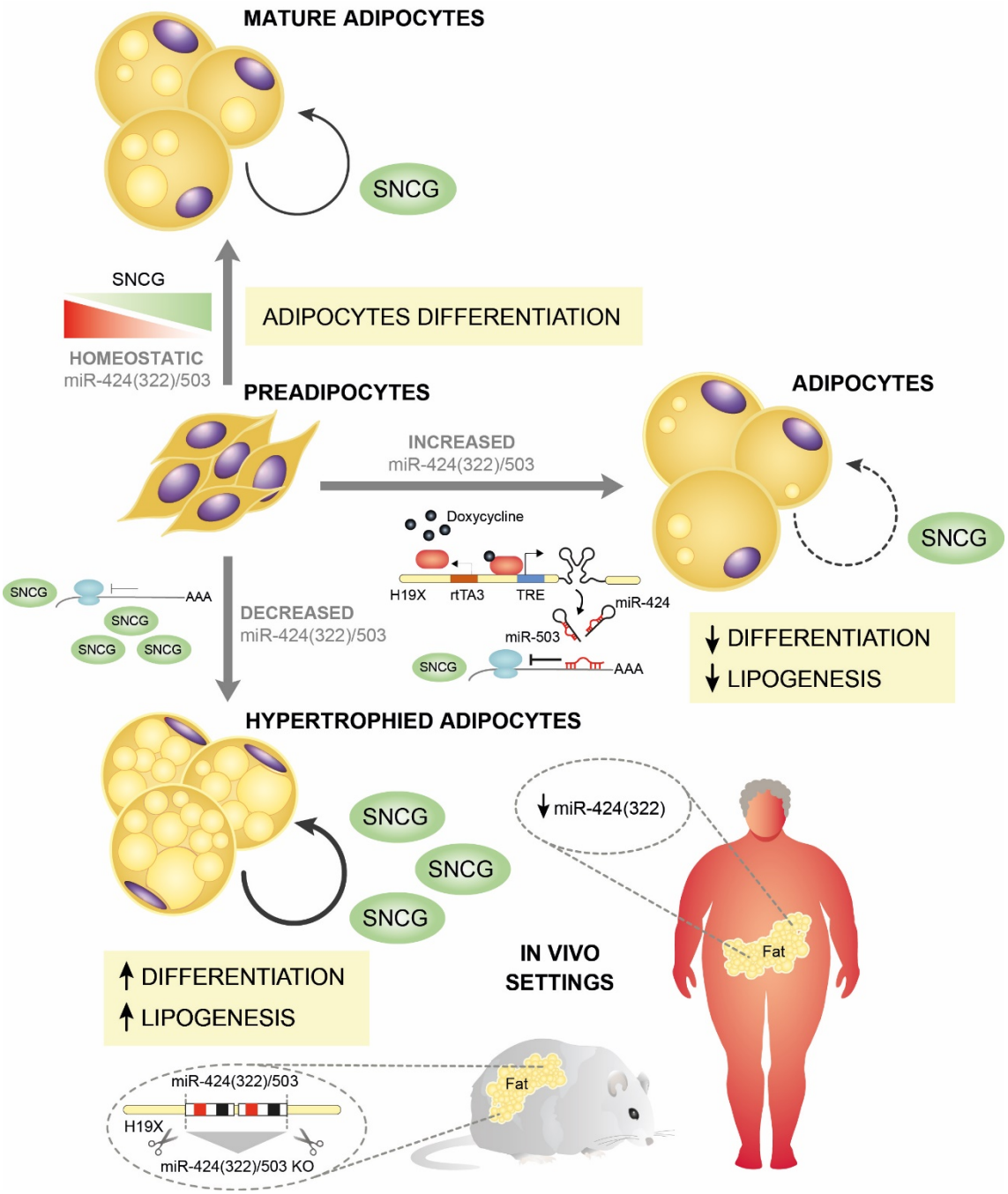
Background and aims. The H19X-encoded miR-424(322)/503 cluster regulates multiple cellular functions. By generating a knockout (KO) mouse model, the role in the mammary gland was first reported. In parallel to its regulatory activity in breast epithelium, intriguing observations made in our laboratory revealed additional alterations, mainly consisting in the significant enlargement of fat depots. Thus, we initiated a research line aimed at defining the role of this set of miRNAs in adipogenesis and its putative implications in obesity.

Materials and methods. We used our KO mouse model exposed to normal chow (NC) and high-fat diet (HFD), engineered cell systems, and human fat samples to investigate the functional roles of adipose miR-424(322)/503 in obesity. Deep-sequencing transcriptomes unveiled mechanistic insights related to its regulatory activity in adipocytes, while additional analyses confirmed the ability of miR-424(322)/503 in orchestrating fat cell commitment by inhibiting γ-Synuclein (SNCG).

Results. Under HFD, increased size and body weight in KO male and female mice was consistent with a significant enlargement of adipose tissue. Together with expanded fat depots, KO females exhibited traits of impaired glucose tolerance under NC (but not upon HFD). We show that this miRNA cluster is negatively regulated during adipogenesis, which in turn allows the expression of genes of relevance in the development of obesity. Ablation of miR-424(322)/503 in mouse embryonic fibroblasts led to enhanced differentiation, while its transient doxycycline-inducible pTRIPz-mediated expression displayed impaired adipogenesis and de-differentiation of human adipocytes. Deep-transcriptomes revealed the impact on many canonical hallmarks, namely adipogenesis and fatty acid metabolism, while 24 common target genes were validated in complementary approaches. Mechanistically, we found that SNCG is a direct target of the miR-424(322)/503 to mediate metabolic functions in adipocytes. Accordingly, diminished adipose miR-424(322)/503 in KO mice and humans co-segregated with increased SNCG in fat and blood as mutually exclusive features of obesity, being normalized upon weight loss.

Conclusion. Our data unveil a previously unknown regulatory mechanism of fat mass expansion tightly controlled by the miR-424(322)/503 through SNCG.

Graphical abstract



2. Main findings

Biological impact and novelty. Since the discovery in 1993 of the first miRNA in the nematode *Caenorhabditis elegans*, the number, function and biological relevance of miRNAs have increased exponentially in almost all science fields; from the control of leaf and flower development to human reproduction, cancer and degenerative diseases, among others. However, often the implications and translational potential of these small non-coding RNAs have been hampered by the inability to **i)** overcome the cross-species conservation gap, **ii)** translate *in vitro* experimental and mechanistic insights into the *in vivo* context by using genetically modified organisms, and **iii)** define the clinical relevance in humans, by accessing and exploring unique patient cohorts. Herein, we combine all three elements in one study where we demonstrate that the new breast tumour-suppressor miR-424(322)/503 displays an unforeseen control over homeostatic adipocyte progenitors, cellular commitment and adipose tissue expansion.

Research in context. Our findings shed light on an intricate molecular, cellular and physiological process. In times of escalating obesity trends, the identification and exploitation of mechanisms allowing adipogenesis and concomitant adipose tissue expansion can encompass unprecedented advances in the field. In line with this, we provide the scientific and biomedical community with a novel molecular regulatory mechanism that brings these objectives one step closer, while broadening our knowledge in this area of research.

Results. We have made seminal contributions that will help to understand the mechanisms that control early progenitor and adipocyte differentiation, obesity, the soluble factors released by these paradigmatic cells, and new auto-paracrine signalling that stems in/from fat depots. Moreover, given the well-known cancer-related functions of the genes disclosed in this work, our study opens new avenues to go more deeply into the obesity-cancer relationship.

Specifically, we have discovered that:
1. The expression of adipose miR-424(322)/503 is related to early progenitor/adipose stem cell contents, and associated with adipocyte hypertrophy and adipose tissue expansion.

2. Adipogenesis requires the transcriptional silencing of the miR-424(322)/503 cluster through DNA methylation.

3. The cluster miR-424(322)/503 inhibits adipogenesis by controlling genetic programs relevant to fatty acid metabolism and differentiation of fat cells by regulating, among others, γ-Synuclein (SNCG), aka Breast Cancer Specific Gene 1 (BCSG1).

4. Opposite expression of adipose miR-424(322) and SNCG/BCSG1 co-segregates with obesity and can be modulated by the weight loss surgically induced by gastric bypass in humans.

5. SNCG is secreted by mature adipocytes, while its paracrine activity also stimulates triglyceride storage and the commitment of these cells.

6. Circulating SNCG levels are increased in peripheral blood from miR-424(322)/503-deficient mice and plasma from obese patients. Noteworthy, SNCG plasma levels are also restored upon weight loss.

3. Relevance and potential future implications

It is well known that adipose tissue expansion in mammals occurs through an increase in the number (hyperplasia) and size (hypertrophy) of existing adipocytes. However, reports have demonstrated differential depot-specific ability to modulate adipocyte number and differentiation. Such differences are linked to the adipose tissue microenvironment and closely related to the hormonal milieu, which also accounts for the sex-specific distribution of adipose tissue and patterns of adipogenesis. For instance, the expansion of subcutaneous adipose tissue in male mice does not exhibit hyperplasia in response to a high-fat fed diet, and studies of human adipose tissue after bariatric surgery indicate that weight loss is primarily mediated by a significant reduction in adipocyte size, which also contributes to the positive effects on energy homeostasis and weight maintenance. Thus, whilst factors determining hyperplasia are not fully understood, increased lipid storage in mature adipocytes is thought to be of the utmost importance for obesity and related diseases. Also, absolute fat cell production and adipocyte turnover show that obese individuals appear to have a

greater number of adipocytes than lean individuals, this difference being set before adulthood. As the turnover of new-born adipocytes added each year is almost the same for both groups, loss of fat cells seems to be largely compensated by the production of new adipocytes, which is twice as high in obese compared with lean individuals. In line with this, lipid removal rate in fat depots decreases with age. Failure to adjust the rate of fatty acid biosynthesis and uptake results in weight gain, whereas substantial surgery-induced weight loss is mainly driven by significant changes in lipid storage. In such scenarios, the pivotal relevance of adipocytes and the number of committed fat precursor cells, as well as the ability to maintain functionality and properly differentiate into adipocytes (adipogenesis), appear to influence physiology to the extent of altering the systemic metabolic state.

A great deal of studies have identified miRNAs expressed in fat depots, being closely related to the adipogenic course, that are aimed to stimulate or inhibit the differentiation of white adipocytes, while orchestrating key metabolic and endocrine functions. However, little is known about the role of miRNAs in the control of fat cell size and function in the burden of obesity. The physiological significance and clinical relevance of miRNAs is often uncertain due to the unavailability of complementary data from engineered mouse models and patient sample analysis. Additionally, while therapeutic modulation of miRNAs provides a promising approach for obesity and impaired metabolism, the promiscuous nature of miRNAs raises many concerns over detrimental off-target effects and the translational utility of experimental results. Systemic dissection of these connections, especially at molecular and organism levels, would likely yield important insights that are currently unavailable.

The H19X-encoded miR-424(322)/503 cluster regulates fundamental cellular processes that include cell proliferation, cellular plasticity, epithelial-to-mesenchymal transition, stress response, metabolic clues, and tissue differentiation and remodelling. In this work, we present a framework endorsing a previously unknown biological function of the cluster miR-424(322)/503. This novel role is supported by an overlap of functional *in vivo* results, as well as an exhaustive assessment of complementary cell systems and clinical data disclosing the translational validity of our experimental observations.

Our study highlights a novel mechanism that regulates the differentiation and commitment of fat cells. These novel findings shed light on the physiological and

molecular mechanisms involved in the occurrence and development of obesity. Our combined results establish that miR-424(322)/503 constitutes an as yet unexplored layer in adipogenesis regulation. The cluster may also promote the "transcriptome shift" from mature adipocyte to partial reversion back to the precursor cell state, as outlined by engineered human adipocytes challenged with increased levels of miR-424(322)/503. Unambiguous target sites of these broadly conserved miRNAs were found throughout major genomic annotations and encompassed adipogenesis-bulged binding sites. Yet, only three 3'UTRs found in canonical miR-424(322)/503 target sites had a significant impact on their respective steady-state mRNA and protein levels, as shown through complementary *in vitro* and *in vivo* observations, and luciferase assays. Our comprehensive studies identified γ-Synuclein (SNCG) as the most robust and consistent target gene of the miR-424(322)/503 cluster in adipose tissue and adipocytes, both in mice and humans. This synuclein family member, also known as breast cancer-specific gene (BCSG1), Synoretin and Persyn, has been predominantly associated with various forms of cancer, as a predictive and diagnostic value, as a marker for assessing tumor grade, and as a potential therapeutic target. High levels of SNCG gene expression have been also reported in adipose tissue, namely in mature adipocytes. This expression is more prominent in obese individuals and situations where adipose plasticity and adipocyte turnover may be engaged. In the current study, we show that adipose tissue and adipocytes from our miR-424(322)/503-null mouse model have steadily increased levels of Sncg, coupled with the higher amounts of this protein in circulation. Lower expression of miR-424(322) in obese subjects correlated with increased levels of SNCG in human fat debris and blood samples, in cross-sectional studies, and in morbidly obese patients following significant weight loss by means of surgical procedures. As results in engineered human adipocytes imply that acute/long-lasting up-regulation of the miR-424(322)/503 cluster specifically and cell-autonomously reduces SNCG, additional experiments performed during this research show the unexpected impact of this synuclein on fat cell differentiation and adipocyte commitment when exogenously administrated. These findings raise the possibility that the cluster miR-424(322)/503 may have a fundamental function in fat depots, by controlling both SNCG-mediated profusion of hypertrophied and inflamed adipocytes and the secretion of this messenger to the media. Of note, overexpression of SNCG has been associated with diseases to which obese individuals are more prone (e.g. esophageal, colorectal, prostate and, in particular, breast cancer, but also some neurodegenerative disorders). Considering this, along with the pioneering impact of

exogenous SNCG on fat cells and circulating measures taken in obese and lean subjects, further investigations and assessment in additional cellular systems and clinical samples are warranted.

4. Generated scientific bibliography

A microRNA cluster controls fat cell differentiation and adipose tissue expansion by regulating SNCG. Ruth RODRÍGUEZ-BARRUECO*[†], Jèssica LATORRE[†], Laura DEVIS-JÁUREGUI[†], Aina LLUCH, Nuria BONIFACI, Francisco J. LLOBET, Mireia OLIVAN, Laura COLL-IGLESIAS, Katja GASSNER, Meredith L. DAVIS, José M. MORENO-NAVARRETE, Anna CASTELLS-NOBAU, Maria ARNORIAGA-RODRÍGUEZ, Wifredo RICART, José M. FERNÁNDEZ-REAL, José M. SILVA*, Francisco J. ORTEGA*[‡], David LLOBET-NAVAS*[‡]. (*Author(s) for correspondence; [†]Same contribution; [‡]Co-leader of the study) – In peer review: **Sci Transl Med** (Impact factor: 16.3 (2019/20)).

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As a result of the research work, the use of accumulated reagents and the application of the knowledge acquired in the course of this line of research, important articles have also been published in indexed scientific journals. All these publications state that the project has been partially funded by the La Marató de TV3 Foundation:

Comparative and functional analysis of plasma membrane-derived extracellular vesicles from obese vs. non-obese women. Fernando SANTAMARÍA-MARTOS, Iván D. BENÍTEZ, Jèssica LATORRE, Aina LLUCH, José M. MORENO-NAVARRETE, Mònica SABATER, Wifredo RICART, Manuel SÁNCHEZ DE LA TORRE, Silvia MORA*, José M. FERNÁNDEZ-REAL*, Francisco J. ORTEGA*. (*****Author(s) for correspondence) – Published: **Clin Nutr**. 2020; 39(4):1067-1076. DOI: 10.1016/j.clnu.2019.04.008. PMID: 31036413 (Impact Factor: 6.4 (2019/20)).

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Decreased TLR3 in Hyperplastic Adipose Tissue, Blood and Inflamed Adipocytes is Related to Metabolic Inflammation. Jèssica LATORRE, José M. MORENO-NAVARRETE, Mònica SABATER, Maria BUXÓ, José I. RODRÍGUEZ-HERMOSA, Jordi GIRONÉS, Josep M. FORT, Ramón VILALLONGA, Wifredo RICART, Rafael SIMÓ, José M. FERNÁNDEZ-REAL, Francisco J. ORTEGA*. (***** Author(s) for correspondence) – Published: **Cell Physiol Biochem**. 2018; 51(3):1051-1068. DOI: 10.1159/000495487. PMID: 30476912 (Impact factor: 5.5 (2017)).

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