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Review

# Regulation of C-type natriuretic peptide expression

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ABSTRACT

C-type natriuretic peptide (CNP) is a member of the small family of natriuretic peptides that also includes atrial natriuretic peptide (ANP) and brain, or B-type natriuretic peptide (BNP). Unlike them, it performs its major functions in an autocrine or paracrine manner. Those functions, mediated through binding to the membrane guanylyl cyclase natriuretic peptide receptor B (NPR-B), or by signaling through the non-enzyme natriuretic peptide receptor C (NPR-C), include the regulation of endochondral ossification, reproduction, nervous system development, and the maintenance of cardiovascular health. To date, the regulation of CNP gene expression has not received the attention that has been paid to regulation of the ANP and BNP genes. CNP expression *in vitro* is regulated by TGF-β and receptor tyrosine kinase growth factors in a cell/tissue-specific and sometimes species-specific manner. Expression of CNP *in vivo* is altered in diseased organs and tissues, including atherosclerotic vessels, and the myocardium of failing hearts. Analysis of the human CNP gene has led to the identification of a number of regulatory sites in the proximal promoter, including a GC-rich region approximately 50 base pairs downstream of the Tata box, and shown to be a binding site for several putative regulatory proteins, including transforming growth factor clone 22 domain 1 (TSC22D1) and a serine threonine kinase (STK16). The purpose of this review is to summarize the current literature on the regulation of CNP expression, emphasizing in particular the putative regulatory elements in the CNP gene and the potential DNA-binding proteins that associate with them.

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## 1. Introduction – natriuretic peptides

The natriuretic peptides, atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) and C-type natriuretic peptide (CNP) constitute a family of small (22–53 amino acid) polypeptides, each of which contains a highly conserved 17-member ring structure [47,59,69]. The founding member of the family, ANP, discovered in the rat atrium and found to possess profound natriuretic properties by de Bold in 1981 [19], is now known to play an important role in maintaining cardiovascular and fluid homeostasis [48]. BNP, synthesized primarily in the heart, not the brain, has similar actions. However, circulating levels of this peptide are quite low in the normal state and are only elevated (as much as 200–300-fold [69]) in chronic disease states, especially in diseases of the ventricular myocardium in which transcription of the BNP gene is reactivated [18].

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ANP is released into the circulation from atrial granules in response to both mechanical and hormonal signals generated by increased blood volume, and targets organs involved in fluid regulation to lower blood volume and blood pressure [50,59]. Much of the action of ANP is a result of its binding to one of a unique group of membrane-bound guanylyl cyclases that catalyze the formation of intracellular cGMP in target cells [30]. The two membrane-bound guanylyl cyclases that act as natriuretic peptide receptors are designated as NPR-A and NPR-B (natriuretic peptide receptors A and B, respectively). NPR-A is highly specific for both ANP and BNP; NPR-B is selective for binding to CNP. Downstream effects of ANP-induced intracellular cGMP are mediated by activation of several of the same protein kinases, phosphodiesterases, and channel proteins that transduce the actions of nitric oxide (NO) in target tissues [69]. Therefore, the natriuretic peptides have been shown to perform many of the same functions as NO in tissues where both signal molecules are expressed (e.g., in the vasculature). However, all three natriuretic peptides also bind with high affinity to a third receptor, NPR-C, which is not a membrane guanylyl cyclase, but a dimer without apparent enzymatic activity. Nonetheless, NPR-C is capable of coupling natriuretic peptide binding to downstream signaling pathways by interacting with G-proteins [4].

## 2. C-type natriuretic peptide

Two years after the discovery of its specific receptor, NPR-B [42,74,75], the third natriuretic peptide was identified as a product of porcine brain in 1990 [79] and following the alphabetical sequence established by the first two natriuretic peptides was designated simply as ‘C-type’ natriuretic peptide. Unlike ANP and BNP, both of which circulate at concentrations that are increased many-fold in response to hemodynamic alterations and cardiac diseases such as congestive heart failure, plasma levels of CNP are low, and show much smaller changes, or no change, with variations in blood pressure and in diseases of the cardiovascular system [20,69,83,96]. These findings have helped establish the major functions of CNP as being either paracrine or autocrine in nature, rather than endocrine. In keeping with its limited range of action, CNP expression is normally confined to those tissues and organs in which it exerts a regulatory role, principally the brain and pituitary [82], cardiovascular system [11,23,38,69,98], endochondral bone [102], and the male [100] and female [95,103] reproductive systems. CNP transcript and/or protein have also been identified in a number of tissues and organs where its functional role has yet to be defined [9,76,77,94]. In contrast with ANP and BNP, whose specific actions in the brain, kidney, cardiovascular system, and endocrine system are collectively responsible for maintaining fluid homeostasis, the specific functions of CNP do not appear to be linked to any overarching physiologic role. Instead, CNP exerts several important but unrelated actions in the body, including regulation of endochondral ossification, reproduction, nervous system function and development, and maintenance of cardiovascular health through regulation of smooth muscle and endothelial function.

Over the past decade, one of the most clinically evident functions of CNP has been recognized as its participation in the regulation of long bone growth [6,29,66,102]. First discovered as a critical factor in endochondral ossification in CNP- or NPR-B knockout mice exhibiting a phenotype of short stature and other skeletal abnormalities [17,101], the CNP signaling system has since been recognized as making an important contribution to the variation in human height [5,27]. On the one hand, the cause of acromesomelic dysplasia, type Maroteaux, a heritable condition associated with short stature has been definitively shown to involve a mutation in the NPR-B, the specific guanylate cyclase receptor for CNP [5]. Conversely, balanced translocations in chromosome 2 near the locus of the CNP gene (Nppc) resulting in excessive CNP production, may be one of the factors contributing to the markedly tall phenotype of certain Northern European populations [27].

Following the early finding of a role in regulating pituitary secretion [49,79], the CNP signaling system has since been identified as having an elemental role in nervous system proper, including the regulation of neuronal development and morphology in both the central [55], and peripheral nervous systems [41,73,105]. CNP also appears to intervene at several stages of the reproductive process, ranging from the maintenance of oocytes in meiotic arrest in the ovary [103], to the maintenance of normal erectile function in the male through its vasodilatory action on cavernosal blood vessels [92].

The mechanisms exploited by CNP to maintain erection [92], specifically the NO-like activation of a cGMP signaling cascade, are also used throughout the cardiovascular system to inhibit smooth muscle contractility [75,98]. In addition, CNP has been proposed to reduce vascular contractility using a novel mechanism of smooth muscle hyperpolarization mediated via NPR-C, rather than NPR-B [72,91].

Since CNP action is predominantly paracrine rather than endocrine, however, it has not been shown to have the same systemic effect as ANP in lowering blood pressure [48], and is likely to play a role in local (e.g. coronary circulation, or microcirculation) rather than systemic control of blood flow [3]. Another important protective action of CNP in the cardiovascular system is its inhibition of the processes leading to cardiac and vascular remodeling and fibrosis after injury [80]. This action of CNP has been widely supported by both *in vivo* [36,45] and *in vitro* [7] studies and involves the ability of CNP to inhibit myocyte hypertrophy (heart) or proliferation (smooth muscle), and to limit the ability of either tissue type to synthesize matrix proteins after tissue damage.

## 3. Hormonal and pathophysiological determinants of CNP gene expression

### 3.1. CNP regulation in the cardiovascular system

Unlike ANP, C-type natriuretic peptide is not stored in large secretory granules that respond to appropriate signals with a massive release of peptide [69]. Therefore, compared with other members of the natriuretic family, the regulation of CNP function is likely to be especially dependent on factors that regulate its transcription rather than its exocytosis. Furthermore, although CNP was first discovered in the mammalian brain, the early identification of CNP as product of endothelial cells coupled with the characterization of one of its receptors as a guanylyl cyclase [74] led to the concept that endothelial CNP performs some of the same functions as NO in the vasculature. Like NO, endothelial CNP leads to increased cGMP production in the underlying smooth muscle, and consequently to the activation of vasoprotective cGMP-dependent (e.g., protein kinase G) pathways downstream of that point [47]. In view of its potential importance as an NO-like factor, many of the initial studies of the hormonal and pathophysiologic alteration of CNP production and secretion were focused on the endothelium.

One of the first of these factors to be studied was TGF-β, an enhancer of CNP expression and secretion in endothelial cells [63,83,84] that has since been shown to have a positive effect on CNP transcript levels in rat [92] and human [52] vascular smooth muscle cells. The stimulatory effect of TGF-β on vascular CNP production would suggest that this key agent in vascular remodeling after injury could constitute part of a feedback loop limiting TGF-β-induced fibrosis through the actions of CNP [83].

Tumor necrosis factor-α (TNF-α) was found to be even more effective than TGF-β at increasing immunoreactive CNP in endothe-

lial cells [83], and interleukin-1 (IL-1) also had a smaller, though still significant effect on production of the peptide, suggesting that macrophage association with endothelial cells as occurs in an area of atheroma formation could result in increased production of the vaso-protective CNP. A positive effect of direct macrophage interaction with endothelial cells on CNP transcription and secretion was further supported in co-culture studies of these two cell types [85].

Several mechanisms regulating endothelial CNP expression may be specific to this tissue type due to its constant exposure to blood flow and plasma content. These include an up-regulation of CNP transcription by shear stress [16,104], and an increase in mRNA levels in human cells by the bioactive lipid, lysophosphatidylcholine, in a process independent of intracellular calcium flux [53]. Similarly, the observed suppression of endothelial CNP by vascular endothelial growth factor (VEGF) might be specific to those cells, such as endothelial cells, that express VEGF receptors in high concentration [26].

Another factor in plasma that has a positive effect on CNP transcript and protein levels in endothelial cells is ANP [58]. Acting through activation of NPR-A and subsequent cGMP signaling, ANP and BNP (whose effect was even greater than that of ANP) were observed to increase CNP secretion as much as 20-fold, and steady-state levels of CNP mRNA by 2–3-fold [58]. This observation invites speculation that the local effects of CNP in the vasculature are linked to blood pressure and plasma volume through the other two members of the natriuretic peptide family.

In addition to its regulation by TGF-β, CNP production in vascular smooth muscle cells has been shown to respond to treatment with several growth factors in the tyrosine-kinase receptor growth factor family. Some of these factors have exhibited remarkable variability in their effects on cardiovascular CNP expression in different species and tissue types. For example, in cultured human aortic smooth muscle cells (AoSMC), CNP mRNA levels were increased as much as 50-fold by platelet-derived growth factor BB (PDGF-BB) [51]. In rat-derived AoSMC however, CNP mRNA levels were *reduced* to only 7% of their control value in the presence of PDGF-BB [51]. Similarly, basic fibroblast growth factor (bFGF) was shown to suppress CNP transcript levels in cultured AoSMC derived from the rat [97], but it elevated CNP transcript levels in cultured smooth muscle cells derived from the human aorta [51]. On the other hand, CNP transcript levels responded positively to basic fibroblast bFGF in smooth muscle cells derived from human aorta, but showed no response to bFGF in human coronary-artery derived smooth muscle cells [52]. Studies such as these underscore the importance of cell phenotype and even species differences in delineating the intracellular transduction pathways controlling CNP production.

### 3.2. CNP regulation in cardiovascular disease

A number of reports have noted changes in CNP expression occurring in cardiovascular disease, some of which could reflect alterations in autocrine/paracrine regulatory factors accompanying disease progression. Naruko et al. [57] used immunohistochemistry to compare CNP expression in normal coronary artery (i.e., having only diffuse intimal thickening) with expression in coronary arteries possessing atherosclerotic lesions of increasing severity. Results showed CNP immunoreactivity confined mostly to endothelial cells in arteries possessing only diffuse intimal thickening, but in endothelium overlying more advanced (hypercellular) lesions and in the most advanced lesions (fibrous, fibro-lipid, and lipid-rich plaques), endothelial CNP positivity was greatly reduced. In contrast, immunoreactive CNP in smooth muscle cells increased with atherosclerotic progression through the hypercellular stage, but decreased in the most advanced lesions. This observation would suggest the existence of cell type-specific differences in the regulation of vascular CNP expression. Subsequently, in a rat model of neointima formation following balloon angioplasty, Brown et al. [8] noted that CNP is expressed in SMC that have invaded and proliferated in the neointima, but not in SMC in the adjacent media or in uninjured arteries. A study of CNP expression in human neointimal SMC following angioplasty [56] pointed to a similar, time-dependent up-regulation of CNP in the human SMC that was maintained for at least six months after surgical intervention. Casco et al. [11], using immunohistochemistry, identified CNP in both medial and intimal SMC of early through advanced atherosclerotic lesions, but the relationship of CNP to lesion stage was not described. CNP expression has also been studied in human aortic valves with increasing severity of stenosis, a condition that has many of the hallmarks of atherosclerosis, including early inflammation and later fibrosis and calcification [68]. The authors showed a significant (92%) decrease in CNP mRNA in stenotic aortic valves compared with valves from patients with aortic regurgitation, a milder form of the disease. Histologically, CNP expression was localized in valvular endothelial cells, myofibroblasts and stromal cells. Taken together, these studies indicate a shifting pattern of CNP expression during the progression of atherosclerotic, restenotic and valvular lesions, characterized by an overall reduction in endothelial CNP and an increase in CNP in other cell types, such as neointimal SMC. Such observations are consistent with a role for smooth muscle CNP in controlling fibrosis and SMC proliferation after vascular damage, possibly induced by the same cytokines (TGF-β, PDGF, FGF) that trigger neointimal growth following endothelial denudation.

In diseases of the myocardium such as heart failure, it has been clearly established that both ANP and BNP expression in the heart are markedly increased, and in the case of BNP, up-regulation of plasma BNP is of such magnitude that assays for this peptide are now routinely used to establish the presence and severity of congestive heart failure (CHF) [50,71]. In contrast, plasma levels of CNP or its amino terminal marker have not shown consistent elevations in human CHF, with some studies showing no difference between control and CHF [10,89,96], and others showing small, but significant increases in heart failure ranging from an ~50% increase [21] to a ~4-fold increase in heart failure of the highest clinical severity [24]. Plasma CNP levels have also been reported as significantly elevated (3.2-fold) in patients with cor pulmonale (right ventricular hypertrophy) [10], and in patients with left ventricular dysfunction irrespective of overt heart failure [22]. Moreover, a study that assessed specifically the myocardial production of CNP in heart failure patients by comparing arterial levels with levels in the coronary sinus, showed significant correlation of patient CNP level with pulmonary capillary wedge pressure [37]. Studies of CNP expression in experimentally induced heart failure in sheep [12] using trans-organ arteriovenous sampling (comparison of carotid artery CNP with CNP in various venous beds) found small, but significant increases in plasma CNP in animals with heart failure. In a study of heart failure in pigs [20], plasma CNP was increased about 2-fold by one week of pacing-induced heart failure, and left ventricular CNP mRNA was increased by ~3-fold over control but did not attain significance [20]. Changes in cardiac CNP expression have also been examined in diabetic cardiomyopathy in a genetic mouse model of the disease (ob/ob genotype). No difference was found in expression levels of cardiac CNP mRNA between the diabetic ob/ob mice and heterozygous controls, despite a greater than 10-fold difference in plasma insulin levels between the diabetic and non-diabetic groups [15]. However, NPR-B expression levels were significantly elevated in the diabetic model, suggesting that diabetes might enhance cardiac sensitivity to CNP despite causing no change in CNP expression.

The results of most of the aforementioned studies suggest that despite the recognized importance of CNP in reducing cardiac fibrosis in diseased hearts [80], myocardial CNP expression itself, unlike

ANP and BNP, is not greatly altered in response to cardiac disease. A possible explanation of these results is that cardiac CNP is not expressed primarily in myocytes, but in the smaller population of cardiac fibroblasts, whose overgrowth and extracellular matrix production are responsible for cardiac fibrosis in pathological states [34]. In support of this hypothesis, Horio et al. [34] in an *in vitro* study of the secretory response of these cardiac fibroblasts to several hormones and cytokines, found that fibroblasts, but not myocytes, expressed CNP mRNA and that they secreted significantly more CNP in the presence of TGF-β or bFGF than they did with control medium. It is also possible that heart-failure induced changes in CNP expression could reflect changes occurring primarily in endothelial or smooth muscle expression of the peptide, rather than in the cardiac myocyte.

### 3.3. CNP regulation in other tissues and organs

Regulation of CNP expression has also been studied in several other CNP target systems, where the observed responses could either reflect a general pathway for CNP gene regulation, or could be restricted to a specific tissue or cell type. In chondrocytes, a major cellular target of CNP involved in long bone growth, glucocorticoid (dexamethasone) was found to increase the expression of CNP mRNA almost 4-fold [2], suggesting the possibility of a CNP role in modulating the effects of glucocorticoid on endochondral ossification. Using plasma levels of amino-terminal pro CNP (NT-proCNP) as a biomarker for hormonal regulation of CNP in the cartilage growth plate, Olney et al. [65] showed evidence that the positive effects of growth hormone and testosterone on bone growth at puberty could be due in part to their up-regulation of CNP production in the growth plate at this time. Also using NT-proCNP as a plasma biomarker of CNP synthesis, Prickett et al. [70] showed that exogenous estradiol administered to female lambs and adult sheep significantly increased CNP expression compared with control animals. The source of the elevated CNP, while not definitively identified, was thought likely to be derived from osteoblasts in bone tissue [70]. In marked contrast to the finding of a positive relationship between testosterone and CNP in pre-pubertal boys [65], Prickett et al. observed no effect of testosterone administration on CNP in male lambs, a species difference in CNP regulation that the authors speculated could be related to the timing of testosterone intervention and the stage of pubertal maturation [70]. These studies point to the possibility that the influence of steroids on bone growth could be mediated, at least in part, through their regulation of CNP synthesis.

Gonadal steroids have also been proposed in the regulation of CNP expression in the female reproductive system, where it was shown that uterine CNP expression in mice varies during the estrus cycle [35], and that pregnancy increases uterine CNP mRNA up to 7-fold [81]. Furthermore, uterine CNP and CNP transcription were observed to increase with the administration of exogenous estradiol to ovariectomized mice [1]. A steroid hormone-responsive consensus sequence has not yet been identified in the CNP promoter [70,89], suggesting that the observed effects of steroids on CNP expression could be secondary to their effects on the transcription of other proteins.

Another potentially important regulator of CNP expression as judged by the magnitude of its effect is bacterial lipopolysaccharide (LPS), which increased CNP mRNA up to 300-fold, and immunoreactive CNP secretion 10-fold in mouse macrophages [93]. Phorbol ester and dexamethasone, in contrast, had no effect. Kubo et al. [43], studying human macrophages in culture, however, found only a modest increase of CNP (25%) in one macrophage cell line, and no change in another cell line after LPS treatment. Similarly, LPS had no effect on CNP secretion from human granulocytes, lymphocytes, monocytes or monocyte-derived macrophages in culture. Also in stark contrast to the mouse macrophages [93], phorbol-ester treatment increased CNP secretion as high as 20-fold in a human macrophage-like leukemia cell line. Whether the contrasting responses of mouse and human leukocyte-derived cells to LPS and to phorbol esters owes more to differences in phenotype or to species differences is a matter for further investigation. However if the manner of CNP regulation in a given cell type is shown to vary between species, it is conceivable that the difference may reside in the structure of the CNP gene itself, especially in the sequence of its 5′ promoter. This region has been examined in several studies, described below.

## 4. CNP gene

The gene encoding human C-type natriuretic peptide (Nppc) was sequenced shortly after the discovery of the peptide [61,87]. Like the genes for ANP and BNP, Nppc has been shown to be composed of three exons, the first two encoding a 126 amino acid pre–pro peptide that contains the two active forms of CNP; a 22-amino acid, and a 53-residue form designated as CNP-22 and CNP-53, respectively. Unlike ANP and BNP, neither form of CNP possesses a carboxy-terminal extension [69]. A third peptide, NT-pro-CNP, derived from the N-terminal of the pre–pro peptide has no known function, but circulating at higher levels than either CNP-22 or CNP-53, it serves as a convenient serum marker of changes in active CNP in response to endocrine alterations and disease [12,25,65]. The proximal 5′-flanking region of the gene was found to contain two GC-boxes (Sp-1 binding sites) in tandem in addition to several other *cis* elements including an inverted CCAAT box (Y box), and a CRE-like (cAMP response-like) element within 50 bp upstream of a Tata box (TATAAA) [62,63,90]. These sequences have been hypothesized to constitute potential targets in the regulation of CNP expression, and are not present in the promoter regions of either the ANP or BNP genes [87].

A detailed functional analysis of the proximal promoter region in the human CNP gene [62] extending to approximately 1400 bp upstream of the transcription start site, demonstrated the existence of a positive regulatory region between positions −54 and −19, a sequence encompassing the tandem GC-boxes. In addition, at least two negative regulatory regions were identified much further upstream. Mutation analysis showed furthermore that 90% of the positive regulatory activity for CNP expression resides in the GC-rich region containing the two GC-boxes. Ogawa et al. [60] then compared the human CNP gene with its mouse equivalent, and found both some interesting similarities and differences between the two. The 5′-flanking regions through about 130 bp upstream of the transcription start site of both CNP genes were shown to have several putative *cis*-acting regulatory elements in common, including a Y-box, a putative CRE-like sequence, and a GC box [60]. However, the human promoter lacks several of the regulatory elements found in the mouse promoter, including a long CA repeat region further upstream and a nuclear transcription factor κβ (NF-κβ) recognition site present in the mouse. This last observation is particularly interesting in light of the reported mouse vs. human differences in CNP response to LPS since the bacterial protein is known to signal through the NF-κβ pathway [95]. In addition, although both species possess at least one GC box in the promoter, the human CNP promoter possesses two GC boxes in tandem to constitute the ‘GC-rich’ DNA-binding site as described above [62]. This could represent a crucial difference in CNP transcriptional regulation between rodent and human. As an example, it has been shown [40] that a Kruppel-like zinc finger transcription factor (Zf9) binds to two tandem GC-boxes in the TGF-β1 promoter with high affinity to up-regulate TGF-β transcription, but to a single GC box with much lower affinity. In addition, Kf9 selectively activates genes

with tandem GC-boxes, but represses genes with a single GC box. It therefore appears possible that either Kf9, or another DNA-binding protein specific for tandem GC could selectively activate the human CNP gene, and repress the rodent gene. These human-rodent differences together with the presence or absence of putative regulatory elements in both the single intron and the 3′-untranslated region identified in one species or the other, could account for some of the species-specific regulation of the CNP gene that has been observed.

## 5. Putative transcription factors

Based on the similarity of specific sequences in the CNP proximal promoter to consensus sequences for the ubiquitous transcription factor Sp-1 and CRE binding protein (CREB), these two transcription factors were among the first to be examined as potential regulators of CNP transcription. Ohta et al. [62] found, however, that neither the Sp-1 nor the CREB consensus binding sequence was able to compete effectively with the GC-rich region of human Nppc for binding of nuclear protein. The consensus sequence for Ap-2 was similarly without effect. Furthermore, a 70 kD putative regulatory protein, identified by its binding to the GC-rich region, did not match the MWs for either Sp-1, CREB or Ap-2 [62]. On the contrary, Thompson et al. [88] have very recently shown evidence using electrophoretic mobility shift assays (EMSA) that both Sp-1 and Sp-3 bind as a complex to the putative GC-rich regulatory site in the proximal Nppc promoter. It might be worth noting that although both studies employed nuclear extract from pituitary cell lines or the pituitary proper, the CNP gene examined in the Ohta et al. study was human, but the gene in the Thompson et al. study was from the mouse [88].

Ohta et al. [63] continued the search for CNP transcription factors using Southwestern screening of a rat pituitary cell-derived library for the ability to bind the GC-rich sequence in the human CNP promoter. Their results identified TSC22D1 (*t*ransforming growth factor β *c*lone, *d*omain *1*), the founding member of a small family of evolutionarily conserved leucine zipper proteins that perform a diverse array of functions in mammalian cells [28,31] as a protein that binds to the human CNP promoter. Although initially cloned using binding to a GC-rich element in the CNP promoter as a selection criterion [63] and despite its leucine zipper-like motif, TSC22D1 lacks the conventional DNA-binding domain of the leucine zipper transcription factors [78]. Therefore its proposed role as a direct enhancer of CNP transcription has remained in question. Recent studies in our laboratory using siRNA gene silencing techniques have shown that TSC22D1-silencing significantly reduces both PDGF-up-regulated CNP transcript in human aortic SMC and TGF-β-up-regulated transcript in coronary artery-derived SMC [52]. These results suggest that in human vascular smooth muscle cells, TSC22D1 expression and activity could represent a critical element in a final common pathway leading to increased CNP expression induced by different stimuli.

Whether TSC22D1 binds directly to promoter DNA, or is instead part of an active regulatory complex remains to be resolved. Several studies support the latter possibility. In a study of the induction of erythroid differentiation, evidence was presented suggesting that TSC22D1 can bind to and modulate the transcriptional activity of two proteins, Smad 3 and Smad 4 that are important in TGF-β signal transduction [14]. Another possible mechanism by which TSC22D1 could regulate gene transcription is its formation of homo- and heterodimers with other members of the TSC22 domain family to form complexes with different transcriptional activity compared with TSC22D1 acting alone [39]. Determination of a specific mechanism of action of TSC22D1 in regulating CNP transcription is further complicated by the existence of TSC22D1 splice variants that result in the formation of proteins of very different MW (18 kD vs. >100 kD) [28]. Recent studies [31,99] suggest that these protein variants could perform unrelated, or even antagonistic functions in the mammalian cell; e.g., although the short form of TSC22D1 has been described many times as a tumor suppressor, the long form in contrast promotes proliferation, growth and cell survival [99]. Interestingly, in our recent studies of the effects of TSC22D1 silencing on CNP expression in vascular smooth muscle cells, [52], we could only identify a long form of the protein (∼120 kD) in Western blots, yet the downregulation of this protein with TSC22D1 siRNA resulted in reduced production of CNP transcript in these cells.

Additional cellular functions of TSC22D1 that have been recently posited include the initiation of apoptosis [46] and the induction of contact inhibition [44], both of which functions may contribute to its postulated role as a tumor suppressor [33]. TSC22D1 also remains a potentially significant factor in cardiovascular fibrosis, since it has been identified as one of only four transcription-related genes that were up-regulated in a pacing-induced model of atrial fibrillation, a condition that can result in cardiac fibrosis [13].

Using the same methodology that they had used earlier to clone transcription factors capable of binding the GC-rich element of the CNP promoter, Ohta et al. [64] identified a second putative CNP activator as a novel protein with both DNA-binding ability and serine-threonine kinase (STK) activity. This protein, designated as TSF-1 by its discoverers and later as STK16, appears to be localized to the Golgi apparatus, but can translocate to the nucleus and modulate the activation of the VEGF gene [32]. It is noteworthy that the VEGF gene possesses a GC-rich putative regulatory sequence in its proximal promoter that is very similar to the GC-rich sequence in the CNP promoter that has been proposed as a binding site for both STK16 [64] and TSC22D1. Aside from this initial report [64], there have been no further studies of STK16 as a transcriptional regulator of CNP. However, a recent report of CNP overexpression and long bone overgrowth in patients with chromosomal translocations in a region (2q37) in the vicinity of the STK16 gene raises the possibility that this CNP-induced overgrowth could be associated with a dysregulation of STK16 expression [54].

More recently, the Wnt4/β-catenin system has been proposed as a transcriptional regulator of CNP in rodent kidney, an organ in which induction of Wnt-4 expression is critical in genitourinary development, and is re-induced following unilateral uretal ligation [86]. The authors of this report noted that cells expressing Wnt-4 in the obstructed kidney also expressed CNP, and hypothesized that increased Wnt-4 protein acting through β-catenin and DNA-binding proteins of the T cell factor/lymphoid enhancer binding factor (TCF/LEF) family activated CNP transcription. Their argument was supported by studies showing that six hypothetical TCF/LEF binding sites are present in the mouse CNP proximal promoter, and that two of these sites are crucial for the activation of a CNP transgene by the Wnt-4 signaling pathway. This study is interesting in showing that sites other than the GC-rich region of the CNP promoter can play an important role in regulating CNP expression. Whether this hypothetical Wnt/B-catenin/CNP pathway is specific for renal tissue, and whether it functions in the human as well as the rodent kidney remains to be determined.

Another possible CNP transcription factor, one specifically involved in the flow-dependent regulation of gene expression in human endothelial cells, has been identified as Kruppel-like factor 2 (KLF-2) [67]. Blockade of flow up-regulated KLF-2 expression significantly reduced the expression of several flow-regulated genes, including CNP, suggesting that KLF-2 could potentially mediate the flow-mediated increases in CNP production *in vivo* that have been described [16,104]. Whether KLF-2 bears any affinity to KLF-9 in binding to a tandem GC-box in preference to a single GC-box has yet to be determined.

## 6. Conclusion

Twenty years after the discovery of CNP and its subsequent identification as a paracrine/autocrine-acting member of the natriuretic peptide family in the cardiovascular and other organ systems, the regulation of CNP transcription remains far from being completely understood. It therefore constitutes a fruitful area for investigation of new therapies targeting CNP production in the treatment of disease. For example, up-regulated CNP acting through cGMP could fulfill vasodilatory and antifibrotic functions in the vasculture very similar to NO, but without incurring the deleterious effects of NO oxidation products. Similarly, diseases of bone growth can potentially be treated by novel means of CNP up-regulation that circumvent the risks of more commonly used hormone replacement therapies.

Studies to date have identified a number of potential regulator sequences in the CNP gene and a handful of transcriptional regulators such as TSC22D1 that may be involved in CNP regulation at the level of its 5′ promoter, but crucial details of the regulatory pathways that lead to suppression of or up-regulation of CNP transcription remain unknown. Understanding how those transduction pathways lead to increased CNP transcription in tissues and organs of clinical relevance is a subject worthy of further investigation.

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