Published in final edited form as:
Endocrinol Metab Clin North Am. 2017 March ; 46(1): 51–62. doi:10.1016/j.ecl.2016.09.014.

Endothelin Signaling in Bone

Jasmin Kristianto, PhD[a,b,c,f], **Michael G. Johnson, PhD**[a,b,c], **Rafia Afzal, MBBS**[d], and **Robert D. Blank, MD, PhD**[a,b,e,f]

[a] Endocrine and Reproductive Physiology Program, University of Wisconsin, Madison, WI 53705

[b] Geriatrics Research, Education, and Clinical Center, William S. Middleton Veterans Hospital, Madison, WI 53705

[c] Department of Medicine, University of Wisconsin, Madison, Wisconsin 53792

[d] Department of Anesthesiology, Aga Khan University Hospital, Karachi 74800, Pakistan

[e] Medical Service, Clement J. Zablocki VAMC, Milwaukee, WI 53295

[f] Division of Endocrinology, Metabolism, and Clinical Nutrition, Department of Medicine, Medical College of Wisconsin, Milwaukee, WI 53226

Keywords

WNT Signaling; endothelin 1 signaling; osteogenesis; mechanotransduction; micro-RNA

Introduction

Endothelin 1 (ET1, see **table 1** for gene and protein abbreviations) signaling has been recognized as a driver of osteoblastic metastasis for more than a decade and recent work points to its having a broader role in bone biology. This review will first outline the ET signaling pathway and ET metabolism. It will next summarize the role of ET1 signaling in craniofacial development. Then, it will discuss observations relating ET signaling to osteoblastic and other osteosclerotic processes in cancer. Finally, it will describe recent work in our laboratory that points to endothelin signaling as the role of as an upstream mediator of WNT signaling, promoting bone matrix synthesis and mineralization. It will conclude with a statement of some remaining gaps in knowledge and proposals for future research. These will be informed by insights gained from study of ET signaling in the development and physiology of the cardiovascular system.

Correspondence to: Robert D Blank, MD, PhD, 9200 West Wisconsin Ave, Milwaukee, WI 53226, USA, 414-955-6722, 414-955-6210 (fax), roblank@mcw.edu.

Disclosure: Dr. Blank is an investigator in a clinical trial sponsored by Novo-Nordisk, a consultant for Bristol-Myers Squibb, and a contributor to UpToDate and receives royalties for this work. Drs Kristianto, Johnson and Afzal have nothing to disclose.

Overview of the ET Signaling Pathway

The ET system includes 3 small peptide hormones[1-3], ET1, ET2, and ET3, 2 G-protein coupled receptors[4,5], EDNRA and EDNRB, and 2 specific converting enzymes[6,7], ECE1 and ECE2. The ETs are synthesized as prepropeptides that are first processed to biologically inactive, 37-41 amino acid propeptides, commonly known as “big ET’s,” by furin-like proteases prior to secretion[8,9]. Following secretion, the big ETs must be converted to their active forms by proteolytic cleavage in the extracellular space. ECE1 and ECE2, which have different pH optima (neutral pH optimal for ECE1, acidi pH optimal for ECE2), catalyze ET activation by cleaving the big ET’s to 21 amino acid active ETs. In addition, big ET’s can be converted by a variety of other proteases (**figures 1 and 2**)[10-12].

The ET system was discovered in arteries, and it has since been shown that various elements of the system are expressed in a wide variety of tissues, but expression is not ubiquitous. Immortalized osteoblasts in culture express ET1, EDNRA, and ECE1, thus having the capacity for autocrine ET signaling within the lineage[13]. Conversely, ET2, ET3, EDNRB and ECE2 are either not detected or expressed at very low levels in these cells[13].

ET1 Signaling in Development

Knockout mice lacking either ET1 or EDNRA have very similar, lethal phenotypes that result from malformations of the craniofacial bones[14,15]. Mice die shortly after birth due to asphyxia, which can be overcome by tracheostomy. They have hypoplastic mandibles, homeotic transformation of the mandible to a maxillary morphology[16,17]. There are multiple defects in other facial and basilar skull bones, and the hyoid bones. Together, these defects obstruct the airway, leading to the observed lethality. Identical craniofacial defects are observed in ECE1 knockout mice[18].

In addition to the craniofacial abnormalities, ET1 and EDNRA knockout mice share defects of the cardiac outflow tract and great vessels[15,19]. These defects are not fully penetrant, and include tubular hypoplasia of the aortic arch, absent right subclavian artery and perimembranous ventricular septal defect. In ECE1 knockout mice, these defects are both more severe and more penetrant than in the ET1 and EDNRA knockouts[18]. **Box 1** provides further information about ET signaling in cardiovascular physiology.

The common element linking the craniofacial and cardiovascular anomalies is that the affected structures are derived from the neural crest. Cranial and cardiac neural crest cells migrate early in development and express EDNRA[15]. It is worth noting that knockouts of ET3 and EDNRB, which are also lethal, affect a different population of neural crest derivatives. Phenotypes of these mutations include colonic aganglionosis (Hirschsprung’s disease) and white spotting[20,21]. As in the case of EDNRA/ET1, EDNRB/ET3 knockouts have very similar phenotypes. ECE1 knockout animals have all the defects characteristic of both the EDNRA/ET1 and EDNRB/ET3 knockouts[18].

ET Signaling in Osteoblastic Metastasis

ET signaling occurs in both mammary and prostate glands, and when it is expressed in neoplasms arising from those tissues, it promotes osteoblastic metastasis[22-26]. Osteoblastic metastasis is very common in prostate cancer and uncommon in breast cancer. In both cases, lesions feature synthesis of sclerotic, woven bone that is mechanically deficient. We recently found that ET1 is highly expressed in the setting of osteosclerosis associated with myelofibrosis[27], with very similar features to those encountered in breast and prostate cancer. Promotion of osteogenesis by ET1 signaling is mediated at least in part by modulation of WNT signaling. In cultured mouse calvarial osteoblasts, ET1 reduces transcription and secretion of DKK1, and concomitantly increases bone formation[28]. In men with metastatic prostate cancer, EDNRA blockade suppressed progression of bone disease as measured by bone turnover markers[29].

Human breast cancer cells can convert big ET1 to active ET1[24,30], suggesting the potential role of ET1 in bone metastasis in breast cancer. EDNRA blockade reduced osteoblastic lesions in mice inoculated with ZR-75-1 human breast cancer cells[24]. Collectively, these results show that ET1 action via EDNRA mediate bone formation in both breast and prostate cancer. The bone cells, in turn, secrete factors that can support proliferation of the tumor cells, such as IGF1, resulting in a vicious cycle of concurrent bone and tumor growth supported by reciprocal paracrine signaling between tumor cells and osteoblasts.

ET Signaling in Bone Physiology

In the course of pursuing our long-standing interest in the genetic basis of bone biomechanical performance, we identified *Ece1* as a candidate gene for a pleiotropic quantitative trait locus (QTL) for bone size, shape, and strength[31-35]. The QTL increases the cross-sectional size and ellipticity of the femoral diaphysis, leading to an increase in the whole bone strength as measured by 3-point bending. This constellation of phenotypes suggested that the primary process affected by the QTL was bone modeling in response to mechanical loading (see **box 2**). Other investigators had determined that our QTL lies within a genomic region that contributes to load-induced bone modeling[36], leading us to hypothesize that both sets of phenotypes were mediated by the same genes. It is worth noting that the mouse QTL corresponds to a confirmed human BMD QTL[37].

In subsequent experiments, we found that murine osteoblasts grown in tissue culture engage in autocrine ET1 signaling via EDNRA[13] (and Johnson *et al.,* unpublished data). They express *Edn1*, *Ednra*, and *Ece1*, but not *Ednrb.* These cells recapitulate maturation of the osteoblast lineage when placed in medium supplemented with vitamin C and phosphate, forming mineralized nodules after 2 weeks in mineralization medium. Supplementation of the medium with big ET1 increases mineralization, which is blocked by pharmacological inhibition of either ECE1 or EDNRA, treatment with SOST, or by transfection of siRNA targeting *Ece1* message. In addition to promoting mineralization, ET1 treatment reduces secretion of SOST and DKK1, in spite of increasing transcription of their mRNAs. These divergent effects on transcription and protein secretion are mediated in part by miR 126-3p, which is increased by ~120-fold via ET signaling, which targets *Sost* message. The effects

of ET1 signaling on mineralization and SOST secretion are mimicked by transfection of a miR 126-3p expressing lentivirus, while the effects of ET1 signaling blockade are mimicked by transfection of a lentivirus expressing a miR 126-3p antagonist.

MiR 126-3p is a critical molecule in angiogenesis[38]. MiR 126-3p ablated mice have a high rate of embryonic lethality with impaired blood vessel formation, while the surviving mice are deficient in the angiogenic response to experimental ischemia[39]. Important endothelial cell targets of miR-126-3p include, but are not limited to, mRNAs encoding a pair of VEGF inhibitors, *Pik3r2* and *Spred1*. It is interesting to note that both in osteoblasts and in endothelial cells, miR 126-3p acts by releasing repression of critical signaling pathways.

Future Considerations

Current understanding of ET biology is very uneven. The developmental roles of ET signaling are relatively well characterized, as is ET signaling within arterial walls. It is also clear that ET drives osteoblastic metastasis in breast and prostate cancer, but little is known regarding the role of ET signaling in the normal function of each of these glands, or of the contribution ET signaling to normal bone physiology. Greater understanding of the ET signaling pathway's role in the normal biology of tissues outside the vasculature is therefore of great importance.

It is important to recall that ET1 signaling is essential in development[14,15,18,19], and that the phenotypes resulting from knockout of *Edn1*, *Ednra*, and *Ece1* overlap some of those arising from mutants affecting WNT signaling. Ablation of *Ece1*, unlike that of either *Edn1* or *Ednra*, leads to significant mid-gestational *in utero* lethality due to heart failure, reflecting a greater severity of cardiac outflow tract abnormalities[18]. In the chick, ET1 signaling mediates essential mechanotransductive signals in Purkinje system development[40-43].

However, the situation is more complex. WNT signaling has an unequivocal trophic impact on cells already committed to the osteoblast lineage; indeed, canonical WNT signaling has been shown by some investigators to inhibit commitment of mesenchymal stem cells to the osteoblast lineage[44,45]. In addition, mice constitutively expressing β-catenin in late stage osteoblasts and osteocytes have *both* increased bone volume and osteomalacia[46]. These findings demonstrate that normal bone mineralization requires down-regulation of canonical WNT signaling at late stages of osteoblast maturation, even while elaboration of bone matrix by less mature cells is promoted.

Down-regulation of SOST, leading to derepression of the WNT pathway has been demonstrated in an *in vivo* experimental mechanical loading protocol[47]. It is unknown at this point whether ET signaling mediates the SOST response in this setting, thus functioning upstream of the WNT pathway in mediating mechanotransduction in bone. Our laboratory's findings that ET1 signaling reduces SOST expression via miR-126-3p provide a mechanistic basis for pursuing this line of investigation[13] (and Johnson *et al.,* unpublished data). Other investigators have identified DKK1 as another WNT-related target of ET1 signaling in cancer[28], further supporting the idea that ET signaling is upstream of the WNT pathway in bone.

More broadly, little attention has been devoted to identifying common mechanisms of mechanotransduction in bones and arteries. Both are tubular organs whose function requires adaptation to highly variable mechanical environments. Bone and vascular biology might both benefit by further work in this area.

Finally, osteoblastic metastases arise in other tumors in addition to prostate and breast cancer. It is worth studying other tumor types to determine whether they share ET1 signaling as the underlying mechanism.

BOX 1

Physiological ET Signaling in the Cardiovascular System

The ET system was first discovered in the vasculature and its biology is best understood in that setting. ET signaling via EDNRB on endothelial cells causes vasodilation and clearance of ETs from the circulation [48-50]. In contrast, signaling via both EDNRA and EDNRB in smooth muscle cells has potent vasoconstrictive effects [1,51]. Endothelial cells are the primary source of ET1 and have a high density of EDNRB, so autocrine signaling favors vasodilation and hypotensive responses, while paracrine signaling leads to hypertensive responses. In experiments we performed using the same mice in which we identified *Ece1* as a candidate gene for bone biomechanical performance, we found that mice harboring a high-expressing *Ece1* allele had larger femoral and arterial cross-sections, greater arterial compliance, and lower BP than mice harboring a low-expressing *Ece1* allele [52].

Shear stress plays a critical role in vascular remodeling, both in developmental [53] and physiological[54-56] settings. NO is known to play a central role in mediating vascular remodeling in pregnancy and its induction by shear stress is well established[57-59]. In this setting, NO is produced by NOS3 and the *Nos3* gene is induced by EDNRB activation in endothelial cells[49]. Insufficient EDNRB function exacerbates inward hypertophic vascular remodeling by low flow[60]. Taken together, these findings suggest that ET signaling via EDNRB could contribute to outward remodeling in response to high shear stress.

While osteoblasts and endothelial cells express all the genes necessary for autocrine ET1 signaling, in blood vessels there is a necessary balance between autocrine and paracrine ET1 signaling. Paracrine signaling contributes to the greater thickness and higher smooth muscle content of arterial *v* venous walls. Even though arterial and venous identities are established prior to the onset of circulation[61], mechanical signals reinforce and amplify those differences [53]. It is presently unknown whether specific mechanical environments lead to differential endothelial cell expression of *Edn1* and of *Ece1*. Should this prove to be the case, differential regulation of these genes by distinct mechanical environments could provide a mechanism by which wall stress and shear stress might lead to distinct adaptive responses.

BOX 2

The Skeletal Mechanostat and the WNT Pathway

The ability of bone to alter its size and shape in response to its habitual mechanical environment is well established. Overloading leads to an increase in long bone cross-sectional size, as was demonstrated in elite racquet sport athletes [62]. Conversely, underloading, as occurs in spaceflight, prolonged bed rest, or spinal cord injury leads to loss of skeletal mass [63-65]. The notion that bone mass is physiologically regulated has been formalized in the mechanostat model of bone modeling [66,67]. Briefly stated, the model holds that bone modeling (change in diameter and/or cross-sectional geometry) occurs to maintain mechanical strain (fractional change in length) within a narrow physiological range. Once such physiological equilibrium is reached, bone size and shape remains stable unless disturbed.

Widely accepted experimental interventions to allow study of defined skeletal loading conditions in experimental animals have been developed. Ulnar loading coupled with dynamic histomorphometry allows the *in vivo* modeling response to loading to be measured, using the contralateral, unloaded limb as a control [12]. Tail suspension [68] and sciatic neurectomy [69] both allow study of *in vivo* unloading. These powerful investigative tools have been used in genetically engineered mice to identify critical molecules mediating mechanotransduction.

The canonical WNT signaling pathway is one of the principal mechanisms by which bone responds to its mechanical environment. Recognition of its central role in bone biology emerged from the recognition that inactivating and activating mutations of *LRP5*, a WNT co-receptor, caused two rare Mendelian conditions, the osteoporosis pseudoglioma syndrome and hereditary high bone mass, respectively [70-72]. Mendelian high bone mass high bone mass disorders, sclerosteosis and Van Buchem's disease, arise as a consequence of mutations in *SOST*, the gene encoding the WNT antagonist sclerostin [73,74]. Overexpression of the WNT inhibitor DKK1 drives bone resorption in multiple myeloma[75].

While human disease provided the first clues that WNT signaling is critical in bone physiology, mechanistic understanding of bone mass regulation has been achieved through study of mouse models. Experiments featuring ulnar loading demonstrated that loss of function *Lrp5* (mouse homolog of *LRP5*) mutations lead to decreased load-induced modeling[76,77], while mutations that mimic human high bone mass variants display increased modeling in response to mechanical loading[77-79]. These modeling responses demonstrate that disruption of the WNT pathway affects physiology as well as development.

SOST is a WNT inhibitor and is produced constitutively by mature osteocytes, but its expression is decreased in the presence of mechanical loading[47]. Mice in which *Sost* (the mouse gene encoding SOST) has been knocked out display increased bone mass, mimicking the human sclerosteosis phenotype[80,81].

Furthermore, SNPs within or near genes involved in the WNT pathway are associated with mass and fractures in humans[37,82-85]. In the case of *LRP5*, there is evidence from the Framingham cohort that the association with BMD is exercise dependent[86]. Anti-SOST antibodies are presently being tested as possible drugs to increase bone mass and prevent fracture[87,88].

Acknowledgments

This work was supported in part by SPiRe Award #1I21 RX1440 from the United States Department of Veterans Affairs Rehabilitation Research and Development Service to Robert D. Blank. In addition, this material is the result of work supported with resources and the use of facilities at the Geriatrics Research, Education, and Clinical Center at the William S. Middleton Veterans Hospital in Madison, WI. The opinions expressed herein are those of the authors, and do not represent the views of the US government.

This work was supported in part by American Heart Association grant 15GRNT25700126 to Robert D. Blank.

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Key Points

- The endothelin system includes 3 small peptide hormones that are secreted as inactive precursors, a pair of G-protein coupled receptors, and a pair of membrane bound, extracellular converting enzymes.
- Endothelin 1/endothelin receptor A signaling is essential for the development of the craniofacial skeleton. Knockouts of the *Edn1*, *Ednra*, and *Ece1* genes have lethal phenotypes.
- Endothelin signaling is osteogenic in the setting of prostate and breast cancer.
- Genetic evidence points to allelic variation of *Ece1* as a mediator of bone biomechanical performance.
- *In vitro* experiments indicate that ET signaling derepresses WNT signaling, and thus may function upstream of WNT in mediating mechanical homeostasis of skeletal mass.

Synopsis

The endothelin (ET) system includes 3 small peptide hormones and a pair of G-protein coupled receptors. All 3 ETs are secreted as biologically inert precursors that must be activated by proteolytic cleavage after secretion. This reaction can be catalyzed by a pair of specific, membrane bound extracellular endothelin converting enzymes or by nonspecific tissue proteases. The ET1/EDNRA axis is essential in development, with knockout mice for either ET1 or EDNRA displaying a similar phenotype featuring multiple defects of the craniofacial skeleton and cardiac outflow tract. Prostate and breast cancers sometimes display osteoblastic metastases driven by high tumor cell expression of ET1. ET1-driven osteosclerosis may also occur in the setting of myelofibrosis. Osteoblasts express ET1, EDNRA, and ECE1, and therefore are capable of autocrine ET signaling. Searches for genes that mediate individual differences in bone biomechanical performance have identified the gene encoding ECE1 as a candidate. Mechanistic studies *in vitro* show that ET signaling in osteoblasts increases matrix synthesis and mineralization and derepresses WNT signaling, acting in part via the micro-RNA miR 126-3p.

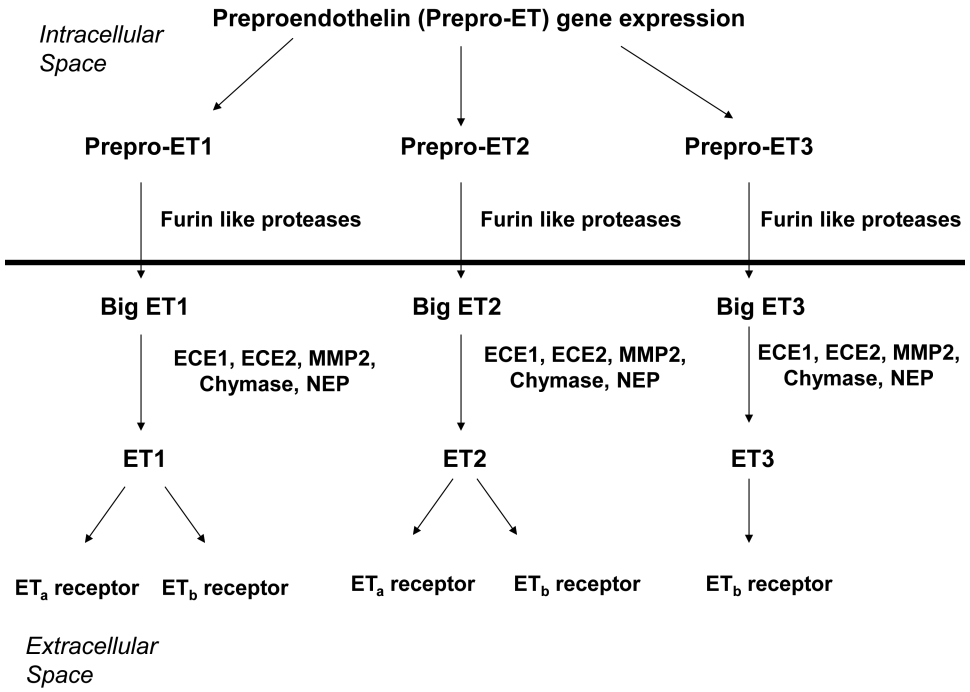

Figure 1. Schematic representation of endothelin synthesis, secretion, and receptor binding

The horizontal line represents the cell membrane, with events above the line occurring intracellularly and those below the line occurring extracellularly. Both the ET receptors and the ECEs are membrane bound, but their ligand binding/catalytic sites are extracellular. ET_A represents the A type endothelin receptor, encoded by *EDNRA* in humans and *Ednra* in mice. ET_b represents the B type endothelin receptor, encoded by *EDNRB* in humans and *Ednrb* in mice. All 3 endothelins are initially synthesized as pre-propeptides, encoded by *EDN1*, *EDN2*, and *EDN3* in humans and *Edn1*, *Edn2*, and *Edn3* in mice. They are processed to the respective big ETs by furin-like proteases prior to secretion. The big ETs are further processed to the mature, biologically active ETs by the ECEs or other extracellular proteases. B type receptors in endothelial cells promote NO synthesis, cell survival, and ET clearance. Both A type and B type receptors promote smooth muscle cell contraction and collagen synthesis by fibroblasts.

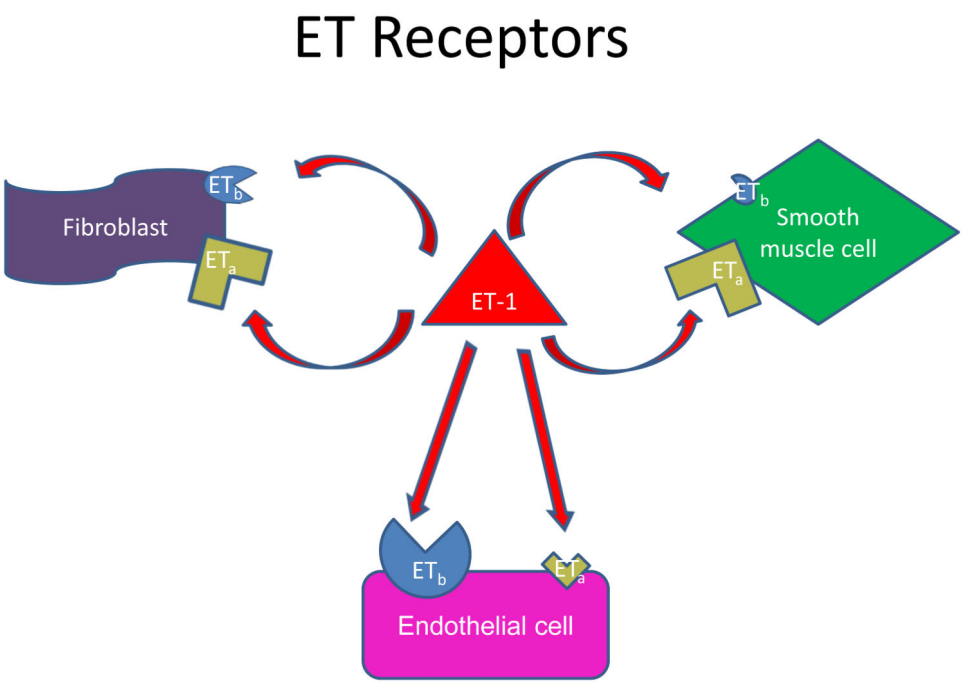

Figure 2. Schematic representation of autocrine and paracrine ET1 signaling

In blood vessels, the balance of autocrine and paracrine signaling is important in determining the biological response to ET signaling. Big ET1 is secreted by endothelial cells. It can be processed to mature ET1 by membrane-bound ECE1 by the endothelial cells and signal in an autocrine fashion via either A type or B type receptors on the endothelial cells. These cells express predominantly B type receptor, thus favoring vasodilatory responses. Alternatively, mature ET1 (21 amino acids) or big ET1 (38 amino acids) can diffuse in the extracellular space. Big ET1 can be activated by other tissue proteases and can signal via ET receptors located on smooth muscle cells, fibroblasts, or other cells present in the vessel wall or perivascular space. Activation of these cell types by ET1 promotes vasoconstriction and thickening/stiffening of the media. The activity of ECE1 can alter the balance of autocrine *v* paracrine ET1 signaling.

Table 1

Genes and Protein Abbreviations

	Protein	Human Gene	Mouse Gene
Endothelin 1	ET1	*EDN1*	*Edn1*
Endothelin 2	ET2	*EDN2*	*Edn2*
Endothelin 3	ET3	*EDN3*	*Edn3*
Endothelin A Type Receptor	EDNRA	*EDNRA*	*Ednra*
Endothelin B Type Receptor	EDNRB	*EDNRB*	*Ednrb*
Endothelin Converting Enzyme 1	ECE1	*ECE1*	*Ece1*
Endothelin Converting Enzyme 2	ECE2	*ECE2*	*Ece2*