The secretome mouse provides a genetic platform to delineate tissue-specific in vivo secretion

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At present, it remains difficult to deconvolute serum in order to identify the cell or tissue origin of a given circulating protein. Here, by exploiting the properties of proximity biotinylation, we describe a mouse model that enables the elucidation of the in vivo tissue-specific secretome. As an example, we demonstrate how we can readily identify in vivo endothelial-specific secretion as well as how this model allows for the characterization of muscle-derived serum proteins that either increase or decrease with exercise. This genetic platform should, therefore, be of wide utility in understanding normal and disease physiology and for the rational design of tissue-specific disease biomarkers.

protein secretion | biotinylation | mouse model

Human plasma contains a complex mixture of thousands of proteins derived from multiple tissues. While the tissue origin of certain abundant proteins, such as albumin, are known, the complete contribution of any given cell type to the plasma proteome currently remains difficult to elucidate. Such information would undoubtedly be useful as there is a growing realization that the abundance of certain serum proteins might provide unique insight into human health (1, 2). Moreover, if one wishes to derive sensitive disease biomarkers, it would be useful to know the total compendium of proteins that derive from a given cell or tissue implicated in the disease process. This tissue-specific in vivo secretome would serve as a rational entry point for subsequent biomarker development.

In an effort to provide a molecular platform to capture the tissue-specific secretome, we have exploited the properties of proximity biotinylation (3). This technique provides a way of using a modified version of the bacterial enzyme BirA to covalently biotinylate proteins within an approximate 10-nm range (4). The utility of proximity-dependent biotin identification (BioID) has been extensively documented as this method allows for a convenient strategy to elucidate protein–protein interactions (3). We realized, however, that this methodology might also provide a method to more generally tag proteins and thereby provide a platform to elucidate tissue-specific secretion.

Here, we describe the construction and analysis of ER-BioID^{HA}, an epitope tagged version of BioID that constitutively localizes to the lumen of the endoplasmic reticulum (ER). Given that most membrane and secreted proteins transit through the ER, expression of ER-BioID^{HA} provides a method of biotinylating those proteins destined for conventional secretion. We have generated a floxed transgenic mouse expressing ER-BioID^{HA} (termed the "secretome mouse") that allows for tissue-specific expression of ER-BioID^{HA}. When crossed with Cre-recombinase-expressing mice and given exogenous biotin in their diet, these animals secrete biotinylated proteins in a tissue-specific fashion. These biotin-tagged serum proteins can be rapidly identified using a simple one-step streptavidin purification followed by mass spectroscopy. Here, we provide initial data on endothelial and muscle in vivo secretion using the secretome mouse. However, we believe this platform should allow for the rapid identification of the secretome of any cell or tissue under basal conditions and following a wide variety of physiological or pathological stresses.

Results

Determining the in Vitro Secretome Using Proximity Biotinylation. In an effort to tag and purify secreted proteins, we constructed ER-BioID^{HA} containing the promiscuous biotinylation enzyme BioID2 in frame with an HA-epitope tag and the KDEL peptide C-terminal ER retention sequence (Fig. 1*A*). To allow the expression of ER-BioID^{HA} in a wide variety of cell types, we incorporated this construct into a lentiviral vector. Subsequent lentiviral-mediated delivery to primary endothelial cells in culture demonstrated ER-BioID^{HA} extensively colocalized with the ER resident protein calnexin (Fig. 1*B*). In cells expressing ER-BioID^{HA}, the addition of exogenous biotin (50 μM) resulted in the accumulation of intracellular biotinylated proteins as evidenced by streptavidin-dependent immunohistochemistry (Fig. 1*C*). As would be expected, this accumulation of biotinylated proteins was not evident when the culture medium was not supplemented with exogenous biotin (Fig. 1*C*). Given that ER-BioID^{HA} localizes to the ER lumen, we predicted that many of these intracellular biotinylated proteins would eventually appear in the conditioned medium of ER-BioID^{HA}-expressing cells. Streptavidin purified supernatants of primary endothelial cells expressing either ER-BioID^{HA} or a green fluorescent protein (GFP) control demonstrated that in the presence of exogenous biotin, only endothelial cells expressing ER-BioID^{HA} secreted multiple

Significance

Serum is a complex mixture of proteins that originate from a wide range of cells and tissues. At present, it is impossible to know what set of proteins any given tissue contributes to the circulating proteome. Here, we describe a genetic model called the secretome mouse. We show this transgenic mouse, which employs proximity biotinylation, provides a robust strategy for delineating the tissue-specific secretome. We demonstrate the utility of the secretome mouse by identifying the set of in vivo proteins secreted by endothelial cells as well as by skeletal muscle with and without exercise. This genetic strategy should, therefore, be useful in delineating a wide range of in vivo physiology and for the rational development of disease-relevant biomarkers.

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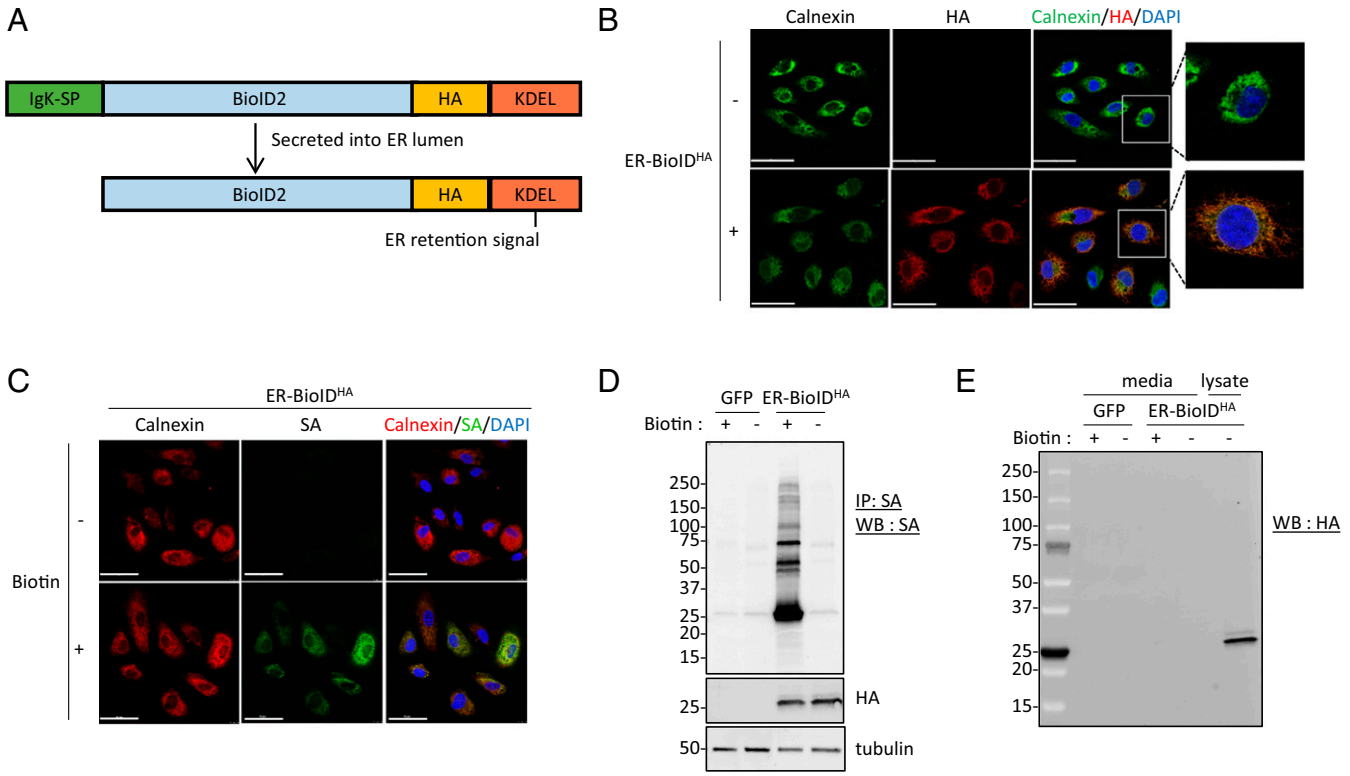

Fig. 1. Expression of ER-BioID^{HA} allows for the rapid purification of secreted proteins from endothelial cells. (*A*) Diagram of the ER-BioID^{HA} construct. (*B*) Expression of ER-BioID^{HA} in endothelial cells colocalizes with the ER resident protein calnexin. Primary endothelial cells were infected with a lentivirus encoding ER-BioID^{HA} or the pLJM1 empty vector lentivirus (–). Expression of BioID was determined using the HA-epitope with 4′,6-diamidino-2-phenylindole (DAPI) used as a nuclear counterstain. (*C*) Detection of biotinylated proteins using streptavidin (SA)-based immunohistochemistry in endothelial cell-expressing ER-BioID^{HA} in the presence (50 μM) or absence of exogenous biotin. (*D*) Detection of secreted biotinylated proteins using SA-based purification and SA-based Western blotting. Conditioned media of endothelial cells expressing ER-BioID^{HA} or GFP in the presence or absence of exogenous biotin was assessed. Initial cell lysate was assessed for ER-BioID^{HA} expression (HA epitope) and tubulin as a loading control. (*E*) ER-BioID^{HA} protein was not detected in the supernatant (media) of ER-BioID^{HA} or GFP-expressing cells as assessed by HA-epitope assessment. Protein lysate from endothelial cells expressing ER-BioID^{HA} serve as a positive control.

biotinylated proteins into the conditioned medium (Fig. 1*D* and *SI Appendix*, Fig. S1). Of note, we did not observe that ER-BioID^{HA} itself was evident in the culture medium, suggesting that biotinylation occurred intracellularly (Fig. 1*E*).

The detection of the secretome of cells in culture is often complicated by abundant serum proteins present in culture medium. While it is theoretically possible to perform these types of experiments in serum-free conditions, this is not a viable strategy for many cell types or for experiments that require extended periods of time in culture. In contrast, ER-BioID^{HA}-expressing cells allow one to rapidly purify secreted proteins using streptavidin thereby largely obviating the concerns about abundant serum proteins (e.g., albumin) present in standard culture medium. To test the utility of this strategy, we took advantage of our recent observation that endothelial cells stimulated with a cytokine mixture of transforming growth factor (TGF)-β1 and IL-1β undergo conversion from an endothelial to mesenchymal (EndoMT) phenotype (5). We envisioned that consistent with this cell fate change, the secretome of endothelial cells before and after undergoing EndoMT would differ and that the strategy outlined above would be useful in identifying such differences. As such, the conditioned medium of ER-BioID^{HA}-expressing endothelial cells was analyzed under basal conditions and 3 d after being stimulated with TGF-β1 and IL-1β. Using this approach, we could rapidly identify a large number of secreted proteins under both basal and EndoMT conditions (Dataset S1). Principal component analysis (PCA) demonstrated that these two cell states could be readily distinguished by their secretome (Fig. 2*A*). We noted a number of specific proteins whose abundance in the culture medium markedly increased following induction of EndoMT (Fig. 2*B* and *SI Appendix*, Table S1). Included in the list of secreted proteins are proteins that might be expected to increase with a transition to a more mesenchymal phenotype including the extracellular matrix protein papilin as well as collagen Type 8 and related proteins (e.g., COL8A1, P3H3, and P4HA2). We next assessed the overall fidelity of this approach by directly analyzing the expression of three proteins identified by the ER-BioID^{HA} approach as being differentially secreted. As seen in Fig. 2*C*, traditional ELISA-based strategies confirmed the ER-BioID^{HA} approach by demonstrating that endothelial cells induced to undergo EndoMT significantly increased their secretion of transforming growth factor-β-induced protein immunoglobulin G (IG)-H3 as well as increasing their secretion of pentraxin 3, a protein previously associated with EndoMT transition (6, 7). We also observed that a similar increase could be observed in cell lysates of endothelial cells stimulated to undergo EndoMT (*SI Appendix*, Fig. S2). This analysis also demonstrated that EndoMT was accompanied by the increased abundance of the cleaved soluble fragment of the VCAM-1 receptor in the culture medium. Soluble VCAM-1 is normally generated by endothelial metalloproteinase cleavage of the extracellular portion of the transmembrane receptor (8). Since most transmembrane proteins, such as VCAM-1 transit through the ER, labeling of this class of proteins was expected. Our results suggest that the conversion from an EndoMT phenotype likely involves accelerated removal of endothelial-specific

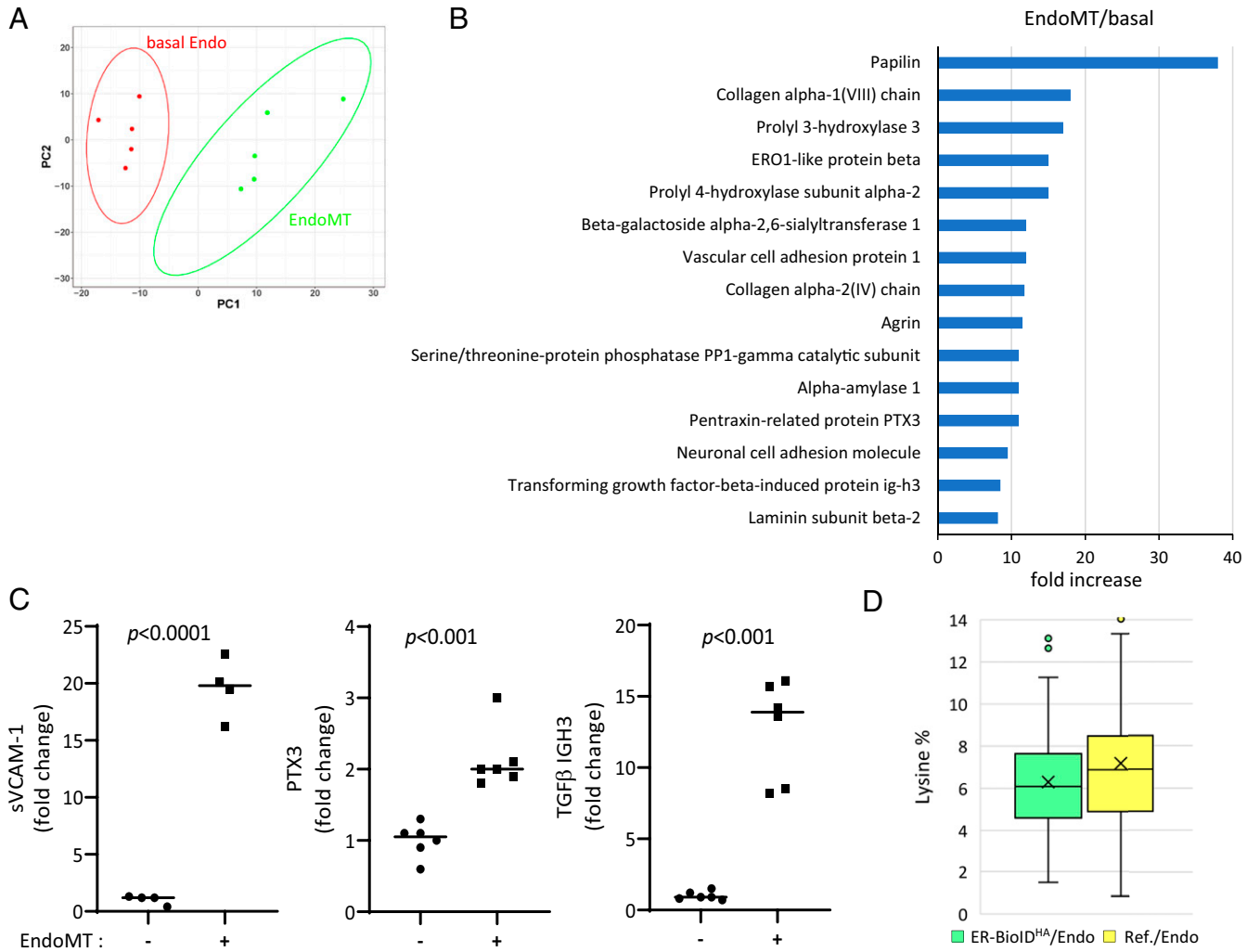

Fig. 2. ER-BioID^{HA} expression provides for a simple method to assess differential secretome of cells in culture. (*A*) PCA analysis distinguishes the secretome of endothelial cells obtained at baseline compared to the secretome of endothelial cells 3 d after induction of EndoMT. Analysis was conducted using five technical replicates per condition. (*B*) Secreted proteins identified as being increased following EndoMT. Proteins are ranked by their fold increase over baseline based on peptide counts recovered. (*C*) Enzyme-linked immunosorbent assay (ELISA)-based determination of the level of three proteins previously determined by the ER-BioID^{HA}–based strategy to increase following EndoMT induction (n = 4–6 replicates per condition). (*D*) Lysine content (percentage of total protein) for secreted endothelial proteins identified by the ER-BioID^{HA}–based strategy compared to a reference (Ref) endothelial cell secretome. See the text for details.

proteins, such as VCAM-1 through an EndoMT-stimulated posttranslational cleavage event. This observation, therefore, highlights one advantage of our experimental approach compared to strategies that solely rely on in silico transcriptomic analysis to predict potentially secreted proteins (9, 10).

Finally, given that biotinylation occurs on lysine residues, we sought to understand whether the ER-BioID^{HA}–based strategy would preferentially label secreted proteins with high lysine content. A previous report had identified 182 proteins secreted into the culture medium using endothelial cells maintained in serum-free conditions (11). We, therefore, compared the lysine content of this reference data set and the lysine content of the top 182 endothelial proteins we detected using our ER-BioID^{HA}–based approach (Dataset S1). This analysis suggested both sets of proteins had similar lysine content (Fig. 2*D* and *SI Appendix*, Fig. S3). As such, this ER-BioID^{HA}–based strategy appears suitable for the rapid identification and characterization of the in vitro secretome of a wide variety of cell types under various experimental conditions.

Generation of the Secretome Mouse. We realized that ER-BioID^{HA} expression could also provide an in vivo method for detecting protein secretion. We generated a transgenic mouse line in which ER-BioID^{HA} expression was initiated by excision of a floxed enhanced GFP (EGFP) cassette (Fig. 3*A*). Crossing this line with a tissue-specific Cre-recombinase allows for cell or tissue-restricted deletion of EGFP and the corresponding expression of ER-BioID^{HA}. We first generated a mouse line for endothelial-specific expression of ER-BioID^{HA} by crossing our transgenic line to mice expressing vascular endothelial (VE)-cadherin Cre (12), hereafter termed secretome^{ENDO}. To confirm the correct subcellular in vivo targeting of the transgene we isolated endothelial cells from the experimental secretome^{ENDO} mice or from corresponding control animals (VE-cadherin Cre only mice). Again, the ER-restricted HA-tagged transgene was readily detectable only in endothelial cells derived from the experimental mice (Fig. 3*B*). Similarly, only these endothelial cells generated intracellular biotinylated proteins when exposed to exogenous biotin (Fig. 3*C*). We next obtained serum from secretome^{ENDO} mice supplemented with biotin or from a similarly treated group of control mice that were positive for either the floxed ER-BioID^{HA} transgene or a Cre-recombinase transgene but not both constructs. Biotinylated proteins were purified using streptavidin and then

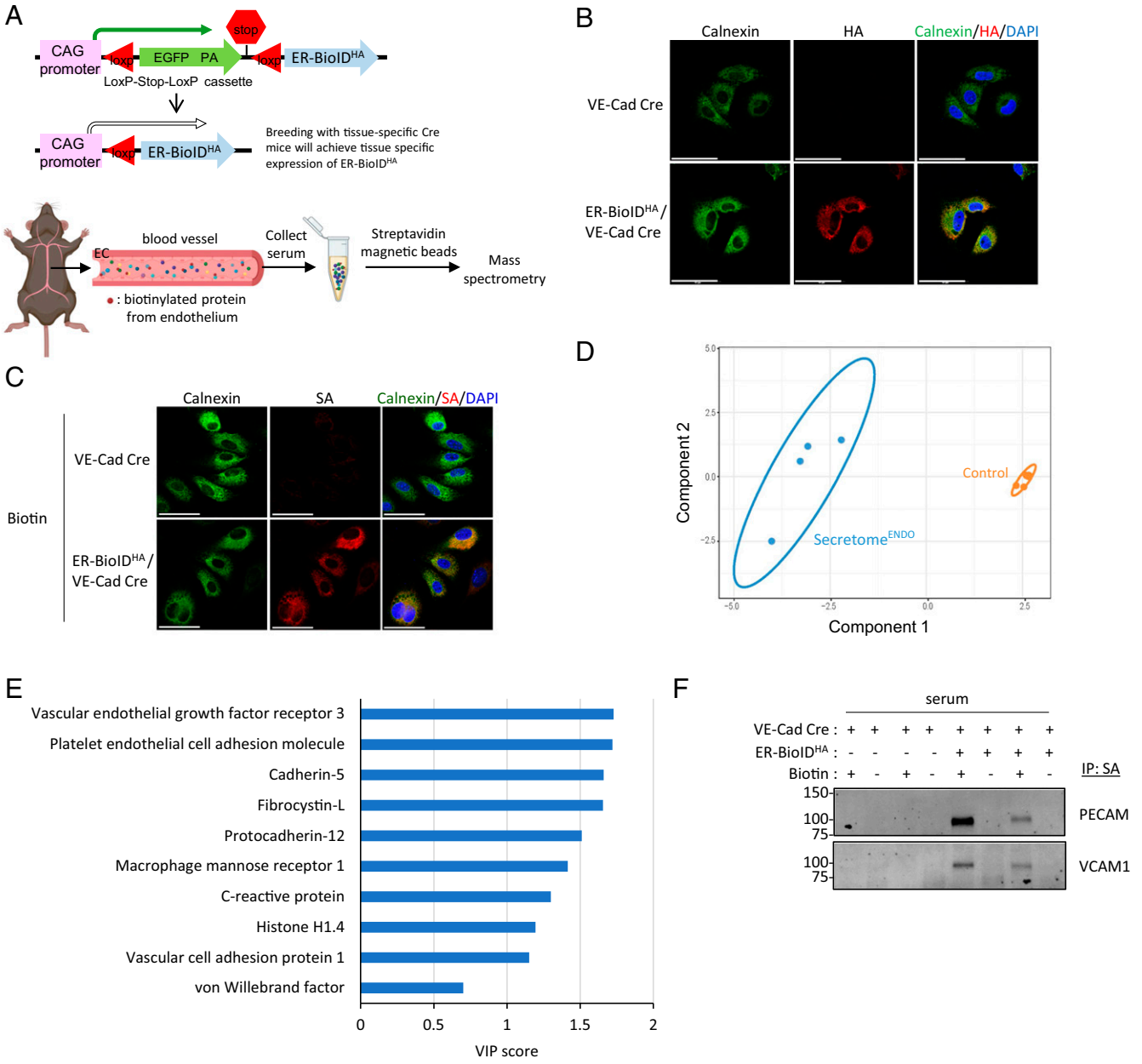

Fig. 3. Generation and characterization of the secretome mouse. (*A*) Diagram of the floxed transgene used. Cre expression leads to tissue-specific expression of ER-BioID^{HA}. (*B*) Primary endothelial cells isolated from control mice (VE-cadherin Cre only) or experimental mice (ER-BioID^{HA}/VE-cadherin Cre). Evidence of transgene expression is only seen in cells isolated from the experimental mice as determined by HA-epitope staining. ER localization is evident using calnexin as an ER-specific marker. (*C*) Biotinylated proteins as evident by SA staining are observed in endothelial cells obtained from the experimental but not control mice. (*D*) Sparse partial least squares discriminant analysis (sPLS-DA) distinguishes the secretome of control mice (ER-BioID^{HA} only or Cre only) from the secretome^{ENDO} experimental animals ($n = 4$ mice per genotype). (*E*) Purified serum proteins with the greatest VIP coefficients between control and experimental mice are shown. (*F*) Western blot analysis of the serum of control and experimental mice with or without biotin supplementation is shown for two proteins with high VIP scores. These proteins are only identified in the setting ER-BioID^{HA} expression and biotin supplementation.

analyzed by mass spectroscopy. As noted in Fig. 3*D*, using sPLS-DA we could readily distinguish control mice from the experimental animals. Purified proteins with the greatest variable importance in projection (VIP) coefficients were subsequently identified (Fig. 3*E* and *SI Appendix*, Table S2). This list included well-known proteins previously identified as deriving from the endothelium included the endothelial cell surface receptors (e.g., VEGFR-3, VCAM1, PECAM1, and cadherin-5) as well as the von Willebrand factor (13). However, we also identified proteins not commonly associated with the endothelium, such as fibrocystin (Pkhd1l1), a surface protein with a large extracellular domain. This protein is known to be secreted and has previously been identified to be expressed in endothelial cells, however, its precise role in vascular function is largely unknown (14). We also detected a C-reactive protein, a well-known modulator of endothelial function, whose expression, although mostly hepatic, has been previously identified as an endothelial secreted protein (15). To provide additional confirmation of our mass spectroscopy results we performed Western blot analysis of purified serum proteins derived from control and experimental animals with and without

biotin supplementation. These results supported the generation of circulating biotinylated proteins in the experimental mice only in the context of biotin supplementation (Fig. 3*F* and *SI Appendix*, Fig. S4).

Analyzing Muscle Secretion In Vivo. To further our understanding of this approach, we next crossed our transgenic line with mice expressing muscle creatine kinase (MCK)-Cre leading to ER-BioID^{HA} expression in skeletal muscle and to a lesser extent in cardiac muscle but not in other tissues (Fig. 4*A*). Analysis of skeletal muscle cell lysate demonstrated that in the presence of biotin supplementation, ER-BioID^{HA} /MCK-Cre^{+} mice (hereafter denoted as secretome^{MUSCLE}) had multiple biotinylated proteins not evident in the skeletal muscle of the control animals expressing only MCK-Cre or ER-BioID^{HA} (Fig. 4*B*). Biotin supplementation in these animals came from a combination of multiple routes of administration (intraperitoneal, subcutaneous, and oral feeding) over a 5-d period. This was based on the observation that although biotin soaked chow was the most important component, the combination approach appeared to increase labeling efficiency (Fig. 4*C*). We also noted that biotin supplementation for 5 d was superior to supplementing mice for just 2 d (Fig. 4*D*).

In an effort to further demonstrate the potential utility of this system, we separated secretome^{MUSCLE} mice into cages with or without a running wheel in order to understand how the muscle-derived secretome would change with voluntary exercise. This voluntary wheel running was of sufficient intensity to effect body weight but not to induce skeletal muscle hypertrophy (*SI Appendix*, Fig. S5). We then compared the secretome of secretome^{MUSCLE} mice with and without exercise. The serum of these experimental animals and the control animals that only expressed a Cre-recombinase transgene were isolated by streptavidin purification followed by mass spectroscopy. As we observed for cells in culture, ER-BioID^{HA} itself was not detectable in the serum, suggesting the biotinylation enzyme is not directly secreted into the circulation (*SI Appendix*, Fig. S6). As noted in Fig. 4*E*, sPLS-DA was capable of delineating secretome^{MUSCLE} mice with and without exercise from each other and from control mice based on their purified secretome. Purified proteins with the greatest VIP coefficients were subsequently identified (Fig. 4*F* and *SI Appendix*, Table S3). As expected, the VIP proteins identified from the secretome^{MUSCLE} mice exhibited no overlap with the proteins previously identified from the secretome^{ENDO} mice.

We noted that a number of the serum proteins we identified as deriving from muscle altered their abundance with exercise (Fig. 4*F*). Among these was the well-known muscle protein myostatin, whose abundance modestly decreased with exercise (average 29 peptide counts basally [n = 4 mice], average 26 peptide counts with exercise [n = 4 mice], and average of two peptide counts in the control group [n = 7 mice]). This observation is in good agreement with the known physiological effects of exercise on myostatin levels (16, 17). We sought to further confirm these observations and subjected a separate group of wild-type mice to similar cage conditions with or without the option for exercise. Analysis of the serum of these animals revealed a similar modest trend of reduced circulating myostatin in the exercise cohort (Fig. 4*G*).

Discussion

To summarize, we describe the generation of the secretome mouse, a genetic platform that allows for the rapid identification of the in vivo cell or tissue-specific secretome under basal conditions or following a physiological or pathophysiological stress. We further demonstrate how these concepts can be used in the in vitro situation for cultured cells. Our experience with cells in culture yielded many more secreted proteins than we observed in the in vivo setting. While there are several possible reasons for this, we feel the most likely explanation revolves around the difficulty of raising serum biotin levels high enough for efficient in vivo biotinylation by BioID. It is estimated that the serum concentration of biotin is roughly 0.5–1.0 ng/mL or roughly 2 nM (18). Although it is well known that oral supplementation with biotin is well tolerated and can substantially increase circulating levels of biotin in humans (18) and rodents (19), the levels routinely used in cell culture (50 μM) are difficult to achieve by simply manipulating the diet. Recent efforts using directed evolution have generated second and third generation BioID molecules, such as TurboID (20). While BioID usually take >18 h to produce a detectable biotinylation signal, TurboID appears to generate a signal in the minute time frame. Of note, there is some evidence that, at least, some low level of this TurboID signal can be seen without biotin supplementation, suggesting that this enzyme might have a lower requirement for exogenous biotin addition (20). As such, using the principles described here with a BioID, a second generation TurboID-expressing transgenic mouse might provide for enhanced in vivo sensitivity. We should also note that since our data suggest that oral biotin supplementation is the most effect route of administration, that it may also be possible to combine biotin in the chow with oral gavage to further boost serum biotin levels.

Our results demonstrate that we can deconvolute the complicated mix of serum proteins to identify the contribution of a given cell or tissue to the serum proteome. We should note that our strategy only captures proteins that transit through the ER lumen and, hence, largely encompass cell surface receptors and proteins destined for conventional secretion. As such, we likely miss most of the serum proteins that enter the circulation through unconventional secretion (21). Unconventional protein secretion (UPS) encompasses several distinct modes of protein secretion including what is sometimes referred to as Type I UPS involving plasma membrane pore formation and subsequent protein release and Type III UPS involving proteins contained within membrane bound organelles/vesicles that subsequently fuse with the plasma membrane and Type IV UPS involving proteins that are synthesized in the ER but which then bypass the Golgi (21). Of note, the latter form of UPS would, in fact, be captured by our current strategy. Type III UPS largely involves structures, such as late endosomes, multivesicular bodies, and autophagosomes whose membrane origin is believed to involve the ER (22). A related phenomenon is lysosomal exocytosis, wherein lysosomes fuse with the plasma membrane to release their contents extracellularly (23). Since lysosomal proteins are largely synthesized in the ER, we anticipate that our strategy would capture some of these types of unconventionally secreted proteins. In addition, recent evidence suggests that the pathway for Type III UPS actually involves entry of the UPS cargo into an ER-Golgi intermediate compartment prior to its packaging into secretory vesicles (24). Again, whether ER-BioID^{HA} is expressed in this newly described subcellular compartment awaits future studies. We should also note that based on our Western blot data (Fig.1*D* lane 3, Fig. 4*B* lanes 5 and 6, and Fig. 4*D* lanes 3–6) a strong biotinylated band corresponding to the approximate 28-kDa molecular weight of ER-BioID^{HA} was routinely noted. This likely suggests that ER-BioID^{HA} is itself biotinylated. Although we were unable to detect the secretion of the ER-BioID^{HA} protein, we cannot formally exclude this possibility. Indeed, the data in Fig. 1*D* appear to provide some support for secretion of ER-BioID^{HA}, at least, under certain conditions.

Our initial analysis determined that when compared to a reference endothelial secretome data set (11), our ER-BioID^{HA} identified secretome appeared to have a lower percentage of proteins containing a predicted signal peptide (31 of the top 182 endothelial proteins identified by ER-BioID^{HA} versus 71 of 182 in the reference data set). Whether this reflects an ability to capture some forms of UPS by our method awaits further analysis. In that context, while in our in vivo analysis, the majority of the proteins we identified as coming from endothelium or skeletal muscle were known to be secreted, we did observe proteins

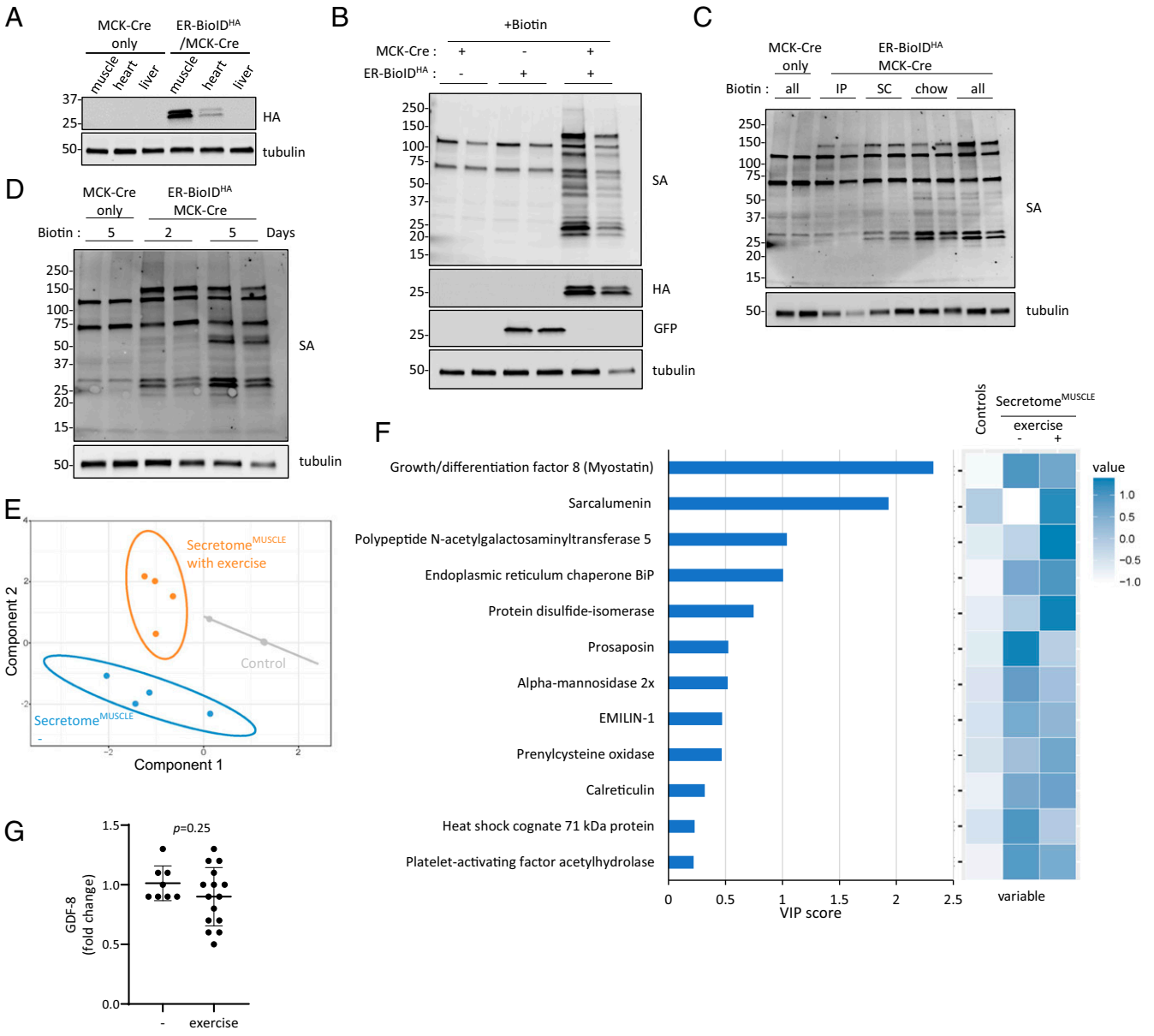

Fig. 4. Analysis of the secretome^{MUSCLE} mice. (*A*) Tissue-restricted expression of ER-BioID^{HA} is confined to skeletal muscle and heart in MCK-Cre positive mice. (*B*) Skeletal muscle-derived lysate from pairs of mice fed biotin with the indicated genotype and assessed using SA detection. Skeletal muscle biotinylation is only observed in experimental animals expressing both MCK-Cre and ER-BioID^{HA}. Of note, within tissues due to Cre-mediated excision HA-expression and GFP expression are inversely related. Tubulin is shown as a loading control. (*C*) Biotinylation in skeletal muscle in mice where biotin is administered by intraperitoneal injection (i.p.), subcutaneous injection (SC), or mixed into the food for 2 d (chow) or by a combination of all three routes of administration (all). Tubulin is shown as a loading control. (*D*) Analysis of control or experimental mice given biotin in their chow for 2 or 5 d. Tubulin is shown as a loading control. (*E*) sPLS-DA distinguishes the secretome of secretome^{MUSCLE} mice with or without exercise ($n = 4$ mice per exercise condition) from each other and from control mice (Cre-recombinase only; $n = 7$). (*F*) Purified serum proteins with the greatest VIP coefficients between control and experimental mice are shown. A heat map of the relative abundance of these VIP proteins with and without exercise is shown. (*G*) ELISA-based serum determination of myostatin, the VIP protein with the greatest coefficient, is shown using a separate group of wild-type mice with or without exercise.

that did not fit this pattern. For instance, we observed that the muscle-specific factor sarcalumenin, which is generally not viewed as a secreted factor, increased following exercise. With that said, examination of serum databases demonstrates that sarcalumenin is readily detected in human plasma (estimated concentration 26 ng/L) (2, 25). Moreover, past analysis has demonstrated that skeletal muscle expression of the protein appears to increase with exercise paradigms (26), and levels of sarcalumenin appear to modulate aerobic capacity (27).

With potential additional refinements, we envision this model to have widespread application as a discovery platform as evident by our initial observations with exercise. The ability to understand what a cell or tissue secretes under basal conditions or following a physiological stimulus should provide significant biological insights. We also suspect that this platform might also aid in the rational design of disease biomarkers. Crossing the secretome mouse where expression is, for instance, restricted to astrocytes or neurons to mice predisposed for Alzheimer's disease may allow for the generation of early serum or cerebrospinal fluid

biomarkers for this condition. Similar approaches could be employed to develop cancer biomarkers, for instance, by using a tissue-specific Cre-recombinase to simultaneously turn on a tissue-specific tumor initiating event (i.e., oncogene [e.g., K-RAS] expression or tumor suppressor [e.g., p53] deletion) in conjunction with ER-BioID^{HA} expression. This might provide a method to develop biomarkers for occult malignancies, such as pancreatic tumors, allowing the sequential analysis of secretome of the target tissue under normal conditions, in the premalignant stage or after the development of a frank tumor. Finally, while we have restricted our analysis to serum, it may be possible to use the secretome mouse and high affinity streptavidin purification to better understand interorgan signaling. For instance, streptavidin purification of the adipose tissue or brain of our secretome^{MUSCLE} mice might provide unique insights as to how factors derived from skeletal muscle communicate with other tissues and organs to mediate important physiological effects.

Methods

Additional methods are included in the *SI Appendix*.

BioID2-ER Construct. To construct an ER lumen-resident BioID expression plasmid (termed ER-BioID^{HA}), we designed the BioID coding sequence with the following features in order: IgK signal peptide, BioID2 coding sequence, HA tag, and the ER retention signal KDEL (Lys-Asp-Glu-Leu) tetrapeptide. In brief, we used the primer listed in *SI Appendix*, Table S4 to amplify BioID2-ER using a plasmid MCS-13XLinker-BioID2-HA (Biofront Technologies) as a template. The resulting DNA fragment was cloned into the Nhe/BsrGI sites of lentiviral vector pLJM1-EGFP (Addgene no. 19319), resulting in pLJM1- ER-BioID^{HA}, which was confirmed by DNA sequencing.

Human Umbilical Vein Endothelial Cell Culture, Transfection, and Expression. Human umbilical vein endothelial cells (HUVECs) (Lonza, Catalog No. C2519A) were grown in endothelial cells growth medium (PromoCell, Catalog No. C22010). To express ER-BioID^{HA} in HUVECs, the cells were infected overnight with ER-BioID^{HA} lentivirus (multiplicity of infection = 10) or a control lentivirus consisting ofeither the pLJM1 empty vector or a similar GFP-expressing lentivirus as described previously (28). For induction of EndoMT, subconfluent HUVECs (ER-BioID^{HA}) grown on fibronectin-coated plates were incubated with 10-ng/mL TGF-β1 (PeproTech, Catalog No. 100–21C) and 1-ng/mL IL-1β (PeproTech, Catalog No. 200–01B) for 3 or 4 d. For protein biotinylation, HUVECs were pulsed with 50-μM biotin for 6 h on day 2 or day 3. After washing to remove remaining biotin in the media, the cells were further cultured overnight. The conditioned medium was then collected and incubated with streptavidin beads (Invitrogen) overnight. Beads were collected using a magnetic stand and washed as described (29). Endothelial cell lysates were prepared in radioimmunoprecipitation assay buffer, analyzed by Western blotting using antibodies against streptavidin, Alexa Fluor 488 (Invitrogen, S32354), anti-pentraxin 3/PTX3 (Abcam, EPR6699), or anti-α tubulin (Santa Cruz Biotechnology, sc-5286). Protein levels of human pentraxin 3, transforming growth factor β (TGFβ)-induced protein IG-H3 (TGFβ IGH3), and soluble VCAM-1 in the above conditioned media were measured by ELISA using the respective commercial ELISA kits flowing the manufacturer manuals (R&D Systems, DPTX30B; Abcam, ab155426; and Invitrogen, BMS232).

Immunostaining. HUVECs were grown on tissue culture treated glass slides (BD Biosciences, 354104), washed with phosphate-buffered saline (PBS), and fixed in PBS supplemented with 4% paraformaldehyde (Electron Microscopy Sciences) for 10 min at room temperature. After permeabilization with 0.3% Triton X-100 (Sigma-Aldrich) in PBS for 20 min at room temperature, the cells were washed with PBS and blocked in fluorescent blocking buffer (ThermoFisher Scientific, Catalog No. 37565) for 1 h at room temperature. To verify the ER localization of ER-BioID^{HA}, cells were then probed with a mouse antibody against the HA-Tag (Santa Cruz Biotechnology, sc-7392) and a rabbit antibody against calnexin (Cell Signaling, C5C9) overnight at 4 °C, followed by labeling with Texas Red conjugated goat anti-mouse and Alexa Fluor-488 donkey anti-rabbit antibodies (ThermoFisher Scientific). To assess the ER localization of biotinylated proteins, cells were then probed with a rabbit antibody against calnexin (Cell Signaling, C5C9) and Alexa Fluor 488 streptavidin overnight at 4 °C, followed by labeling with a Texas Red goat anti-rabbit antibody.

For the in vivo localization of ER-BioID^{HA}, primary endothelial cells were isolated from the lungs of secretome^{ENDO} mice or control (VE-cadherin Cre only) mice, using our previously described method (30). Briefly, two mouse lungs per genotype were digested with type I collagenase and plated on gelatin, fibronectin, and collagen-coated flasks. The cells were then subject to sequential negative sorting by magnetic beads coated with a sheep anti-rat antibody using a Fc blocker (rat anti-mouse CD16/CD32, BD Pharmingen Catalog No. 553142) to remove macrophages and positive sorting by magnetic beads using an anti-intermolecular adhesion molecule 2 (ICAM2 or CD102) antibody (BD Pharmingen Catalog No. 553326) to isolate ECs (ICAM2 positive cells). These primary ECs were used within five passages after isolation for immunostaining. Similarly, to confirm the ER localization of ER-BioID^{HA}, the fixed cells were probed with a mouse antibody against the HA-Tag (Santa Cruz Biotechnology, sc-7392) and a rabbit antibody directed against calnexin (EMD Millipore Catalog No. AB2301) overnight at 4 °C, followed by labeling with a goat anti-mouse Texas Red and Alexa Fluor-488 donkey anti-rabbit antibody (ThermoFisher Scientific). To verify the ER localization of biotinylated proteins, cells were probed with a rabbit antibody against calnexin (EMD Millipore Catalog No. AB2301) and Alexa Fluor 647 streptavidin overnight at 4 °C, followed by labeling with an Alexa Fluor-488 donkey anti-rabbit antibody.

Generation of Tissue-Specific Secretome Transgenic Mice. The ER-BioID^{HA} coding fragment was cloned into EcoRI/NheI sites of the pCLE vector (31), resulting in a transgenic vector pCLE-ER-BioID^{HA} in which the ER-BioID^{HA} coding sequence was under the control of the CAG promoter (P_{CAG}, a combination of the cytomegalovirus early enhancer element and chicken β-actin promoter). A loxP-Stop-loxP (LSL) cassette (a DNA fragment containing an EGFP coding sequence followed by a poly[A] terminator) was placed between P_{CAG} and BioID2-ER^{HA} (Fig. 3*A*). After removing the nonrelevant sequences using Xho I and Dra III digestion, the transgenic vector was microinjected into the pronuclei of fertilized eggs to generate a transgenic mice (C57BL/6 background). The founder lines were genotyped by PCR analyses using the primers listed in *SI Appendix*, Table S4. These animals do not express the ER-BioID^{HA} transgene until crossed with cell-type-specific *Cre*-transgenic mice to remove the LSL cassette and place P_{CAG} adjacent to the ER-BioID^{HA} coding sequence (secretome mouse; Fig.3*A*). Thus, endothelial-specific-expressing mice, secretome^{ENDO} mice, were obtained by breeding the secretome mouse with *Cdh5-Cre* (Cre-recombinase under the VE-cadherin promoter) mice (The Jackson Laboratory no. 006137) to delete the LSL cassette only in endothelial cells. Secretome^{MUSCLE} mice were generated similarly using the corresponding muscle-specific MCK-Cre mice (The Jackson Laboratory no. 006475). Secretome transgenic mice were generated by the University of Pittsburgh Transgenic and Gene Targeting Laboratory. The raw proteomic data are deposited in the ProteomeXchange repository of Proteome Central.

Animal Studies. All animal studies were carried out in accordance with protocols approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh. Except when noted, an equal number of male and female mice 8–12-wk-old mice were used in this analysis. For biotin labeling of the in vivo secretome, prior to serum collection, the mice were administered both i.p. and subcutaneously with 500 μL of a 2-mg/mL biotin solution (Sigma-Aldrich) once a day for five consecutive days. Except when noted, the animal's chow was also soaked in a solution of 2-mg/mL biotin (2-mg biotin per gram of chow) for five consecutive days before harvest. The sera were collected after terminally bleeding. Approximately 300-μL serum from each mouse was incubated with 100-μL streptavidin beads overnight. The beads were collected using a magnetic stand, and the biotinylated proteins were collected in sodium dodecyl sulfate (SDS) loading buffer and analyzed by mass spectrometry. For the exercise paradigm, mice were provided with InnoWheel on top of an InnoDome for a month. In each cage, there was one apparatus for every two mice. Serum myostatin levels were assessed in a separate set of 3-mo-old wild-type male C57BL/6 mice either exercised (n = 15) or nonexercised mice (n = 8) using a GDF-8/Myostatin Quantikine ELISA Kit (R&D Systems, DGDF80). For confirmation of endothelial biotinylated proteins, 200 μL of serum per mouse (n = 4 per genotype) were incubated with streptavidin beads overnight. The beads were collected using a magnetic stand, and the biotinylated proteins were collected in SDS loading buffer and analyzed by Western blotting using an anti-PECAM antibody (Abcam, ab222783) or an anti-VCAM-1 antibody (Abcam, ab134047). For assessing whether ER-BioID^{HA} was secreted into the circulation, 20 μL of serum from either secretome^{MUSCLE} mice or control mice were analyzed by Western blotting using an anti-HA-tag antibody.

Data Availability. All study data are included in the article and supporting information. The raw proteomic data are deposited in the ProteomeXchange repository of Proteome Central with the identifier PXD022694.

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