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Role of gremlin-1 in the pathophysiology of the adipose tissues

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ABSTRACT

Gremlin-1 is a secreted bone morphogenetic protein (BMP) antagonist playing a pivotal role in the regulation of tissue formation and embryonic development. Since its first identification in 1997, gremlin-1 has been shown to be a multifunctional factor involved in wound healing, inflammation, cancer and tissue fibrosis. Among others, the activity of gremlin-1 is mediated by its interaction with BMPs or with membrane receptors such as the vascular endothelial growth factor receptor 2 (VEGFR2) or heparan sulfate proteoglycans (HSPGs). Growing evidence has highlighted a central role of gremlin-1 in the homeostasis of the adipose tissue (AT). Of note, gremlin-1 is involved in AT dysfunction during type 2 diabetes, obesity and non-alcoholic fatty liver disease (NAFLD) metabolic disorders. In this review we discuss recent findings on gremlin-1 involvement in AT biology, with particular attention to its role in metabolic diseases, to highlight its potential as a prognostic marker and therapeutic target.

1. Gremlin-1

1.1. Gremlin-1 protein

Gremlin-1 gene is localized on chromosome 15q13.3 and it encodes for a highly conserved 184 amino acid-long protein, whose theoretical molecular weight is 20.682 Da [1]. Gremlin-1 is a highly basic protein (pI=9.53). Its sequence contains a N-terminal signal peptide for protein secretion. It is localized in the endoplasmic reticulum and it can be found adsorbed to the cell surface, where it binds to heparan sulfate proteoglycans (HSPGs), once secreted [2]. The gremlin-1 sequence also contains two nuclear localization sequences, even though nuclear localization has not been reported so far. Gremlin-1 undergoes several post translational modifications, including the phosphorylation of serine and tyrosine residues (Ser77, Ser140 and Ser142), N-glycosylation on asparagine 42 [2] and O-glycosylation on threonine 66. While the function of gremlin-1 phosphorylation is not known to date, Chen B and colleagues have shown that gremlin-1 glycosylation at Asn 42 is required for its interaction with Slit1 protein ([3]; see below).

Gremlin-1 structure contains the characteristic cystine-knot domain [4], a structural motif composed of three intertwined intramolecular disulfide bridges that confer high thermal, chemical and proteolytic stability. Like most cystine-knot proteins, gremlin-1 dimerizes. Structural data suggested that gremlin-1 forms head-to-tail non-covalent homodimers stabilized by hydrogen bonds [5]. However, functional studies revealed that gremlin-1 forms covalent homodimers, and that Cys141 residue is essential for this to occur, possibly involved in the formation of a Cys141-Cys141 intermolecular disulfide [6]. Remarkably, gremlin-1 exists in a redox-dependent monomer/covalent-dimer equilibrium *in vitro* and *in vivo*, and the monomeric and dimeric forms have differential biological activities [6] (see below). This is of particular interest as regards the pathological roles of gremlin-1. Local conditions (pH, redox state) can indeed influence the activity of the protein and this may have significant functional implications that will be discussed in the next paragraphs.

1.2. Gremlin-1 interactions

1.2.1. Bone morphogenetic proteins (BMPs)

Gremlin-1 was originally described as a BMP2 and BMP4 antagonist in a Xenopus model [7]. Later, gremlin-1 was shown to also block BMP7 activity [8] (Fig. 1A). Up to date, no other BMPs have been shown to interact with gremlin-1. The oligomeric state of gremlin-1 (monomer *vs* dimer) does not affect BMP-antagonism [6]. Unlike other BMP/BMP antagonist complexes [9], the stoichiometry of gremlin-1/BMP2 interaction is surprisingly not 1:1, and different arrangements of oligomers

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can be found [5]. This peculiar mode of action of gremlin-1 could be exploited to design therapeutics to specifically block gremlin-1 without affecting other BMP antagonists.

1.2.2. Slit proteins

Gremlin-1 is also endowed with BMP-independent activities. Among these, gremlin-1 has been shown to physically and functionally interact with slit1 and slit2 proteins using the yeast two-hybrid screening approach. Slit proteins are a family of secreted extracellular matrix (ECM) proteins involved in the regulation of neural development and inflammation. When interacting with slits, gremlin-1 potentiates slit functions, inhibiting monocyte chemotaxis [3]. A more recent study showed instead that gremlin-1 can block slit2-mediated signaling. Remarkably, gremlin-1/slit2 interaction interferes with BMP antagonism [10] (Fig. 1B). Further research will be necessary to clarify these contradictory findings and to investigate the biological implications of gremlin-1/slit interaction under physiological and pathological conditions.

1.2.3. Vascular endothelial growth factor receptor 2

As early as 2007, a BMP-independent pro-angiogenic activity of gremlin-1 has been found and characterized *in vitro* and *in vivo* [11]. This activity was subsequently linked to gremlin-1 ability to bind the vascular endothelial growth factor receptor 2 (VEGFR2) [12] and the HSPGs on the endothelial cell (EC) surface [13]. Of note, gremlin-1/VEGFR2 interaction is not affected by the presence of BMPs [12]. Since then, several groups have reported the capacity of gremlin-1 to bind and activate VEGFR2 [6,14–17] (Fig. 1C).

Structurally, gremlin-1 resembles the vascular endothelial growth factor A (VEGF-A), a well-known cystine-knot homodimeric secreted ligand of VEGFR2. Consistent with this, gremlin-1 and VEGF-A bind VEGFR2 with a similar high affinity (respectively 47 nM and 3 nM) [17]. Like VEGF-A, gremlin-1 induces the formation of VEGFR2/αvβ3 integrin supramolecular receptor complexes. The engagement of αvβ3 sustains the prolonged activation of VEGFR2 induced by gremlin-1. Consistently, the inhibition of αvβ3 prevents VEGFR2 activation and reduces gremlin-1 pro-angiogenic activity *in vitro* and *in vivo* [18,19]. However, gremlin-1 does not directly interact with αvβ3 integrin [18]. Unlike VEGF-A, gremlin-1 does not bind other VEGFRs (VEGFR1 and VEGFR3) [12] nor neuropilin 1 receptor [13].

Remarkably, gremlin-1 monomer and dimer elicit opposite effects on VEGFR2. Although both forms bind VEGFR2, dimeric gremlin-1 fully activates it, while the monomers bind to receptor, impede receptor dimerization, thus acting as receptor antagonists [6,20,21]. Considering that gremlin-1 dimerization occurs *via* the formation of redox/pH-sensitive disulfide bridges, the microenvironment could tune gremlin-1 action as a VEGFR2 ligand. Thus, therapeutic strategies could be envisioned to switch gremlin-1 from being an agonist to being an antagonist of VEGFR2.

The VEGFR2 activation capacity of gremlin-1 has been debated [22]. A high monomer/dimer ratio of recombinant gremlin-1 preparations may result in low-absent VEGFR2 activation. Thus, caution should be used to interpret results when using recombinant sources of gremlin-1 with unknown monomer/dimer ratios.

1.2.4. Heparan sulfate proteoglycans

The high content of basic residues determines gremlin-1 ability to interact with heparin and HSPGs on cell surface and into the ECM [11] with high affinity (Kd = 20 nM) [13,19,23]. This ability has been exploited for the purification of the recombinant protein by heparin affinity chromatography [6,21]. The heparin binding site of gremlin-1 consists of a non-linear 11-amino acid arrangement of lysine and arginine residues [24] and the interaction with heparin involves sulfated groups of the polysaccharide[13]. The gremlin-1/heparin binding can be competed with heparan sulfate (HS), glycosaminoglycan (GAG), but not with dermatan sulfate, chondroitin-4-sulfate, chondroitin-6-sulfate, or hyaluronic acid GAGs. These results show that gremlin-1/heparin interaction depends, at least in part, on differences in GAG structure and degree of sulfation.

ECM acts as a localized reservoir of gremlin-1 that can regulate protein activity in a spatially and temporally restricted manner. Also, gremlin-1 accumulation into ECM can change under pathological

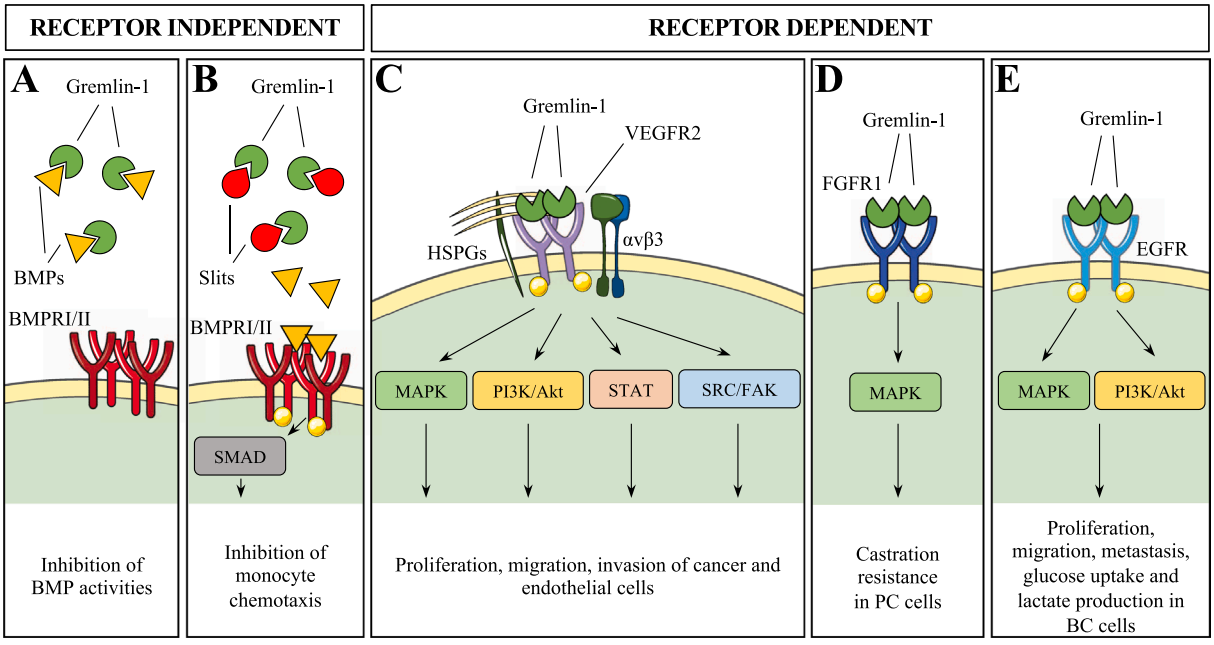

Fig. 1. Receptor-independent and receptor-dependent molecular pathways affected by gremlin-1. A, Gremlin-1 sequesters BMP2/4/7 from binding their cognate cell surface receptors. This mechanism blocks BMP function in embryogenesis, cell differentiation (including adipogenesis, see below) and cancer. **B**, Gremlin-1 also sequesters Slit proteins blocking monocyte chemotaxis. Slit inhibition may interfere with BMP-antagonism. **C,** gremlin-1 engages a multi-molecular receptor complex comprising VEGFR2, HSPGs and the integrin receptor αvβ3 in ECs and cancer cells. Thus, gremlin-1 activates cell proliferation, migration and invasion and promotes tissue fibrosis. **D-E,** Gremlin-1 has been shown to bind to and activate FGFR1 and EGFR in cancer cells, controlling tumor cell behavior.

conditions due to changes in the availability of gremlin-1 binding sites, as previously shown in fibrotic kidney disease [23].

While gremlin-1 interaction with HS seems dispensable for BMP antagonism [23], it is necessary for the interaction with VEGFR2 and for its pro-angiogenic activity [13] (Fig. 1C). The inhibition of gremlin-1/HS interaction using heparin-like *E. coli* K5 derivatives prevents gremlin-1 from binding and activating VEGFR2 and restricts angiogenesis *in vitro* and *in vivo* [13]. We speculate that this interaction could become a therapeutic target to block HSPGs-dependent functions of gremlin-1.

Scattered studies have suggested that gremlin-1 also binds tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein (YWHAH) [25], macrophage migration inhibitory factor (MIF) [26], epidermal growth factor receptor (EGFR) [27], transforming growth factor β [28], it activates the notch pathway [29] and recently it was shown to bind fibroblast growth factor receptor 1 (FGFR1) [30] (Fig. 1).

2. Gremlin-1 function

Gremlin-1 has pleiotropic functions. Through the interaction with all different molecular partners described above, gremlin-1 acts in different physiological and pathological settings regulating fundamental processes. The main functions of gremlin-1 are discussed in the next paragraphs.

2.1. Embryonic development

As a BMP antagonist, gremlin-1 is a central player in tissue formation during embryonic development. It controls *Xenopus* dorsalization [7], the formation of the proximal-distal axis during lung development [31], kidney development [32] and it drives limb outgrowth *via* the modulation of sonic hedgehog (shh)/FGF axis [33,34]. During embryonic brain development, gremlin-1 is co-expressed with BMPs [1], controlling the maturation of the developing brain cortex [35]. In keeping with its role during embryonic development, gremlin-1 knockout mice die within 48 h after birth [36] due to early development abnormalities, such as bilateral agenesis of kidneys and lung and limb defects.

2.2. Inflammation

Gremlin-1 regulates inflammation. Its expression is modulated during inflammatory events and correlates with tissue damage in different diseases. Gremlin-1 is significantly upregulated in osteoarthritis both in humans and animal models [37,38]. Also, gremlin-1 promotes renal inflammation and damage in mice, through the activation of NOTCH pathway [29].

In ECs, gremlin-1 induces the expression of pro-inflammatory chemokines (i.e., CCL2, CCL7, CXCL1, and CXCL2) and adhesion molecules (i.e., ICAM1 and VCAM1) *via* the activation of CREB and NF-κB pathways [39]. Consistent with its pro-inflammatory role, gremlin-1 is upregulated in ECs in response to hypoxia [20], a process interdependent with inflammation. At the same time, gremlin-1 seems to have a protective role in vascular inflammation and atherosclerotic plaque progression. Different studies have shown that gremlin-1 inhibits MIF (macrophage migration inhibitory factor)-dependent monocyte activation and chemotaxis [3,26] and it reduces leukocyte recruitment, attenuating the growth of atherosclerotic plaques [40]. Beside this, gremlin-1 has been suggested to restrain tumor inflammation, for example through the inhibition of the proinflammatory/pro-tumoral role of MIF [41].

Overall, gremlin-1 regulates inflammation in a tissue-dependent manner. Further studies are warranted to better understand the molecular/cellular bases of gremlin-1 role in inflammation.

2.3. Angiogenesis

After the first evidence in 2007 [11], several studies demonstrated that gremlin-1 is endowed with a pro-angiogenic activity. As such, gremlin-1 is involved in various angiogenesis-dependent human diseases, including osteoarthritis [37,42], endometriosis [43], diabetic retinopathy [44], and cancer [45,46].

By binding to, recruiting and activating the VEGFR2/HSPGs/αvβ3 supramolecular receptor complex on the cell surface [12,13,18,19], gremlin-1 induces the activation of EC migration and proliferation, formation of tube-like structures, and EC sprouting in 3D gels *in vitro* [11,47,48]. Also, gremlin-1 induces angiogenesis *in vivo* in the chorioallantoic membrane (CAM) of the chick embryo and in the mouse matrigel plug assay [11]. Similarly to other proangiogenic stimuli, gremlin-1 activates a Ang-1/Tie-2 autocrine loop, which modulates EC behavior *via* NF-κB pathway [49]. Also, gremlin-1 transactivation by the Nox1-PKA-CREB/Ref-1 signaling pathway promotes angiogenesis in pulmonary ECs [48].

Although the majority of the literature agrees to consider gremlin-1 a pro-angiogenic factor, scattered papers have suggested that gremlin-1 can instead inhibit angiogenesis [6,20]. In this regard, we have demonstrated that the activity of gremlin-1 on ECs depends on its oligomeric state, as for its other VEGFR2-dependent functions. The dimeric form is responsible for the pro-angiogenic activity of gremlin-1. Instead, monomeric gremlin-1 is anti-angiogenic, hampering angiogenesis driven either by dimeric gremlin-1 or VEGF-A [6,20]. This could explain the controversial data about gremlin-1 not being a pro-angiogenic stimulus. Considering that gremlin-1 dimerization is redox-dependent [6], there is the possibility that gremlin-1 function on blood vessels could be differentially modulated *in vivo* by extracellular cues (e.g., hypoxia/redox state). Also, there is space for therapeutic interventions to modulate gremlin-1 oligomeric state and function.

In addition to the VEGFR2/HSPGs/αvβ3 receptor complex, gremlin-1 may regulate angiogenesis through the inhibition of BMPs. Accordingly, BMP2 [50] and BMP4 [51] regulate angiogenesis through the interaction with BMP receptors. It is reasonable to think that gremlin-1 may also regulate angiogenesis through alternative molecular mechanisms (e.g., NOTCH pathway, binding to FGFR1) that remain to be better elucidated.

2.4. Cancer

Gremlin-1 was initially identified as an onco-suppressor [52–54]. However, after that, a significant body of evidence has clearly demonstrated a pro-oncogenic role of gremlin-1 [55,56]. Gremlin-1 is found overexpressed in cancer. It is expressed both in stromal and parenchymal cells [25,57]. Gremlin-1 promotes tumor growth, metastasization, stemness, angiogenesis and rewires cell metabolism in different types of cancer including breast, cervical, lung and pancreatic cancer [6, 55,58–62] (reviewed in [41]). Also, the expression of gremlin-1 in cancer correlates with tumor angiogenesis [45,46]. Finally, gremlin-1 is a predictive prognostic biomarker in breast cancer patients [63] and its detection in blood samples (e.g., in circulating extracellular vesicles) has suggested the possibility to exploit circulating gremlin-1 as a diagnostic tool [64].

2.5. Tissue fibrosis and cell transdifferentiation

Fibrosis, that is the replacement of healthy tissue with a fibrotic and rigid tissue, is typically characterized by aberrant inflammation, cell differentiation processes, excessive deposition of ECM and chronic tissue injury. This leads to the loss of the original tissue architecture and function. Fibrotic diseases include a wide spectrum of pathologies that can be multisystemic like systemic sclerosis, multifocal fibrosclerosis and nephrogenic systemic fibrosis, or can be organ-specific such as pulmonary, liver, and kidney fibrosis.

Gremlin-1 is an important mediator of fibrosis in numerous diseases including chronic pancreatitis [65], peritoneal fibrosis [66], proliferative vitreoretinopathy [67] and heart failure-associated endomyocardial fibrosis [68] (reviewed in [69]). In the kidney, gremlin-1 drives renal inflammation, fibrosis [16,32] and diabetic nephropathy [70]. Here, its expression is augmented by fibrosis-inducing high glucose conditions [69,71] and its role is mediated by the activation of VEGFR2/NOTCH signaling pathways [29,72,73].

Gremlin-1 is transcriptionally activated by TGF-β, a major pro-fibrotic factor [74,75]. Also, gremlin-1 induces the expression of ECM proteins such as fibronectin [76] and it associates with collagen ECM deposition [65,71,77,78,75]. Besides ECM production, gremlin-1 plays additional roles in the fibrotic process, including the activation of myofibroblasts through a TGF-β positive loop and inhibition of BMPs [74], the induction of a pro-inflammatory response (see above) and the modulation of cell transdifferentiation processes towards the mesenchymal phenotype, such as epithelial-to-mesenchymal (EMT) and endothelial-to-mesenchymal (EndMT) transitions [79]. Gremlin-1 expression is linked to EMT/EndMT in diabetic nephropathy [80–82], and pulmonary [79,83,84], hepatic [85] and cutaneous [69] fibrotic disorders. Gremlin-1 induces EMT acting as a downstream mediator of TGF-β/Smad pathways in renal fibrosis [74,75]. Also, it promotes epithelial cell proliferation and EMT (characterized by gain of α-SMA, fibronectin and type I collagen, loss of ZO-1, and up-regulation of MMP activity) *via* TGF-β/Smad, ERK/AKT and BMPs/Smad1/5 pathways in the posterior capsular opacification and proliferative vitreoretinopathy [86,87]. Finally, gremlin-1 induces EndMT in a phospho-Smad2/3-dependent manner in pulmonary ECs. However, one study has shown that gremlin-1 can counteract the pro-fibrotic effects of TGF-β in myocardium [28].

Together all these data indicate that gremlin-1 is a promising therapeutic target for tissue fibrosis. Consistent with its pro-fibrotic role, gremlin-1 is found in the serum of patients with fibrotic diseases as idiopathic pulmonary fibrosis (IPF) [88,89] and systemic sclerosis [78,89,90] and it has been proposed as a prognostic/diagnostic biomarker.

3. Gremlin-1 in the adipose tissue

3.1. The adipose tissue

Adipose tissues (ATs) are organized to form a highly dynamic endocrine organ which plays a pivotal role in the maintenance of the whole-body homeostasis. The adipose organ is composed by white and brown ATs that closely collaborate to regulate energy homeostasis, thermogenesis, lactation, and immune response. Due to their ability to respond to environmental stimuli, the ATs are highly plastic and are necessary to adapt to different external triggers [91].

In humans, AT develops during the early stage of gestation (week 14) and comprises multiple depots distributed at specific anatomical sites [92,93]. Although all AT depots are involved in the regulation of the energy homeostasis, they differ in the size of adipocytes, lipid composition, secretome (i.e., adipokines and cytokines), ECM composition, mechanical properties, and gene expression pattern [94,95].

The white AT (WAT) is localized in either subcutaneous or visceral compartments and is recognized as the most abundant type of AT. Within WAT, white adipocytes represent more than 30% of all cells. ECs, fibroblasts, macrophages, immune cells, and pre-adipocytes (generally referred to as the stromal vascular fraction, SVF) are also present. White adipocytes derive from myogenic factor 5 $(Myf5)^-$ cells upon the increase in the expression of BMP2/4, peroxisome proliferator-activated receptor-ɣ (PPARɣ), and CEBPα/β/δ and in part from the $Myf5^+$ mesenchymal stem cells that lose the expression of PTEN and activate the expression of PPARɣ and CEBPα/β/δ [96–98]. However, Myf5 lineage distribution in adipose tissues changes in response to both non-modifiable (e.g., age) and modifiable (e.g., diet) factors, suggesting that adipocyte lineages could have context-dependent plasticity [99]. In white adipocytes the energy is stored in the form of triglycerides, which are in turn organized to form a single unilocular lipid droplet (LD). LDs occupy most of the cell volume and push the nucleus and other organelles (including the few mitochondria, the Golgi complex, endoplasmic reticulum, and vesicles) to the closed proximity of the plasmatic membrane [100]. WAT responds to nutritional and metabolic stimuli by accumulating/releasing lipids from/into the bloodstream, ability that makes this tissue a critical regulator of the whole-body energy homeostasis. Importantly, the subcutaneous WAT (sWAT) depot is the primary storage of the excess of lipid and plays a critical function in the control of glucose homeostasis [100]. When the lipid storage capacity of sWAT is exceeded (in case of chronic positive energy balance), lipids are stored into visceral adipose depots, such as epicardial, omental, perivascular, and perirenal compartments, and ectopically (e.g., liver and skeletal muscle). Here, the fat excess strongly contributes to the pathogenesis of obesity comorbidities such as type 2 diabetes, NAFLD and cardiovascular diseases [100].

Brown adipose tissue (BAT) is abundant during the early post-natal life in humans, where it helps maintaining the body temperature. In adults, small and metabolically active depots of BAT have been documented in the cervical, supraclavicular, axillary, periaortic, paravertebral, and suprarenal regions [101,102]. Brown adipocytes derive from $Myf5^+$ mesenchymal precursor cells which start expressing BMP7, PMDR16 followed by PPARɣ, CEBPα/β/δ, and PGC-1α [96,97,99,103]. Brown adipocytes contain multiple, small LDs and a high number of mitochondria expressing the uncoupling protein 1 (UCP1), also known as thermogenin. UCP1 is a fatty acid anion/H^+ symporter located in the inner membrane of mitochondria whose main role is to dissipate the proton gradient generated by the electron transport chain. UCP1 hence uncouples the cellular respiration from the mitochondrial ATP synthesis, generating heat (thermogenesis). In brown adipocytes the lipids contained in the small LDs are hence used to produce heat in a process known as non-shivering thermogenesis [104,105]. The plasticity of the adipose organ is well documented by the ability of adipocytes to undergo specific morphofunctional modifications to respond to diverse stimuli. The phenomenon of browning, occurring in specific white adipose depots, consists of the differentiation of adipocytes progenitors in brown adipocytes [97] and of the transdifferentiation of white adipocytes into brown-like cells (beige) upon cold-exposure [106]. Such ability holds critical implication in the formulation of therapies to counteract obesity. Beige adipocytes display an unilocular lipid droplet and a gene expression pattern like white adipocytes. However, they are able to switch to brown-like cells for thermogenetic purposes [107]. Importantly, brown adipocytes whitening has also been documented in obesity and consists in their conversion into white-like unilocular cells, a phenomenon that ultimately results in cellular stress and death [108]. It is worth noting that additional, distinctive adipocyte subpopulations displaying peculiar functions have been recently documented thanks to the advent of the single-nucleus RNA-seq approaches, findings that open critical, new research directions in the study of the AT function [109,110].

Adipocytes are secretory cells. They produce a plethora of cytokines and hormones, known as adipokines, which regulate inflammation, angiogenesis, and the local and systemic glucose-metabolic homeostasis [111,112]. In addition, secreted metabolites (e.g., acetate) can act as paracrine factors regulating AT functions [113]. One big challenge for future research is to reach a comprehensive understanding of the integrated role of all AT-secreted adipokines/factors in different AT depots, and their mutual regulation. Single-cell analyses have the potential to provide pivotal insights into this and will help designing effective pharmacological treatments to tackle obesity and its comorbidities.

During the continuous AT remodeling in response to environmental/metabolic cues, adipocytes can enlarge, proliferate, or transdifferentiate into other types of adipocytes or into myofibroblasts/mesenchymal cells [114]. Changes in ECM accompany AT remodeling. The ECM provides adipocytes with mechanical cues that promote survival. These processes are strictly dependent on the remodeling of the vascular network *via*

finely regulated angiogenesis [115,116]. ECs can also transdifferentiate into adipocytes through the endothelial-pericyte-preadipocyte differentiation, participating in AT turnover [92]. All these processes (i.e., cell transdifferentiation, angiogenesis, ECM remodeling) are likely to be affected by gremlin-1. In addition, most of the molecular partners of gremlin-1 are involved in AT remodeling and homeostasis. BMPs (mainly BMP4 and BMP7) are master regulators of both white and brown adipogenesis and AT maintenance. Also, slit2 is expressed in beige adipose cells and its C-terminal fragment promotes thermogenesis and energy expenditure [117]. Finally, the VEGF/VEGFR2 system is an important regulator of AT angiogenesis and function [115,118]. Considering that gremlin-1 is expressed in AT both in humans and in mice and that gremlin-1 levels increase in patients with obesity, all these bodies of evidence point to a pivotal, complex role of gremlin-1 in AT function and dysfunction that is addressed in the next paragraphs.

3.2. Gremlin-1 in adipose tissue development and function

Gremlin-1 is expressed in human AT, at higher levels in omental and visceral fat than in subcutaneous AT (SAT) [119–121]. The expression of gremlin-1 is modulated during human preadipocyte adipogenic differentiation *in vitro*. Gremlin-1 is expressed in human preadipocytes *in vitro*, it decreases in the early phases of adipogenesis and it is restored at later stages [119]. Similarly, the expression of gremlin-1 mRNA in murine 3T3-L1 preadipocytes decreases in the early phases (d3-d10) of adipogenesis [122], and the secreted protein is found in the culture medium of terminally differentiated adipocytes (personal observation, July 2022). Altogether, *in vitro* and *in vivo* data suggests a central role of gremlin-1 during adipogenesis.

As expected, gremlin-1 expression in AT biopsies and preadipocytes shows a positive correlation with the expression of BMP2, 4 and 7 [123]. BMP4 stimulates precursor cells to undergo adipogenic commitment, promoting the formation of white and brown preadipocytes, and it drives terminal differentiation into mature white adipocytes [119,124]. Accordingly, constant BMP stimulation, either by BMP4 addition or by silencing BMP4 antagonists promotes the adipogenic differentiation while the overexpression of BMP antagonists leads to resistance to BMP administration and impaired white and brown adipogenesis. Consistent with its BMP antagonist activity, gremlin-1 knock-down in preadipocytes potentiates the adipogenic response (i.e., PPARγ; ZNF423 expression) to BMP4 stimulation [119]. This data confirms that gremlin-1 regulates adipogenesis, at least in part, through its BMP antagonist activity (Fig. 2). However, further studies are needed to better elucidate how gremlin-1 role in adipogenesis relates to other mechanisms, including the activation of VEGFR2.

Gremlin-1 also plays a role in adipose cell browning by inhibiting BMPs. Gremlin-1 silencing in preadipocytes upregulates several BAT markers such as ZIC1 and UCP1 and increases the number of mitochondria [119]. Gremlin-1 has been suggested to prevent AT browning by inhibiting BMP4. However, the browning effect of BMP4 remains debated and various studies have shown that this factor inhibits rather than induce thermogenesis and AT browning [124]. Despite this, gremlin-1 may also impair brown fat cell development or browning processes by blunting the capacity of BMP7 to guide cell fate towards brown adipocytes [123,125,126] (Fig. 2). Thus gremlin-1 represents an attractive target that could be exploited to promote AT browning or to overcome BMP resistance of AT occurring in metabolic disorders.

The role of gremlin-1 in AT homeostasis is far from being elucidated. However, one can speculate that gremlin-1 secreted by adipose cells and by the SVF accumulates in the ECM through the binding of HSPGs. Here it can act as an autocrine/paracrine stimuli for the different cell types resident in the AT. It is reasonable to hypothesize that gremlin-1 may contribute to various processes during the homeostatic remodeling of AT. Gremlin-1 is a pivotal regulator of angiogenesis, through both inhibition of BMPs and activation of VEGFR2. Thus, once secreted, it could regulate the angiogenic processes occurring during AT remodeling. In this respect, the extracellular redox state of AT could tune gremlin-1 function as a VEGFR2 agonist. Also, gremlin-1 may influence the activity of resident immune cells [26,127]. Furthermore, gremlin-1 might intervene in the maintenance of the adipose phenotype of adipocytes and in the reversible endothelial-pericyte-preadipocyte conversion by antagonizing BMP action. Overall, all available data suggest that gremlin-1 could orchestrate AT development and homeostasis. However, further efforts will be necessary to clearly confirm the role of gremlin-1 in these processes. Such studies will need to focus on the diverse cell types, molecular and biological processes that can be affected by gremlin-1.

3.3. Gremlin-1 in AT dysfunction

AT dysfunction is a pathogenic event in the development of metabolic syndrome, obesity and lipodystrophies, all conditions associated with perturbation of lipid storage and of metabolic homeostasis [100,128,129]. Gremlin-1 secreted by the AT has been shown to control adipocyte function and metabolism under different pathological conditions, suggesting that this protein could participate in the pathogenesis

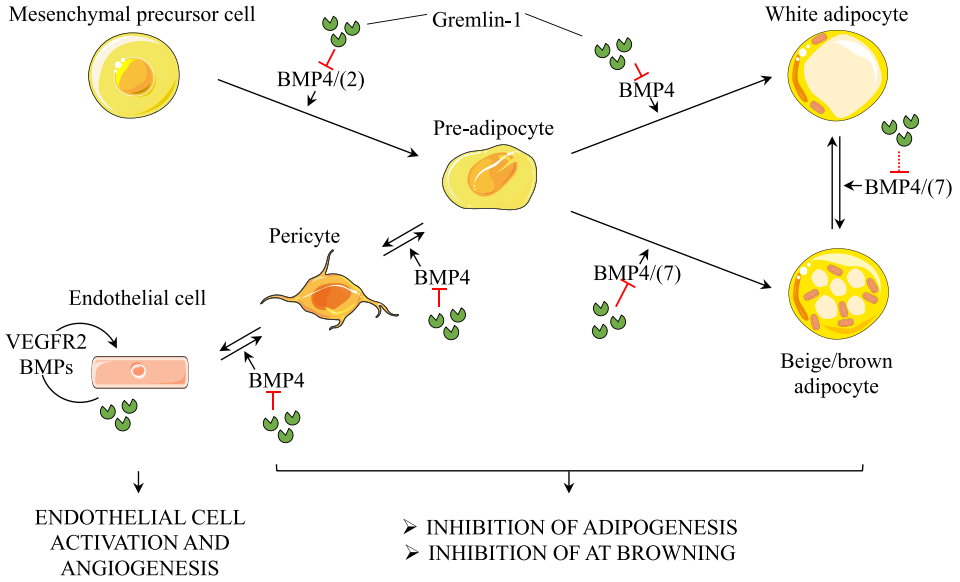

Fig. 2. Role of gremlin-1 in adipogenesis, endothelial-pericyte-preadipocyte conversion and AT homeostatic remodeling. Gremlin-1 produced by ECs autocrinally activates angiogenesis. Gremlin-1 produced in AT also counteracts the pro-adipogenic role of BMP4. Dashed lines and brackets indicate possible alternative mechanisms of action of gremlin-1 during adipogenesis. Other mechanisms (e.g., VEGFR2) could intervene in these processes, but remain to be elucidated. Together, these mechanisms are necessary for balanced adipogenesis and maintenance of AT homeostasis.

of AT dysfunction. In the next paragraphs we address the involvement of gremlin-1 in AT-related disorders.

3.3.1. Gremlin-1 in obesity

In obesity, the chronic positive energy balance results in rapid WAT expansion. Adipocyte hypertrophy increases cellular stress, deregulates fatty acid flux, it alters the expression of adipokine/chemokines and attracts immune cells. Dysfunctional AT is characterized by the formation of crown-like structures, i.e., macrophages surrounding dead/dying adipocytes and cleaning their remnants/debris [130]. Moreover, macrophages secrete TNFα, iNOS, and IL-6 cytokines inducing adipose stem cells to differentiate into proinflammatory cells [131]. During the rapid expansion of AT, hypoxia can occur. In this condition, the hypoxia inducible factor-1α (HIF-1α) induces a transcriptional program that upregulates pro-fibrotic genes such as ECM components (including collagens) and ECM modifying enzymes [132,133]. Also, adipocytes and resident ECs can undergo adipocyte-to-myofibroblast transition (AMT) [134] and EndMT [135], respectively. All these events ultimately lead to AT fibrosis [136]. Fibrosis reduces the plasticity of AT, leading to tissue stiffening and mechanical stress of adipocytes which, in turn, drive profound metabolic changes [136,137]. The inflamed/fibrotic state of AT decreases insulin sensitivity and is associated with poor bariatric surgery outcomes and difficulty in weight loss [138]. Consistent with the pathogenesis of AT dysfunction, adipose cells from patients with obesity have a gene expression pattern correlated with inflammation, senescence, impaired angiogenesis and fibrosis [139].

Various classes of molecules contribute to the pathogenesis of AT dysfunction and control differentiation and transdifferentiation processes. Among these, the available literature has highlighted the central role of gremlin-1. Gremlin-1 expression in AT is markedly increased in patients with hypertrophic obesity [119]. Accordingly, its expression positively correlates with adipocyte size both *in vitro* and *in vivo* as well as with body mass index (BMI) [119] and percentage of body fat [121].

In patients with obesity, circulating gremlin-1 increases [119] and its level is a good indicator of the metabolic health. Indeed, gremlin-1 levels are normalized by physical exercise in these patients and this correlates with a reduced risk of cardiovascular/metabolic complications [140]. Thus, serum gremlin-1 may be exploited to monitor patient's metabolic function in response to therapeutic interventions.

In AT, gremlin-1 counteracts the activity of BMPs, which have been repeatedly shown to promote physiological adipogenesis, induce AT browning eventually preventing AT dysfunction. For example, BMP7 gene therapy reduces body weight, and insulin resistance [141]. Also, reduction of BMP4 signaling can contribute to the development of obesity and associated metabolic disorders. Accordingly, BMP4 gene therapy improves insulin sensitivity and increases energy expenditure by inducing the browning of sWAT [142]. Thus, increased levels of gremlin-1 found in the ATs of obese individuals may hamper beneficial BMP functions.

During AT dysfunction, VEGFR2 activation/inhibition by gremlin-1 could also play substantial roles. The VEGF/VEGFR2 system plays a crucial role regulating angiogenesis, inflammation and browning in AT [118]. Perturbation of this system results in significant changes in AT pathophysiology [143,144]. Thus, gremlin-1 overexpression in the AT of patients with obesity may lead to alterations of VEGFR2 signaling with significant consequences on AT function. Also, one could speculate that the altered redox state (i.e., low oxygen levels) found in dysfunctional/hypoxic AT may favor gremlin-1 to be monomeric thus acting as an anti-angiogenic stimulus and worsening AT dysfunction. Also, the capacity of gremlin-1 to regulate leukocyte recruitment through VEGFR2 activation could also play a role in AT dysfunction.

ECM components and modulators of ECM remodeling drive AT fibrosis. For instance, type VI collagen and MMP14 are upregulated in obese individuals and control AT expansion and metabolic alterations in mice fed with a high fat diet. Also, collagen VI knockout or targeting of endotrophin, a cleavage product of type VI collagen, reduces AT fibrosis and ameliorates the metabolic function of mouse models of obesity. The potent pro-fibrotic role of gremlin-1 points to the possibility that this factor could modulate the fibrotic processes during AT dysfunction of patients with obesity. A hypothesis of gremlin-1 role in AT dysfunction is illustrated in Fig. 3. Future studies will be necessary to confirm this hypothesis and must address possible differences in the role of gremlin-1 between humans and mice, as previously found [119].

AT dysfunction/fibrosis is an interesting therapeutic target for the treatment of obesity and metabolic disorders. Various studies have highlighted that targeting AT fibrosis may not reduce obesity *per se* (excess of body weight). However, fibrosis reduction has the critical potential to revert the AT dysfunction and improve the overall metabolic health in obesity, hence reducing the risk of complications, including type 2 diabetes, cardiovascular events and different types of cancer. To reach this goal, further knowledge and novel models of AT dysfunction and fibrosis are needed to identify and characterize the driving factors. Given its central role, gremlin-1 could become a biomarker of obesity-associated AT dysfunction. Also, it is an attractive therapeutic target that could be exploited to improve the cardio-metabolic risk of obese patients. However, gremlin-1 potentially triggers a multifaceted, complex response during AT dysfunction acting on different cell types with different mechanisms. Thus, only a clear elucidation of all gremlin-1 roles in AT will help identifying potential windows of intervention to treat obesity and other AT-related disorders.

3.3.2. Gremlin-1 in AT-related metabolic disorders

AT dysfunction is a key factor in the pathogenesis of many metabolic

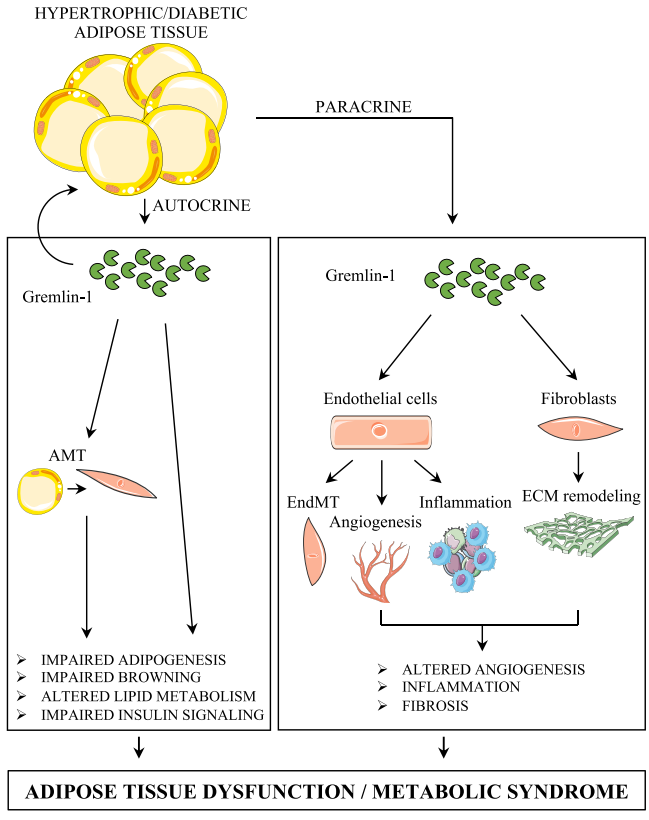

Fig. 3. Hypothesis of the pathogenetic role of gremlin-1 in AT dysfunction. Gremlin-1 is upregulated in the AT during obesity and metabolic disorders. Through autocrine mechanisms, gremlin-1 may regulate adipocyte size, impair white/brown adipogenesis and alter lipid and glucose metabolism. Simultaneously, gremlin-1 may act paracrinally on AT resident ECs and fibroblasts, triggering angiogenesis, inflammation and ECM remodeling. In turn, these alterations could contribute to AT fibrosis, eventually leading to AT dysfunction and metabolic syndrome.

disorders other than obesity, such as type 2 diabetes, fatty liver disease (e.g., NAFLD), polycystic ovary syndrome (PCOS) and cardiovascular diseases. Scattered studies have reported the involvement of gremlin-1 in multiple hormonal/metabolic disorders. Therefore, one can speculate that gremlin-1 may be a central driver of metabolic alterations and in turn of AT dysfunction under various pathological conditions. For example, gremlin-1 serum levels are significantly increased in patients with PCOS [145], a disorder characterized by multiple metabolic and hormonal alterations, including insulin resistance and type 2 diabetes. Several groups have demonstrated that gremlin-1 expression is strongly induced by high-glucose exposure in different cell types, including retinal pigment epithelial cells [44], retinal pericytes [146] and kidney mesangial cells [70] where it triggers pathogenetic events leading to diabetic retinopathy and nephropathy. Accordingly, the expression of gremlin-1 in the AT (both visceral and subcutaneous), in the liver and its plasmatic levels increase in insulin resistance and patients with type 2 diabetes [121,147]. Moreover, increased AT, liver and serum gremlin-1 is associated with markers of NAFLD/NASH, such as degree of steatosis, fibrosis, inflammation, liver fat content, circulating free fatty acids, serum PCR, ALAT and ASAT [121]. Thus, gremlin-1 is an attractive target for obesity-associated complications, such as type 2 diabetes and NAFLD/NASH.

3.3.3. *Gremlin-1 as an endocrine adipokine*

Multiple studies have reported the association of circulating gremlin-1 levels with a variety of pathological conditions. In most cases, gremlin-1 has been seen as a biomarker that, when detected in the bloodstream, indicates its massive production in peripheral tissues (e.g., kidney, AT). However, the role of circulating gremlin-1 and its possible endocrine function have remained unclear until recent studies [121]. In this work, Smith and colleagues showed that gremlin-1 is an endocrine adipokine involved in the regulation of glucose metabolism and insulin sensitivity.

Circulating gremlin-1 and its expression in AT correlate with insulin resistance assessed by euglycemic clamps and HOMA-IR index [121]. Consistent with this, circulating gremlin-1 was found to be associated with HOMA-IR index in women with PCOS [145]. In addition, secreted extracellular gremlin-1 impairs insulin signaling in adipose cells, hepatocytes and muscle cells and strongly reduces glucose uptake upon insulin stimulation in all three cell types [121]. This suggests that circulating gremlin-1 is involved in the systemic control of glucose metabolism, targeting the major insulin-sensitive tissues.

However, whether AT is a major source of circulating gremlin-1 and the exact molecular mechanism of gremlin-1-driven control of insulin sensitivity remains to be better investigated. Its definition would reveal further insights on the endocrine roles of the adipokine gremlin-1.

4. Concluding remarks

As obesity and its associated complications acquire pandemic proportions, defining the molecular mechanisms underlying the onset and progression of AT dysfunction is of growing interest. In this context, gremlin-1 is emerging as a central regulator of AT pathophysiology, gaining increasing attention in the adipose tissue research field.

As reviewed in the present work, gremlin-1 regulates, *via* different molecular pathways, inflammation, angiogenesis, fibrosis and cell differentiation, all processes that contribute to the pathogenesis of AT dysfunction. Gremlin-1 directly controls adipogenesis and insulin sensitivity of muscle, adipose and liver cells. Recent advances have pointed to a central role of gremlin-1 in AT browning. Remarkably, gremlin-1 is found overexpressed in obesity, where it positively correlates with adipocyte size, BMI and percentage of body fat. Also, gremlin-1 is upregulated in AT-related metabolic disorders such as type 2 diabetes and NAFLD. Thus, it is reasonable to speculate that gremlin-1 may be a master regulator of AT dysfunction.

Despite these studies, some crucial aspects of the involvement of gremlin-1 in AT dysfunction still deserve future research efforts. First, the pathogenetic role of gremlin-1 as a driver of the AT dysfunctional state (both in obesity and lipodystrophies) needs further confirmation. Also, whether the SVF is a source of AT gremlin-1 and how gremlin-1 production in stromal cells is regulated under pathological conditions need to be elucidated. Finally, *in vivo* studies will be necessary to reveal how AT dysfunction develops in animal models where gremlin-1 expression has been specifically knocked out in AT. Possible differences between humans and mouse models could arise and need to be addressed to better understand the species-specific effects of gremlin-1. These studies could confirm gremlin-1 as an attractive therapeutic target to promote AT browning, improve AT function and fight the metabolic syndrome associated with altered AT homeostasis occurring in obesity and lipodystrophies.

Treating obesity has proven to be a complex and mostly unsuccessful endeavor. Lifestyle interventions, bariatric surgery and pharmacological treatments are typically combined with psychological support. However, obesity relapsing nature has made the identification of long-term effective strategies extremely challenging. In this framework, the identification of novel strategies to improve the metabolic function of patients, thus reducing the risk of the associated complications, would be highly beneficial. Based on the data collected so far, gremlin-1 is an attractive target to achieve this goal. However, no drug has been developed to target gremlin-1 in any tissue/organ context yet. From a pharmacological perspective, a clear elucidation of the molecular mechanism of gremlin-1 role in AT would reveal novel druggable nodes to target AT dysfunction in obesity and metabolic disorders. For example, if a prominent role of the gremlin-1/VEGFR2 axis is found, inhibitors of VEGFR2 may be beneficial.

Gremlin-1 is a leading adipokine secreted by adipocytes and found in the plasma of humans and mice where it acts as an endocrine regulator. Beside this, circulating gremlin-1 may become a biomarker of AT dysfunction. In this respect, serum gremlin-1 is a biomarker for other diseases, such as renal damage, but its prognostic value remains unknown. Future studies will assess whether the plasmatic levels of gremlin-1 could become a diagnostic/prognostic marker of AT dysfunction useful to monitor the progression of metabolic disorders and the response to therapeutic interventions.

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Statements and Declarations

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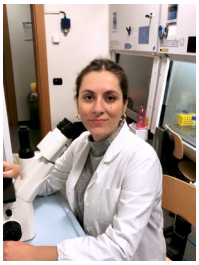

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