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Myoferlin gene silencing decreases Tie-2 expression in vitro and angiogenesis in vivo

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ABSTRACT

Angiogenesis consists in the growth of new blood vessels from pre-existing ones. Although anti-angiogenesis interventions have been shown to have therapeutic properties in human diseases such as cancer, their effect is only partial and the identification of novel modulators of angiogenesis is warranted. Recently, we reported the unexpected proteomic identification in endothelial cells (EC) of Myoferlin, a member of the Ferlin family of transmembrane proteins. Ferlins are well known to regulate the fusion of lipid vesicles at the plasma membrane in muscle cells, and we showed that Myoferlin gene knockdown not only decreases lipid vesicle fusion in EC but also attenuates Vascular Endothelial Growth Factor (VEGF) Receptor-2 (VEGFR-2) expression. Herein, we show that Myoferlin gene silencing in cultured EC also results in attenuated expression of a second tyrosine kinase receptor, Tie-2, which is another well-described angiogenic receptor. Most importantly, we provide evidence that delivery of a low-volume Myoferlin siRNA preparation in mouse tissues results in attenuated angiogenesis and edema formation. This provides the first evidence that acute Myoferlin knockdown has anti-angiogenic effects and validates Myoferlin as an anti-angiogenesis target. Furthermore, this supports the unexpected but increasingly accepted concept that proper tyrosine kinase receptors expression at the plasma membrane requires Myoferlin.

1. Introduction

Angiogenesis is well known to impact the outcome of a range of disorders, such as cancer and ischemic heart disease (Carmeliet, 2000). In settings of cancer, preventing angiogenesis decreases tumor size and favors patient survival in a synergistic fashion with chemotherapeutic agents (Folkman, 1975). The most common theory behind this synergy stipulates that anti-angiogenesis drugs not only prevent growth of new blood vessels, effectively starving tumors (Folkman, 1975), but also create a window of vessel stabilization which allows for better delivery of chemotherapeutic drugs (Hurwitz et al., 2004). However, the anti-tumor effect of angiogenesis blockers is partial and therefore the identification of novel angiogenesis-regulating pathways is essential.

Through their tyrosine kinase receptors, members of the Vascular Endothelial Growth Factors (VEGFs) family of proteins have been described as the main endogenous regulators of angiogenesis. VEGFs' main isoform VEGF-A (VEGF) is up-regulated by ischemia, stimulates the proliferation and migration of endothelial cells (EC) (Ferrara, 2004), and in contrast to virtually any other growth factor, VEGF increases plasma protein permeability (Bernatchez et al., 1999, 2001; Sirois and Edelman, 1997). VEGF promotes angiogenesis in a synergistic fashion with other growth factors, such as angiopoietins (Ang), which are growth factors believed to regulate vascular quiescence. Ang-1 mediates its activity through its mostly EC-specific tyrosine kinase receptor Tie-2 (tyrosine kinase with Ig and epidermal growth factor homology domains-2), and downstream signaling results in greater vessel maturity and impermeability to vascular macromolecules (Jain and Munn, 2000). Hence, due to the importance of VEGFR-2 and Tie-2 for blood vessel growth, modulation of their surface expression is likely to have an effect on the outcome of angiogenesis-driven diseases, which is of therapeutic interest.

Recently, by performing proteomics analysis of endothelial cholesterol-rich membrane microdomains (CEM), often referred to as caveolae/lipid rafts (Sharma et al., n.d.), we reported the unexpected detection in EC of muscle repair protein Myoferlin (Bernatchez et al., 2007, 2009), a member of the Ferlin protein family linked to muscular dystrophy (Bansal et al., 2003; Davis et al., 2000). Myoferlin exhibits a high degree of homology with the *Caenorhabditis elegans* FER-1 gene product that is essential for certain aspects of membrane fusion events at the plasma membrane (Argon and Ward, 1980; Ward et al., 1981; Achanzar and Ward, 1997). All Ferlins contain calcium-sensing and phospholipids-binding C2 domains that allow them to regulate membrane trafficking events at the plasmalemma (Glover and Brown, 2007; Washington and Ward, 2006). In EC, we found that Myoferlin gene silencing results in decreased EC proliferation, endocytosis and most importantly decreased translocation of VEGFR-2 to the plasma membrane resulting in its poly-ubiquitylation and proteasomal degradation (Bernatchez et al., 2007). This unexpectedly documented not only the presence of Ferlin in EC but also the fact that

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they regulate trafficking of membrane-bound receptors. These contentions were recently confirmed by reports documenting deficient Insulin-like Growth Factor (IGF) receptor in cells with blunted Myoferlin expression (Demonbreun et al., n.d.). Herein, we report that Myoferlin gene silencing also induces down-regulation of Tie-2 in EC in vitro. Most importantly, in vivo delivery of a low-volume Myoferlin siRNA preparation results in decreased angiogenesis and permeability in mice. These data not only broaden the scope of Ferlin involvement in endothelial activity but also indicate that down-regulation of EC angiogenic receptors through acute Myoferlin gene silencing is a feasible approach to modulate the growth of new blood vessels.

2. Materials and methods

2.1. Cell culture and in vitro siRNA treatment

Native bovine aortic endothelial cells (BAECs) between passages 5 and 15 were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen), supplemented with 5% Fetal Bovine Serum (FBS, Hyclone) and 1× penicillin/streptocycin (Sigma), whereas HUVEC were grown in M199 media as previously described (Bernatchez et al., 2007). Plasticware was from BD Biosciencs. Cells were placed in a 37 °C humidified incubator supplied with 7% CO_2. siRNA treatment was performed as previously described by our group (Bernatchez et al., 2007, 2009). Oligofectamine was purchased from Invitrogen.

2.2. RNA isolation and Quantitative polymerase chain reaction (qPCR)

Primers of Tie-2 and GAPDH (internal control) were designed using the Primer Express software (Applied Biosystems) and the recommended criteria. Selection of amplicons was based on their length—chosen at around 150 base pairs to allow for rapid amplification and a G-C content between 20%and 80%. The Tie-2 forward primer was 5′ACTCAAGATGY-GACCAGAGAA and reverse primer was 5′CCTCGAACTCGCCCTTCAC. The GAPDH forward primer was 5′ACAGTCAAGGCAGAGAACGGG and reverse primer was 5′CACATACTCAGCACCAGCATCAC. Total RNA was isolated from BAECs with the use of the RNeasy kit (Qiagen) and the protocol as described by the manufacturer. Complementary DNA (cDNA) was produced by reverse-transcription with the use of Superscript II Reverse Transcriptase (Invitrogen) protocol. Synthetic standards for Tie-2 and GAPDH by the use of the TOPO TA Cloning kit (Invitrogen) were produced to allow generation of a standard curve, from which efficiencies for each sets of primers could be deduced. Treated BAEC samples or standards

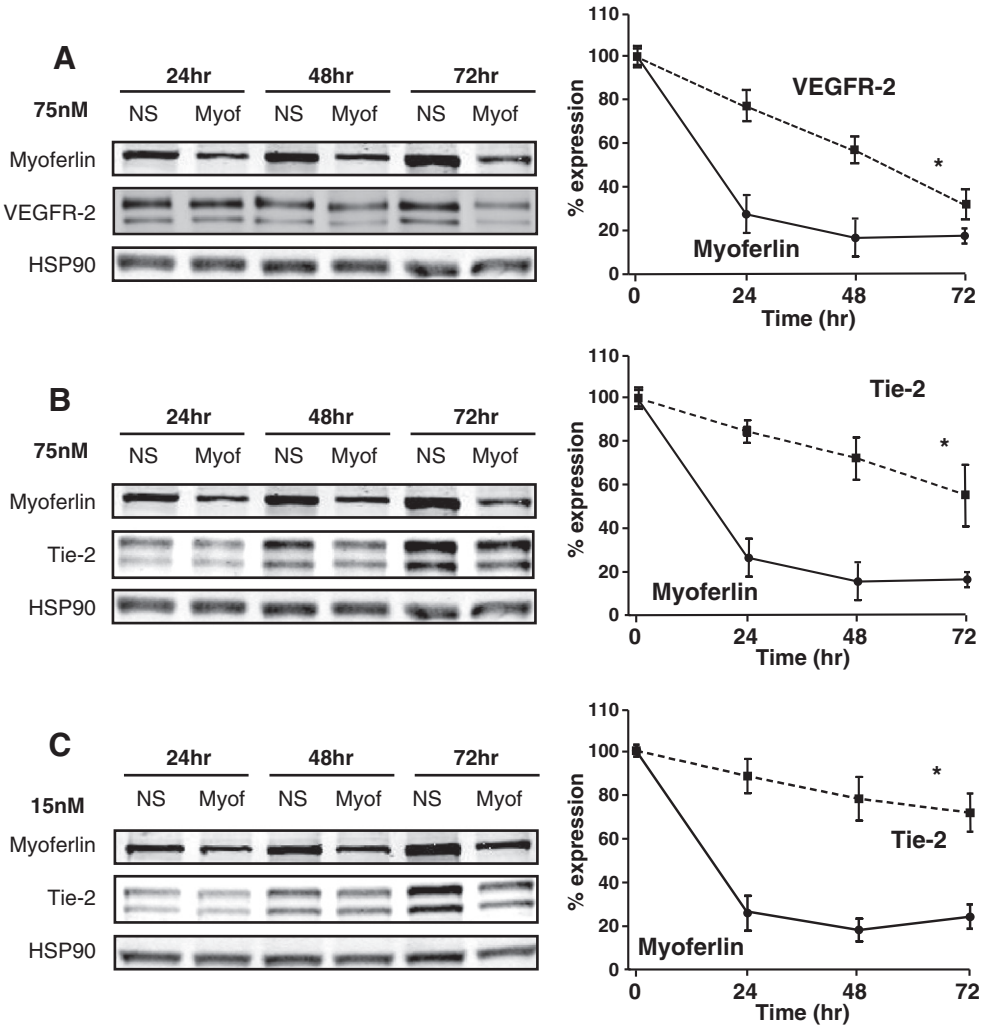

Fig. 1. Myoferlin gene silencing decreases VEGFR-2 and Tie-2 protein expression. Ninety percent (90%) confluent BAECs were treated with Myoferlin (Myof) siRNA (15 and 75 nM) or scrambled non-silencing (NS) siRNA controls, respectively, for 24, 48 and 72 h (A, B, C, respectively). Western blot analysis was performed using antibodies against Myoferlin, VEGFR-2, Tie-2 and HSP90 (loading control). Right, %protein expressions are calculated by densitometry as the VEGFR-2 or Tie-2 expression vs. HSP90 and comparing the Myof siRNA condition against the NS (100%) control. Representative Western blots are shown and graphs represent mean ± SEM ($n \geq 4$). *$P<0.05$ when comparing 72 hr Myof siRNA vs. NS siRNA for VEGFR-2 or Tie-2 by Student's *t* test for unpaired values.