

The Neural Cell Adhesion Molecule and Heart Development: What is NCAM Doing in the Heart?

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Abstract

The neural cell adhesion molecule (NCAM) may be as important in the heart as it is in the nervous system. NCAM is a cell surface glycoprotein which manifests great diversity in polypeptide isoform and glycosylation throughout cardiogenesis as well as neurogenesis. It is expressed on cardiac myocytes in the myocardium and on epicardial and endocardial cells as well as on the components that innervate the heart. The patterns of its expression suggest that it could be modulating a number of events including cardiomyocyte interactions from initial epithelialization and tubular heart stages, interactions of the developing His-Purkinje fiber myocytes, endocardial mesenchymal transformation, and neural crest cell migration into the heart. Approaches that can be pursued to support these hypotheses include taking advantage of transgenic mouse lines and utilizing the information available about NCAM function from studies of the nervous system and skeletal muscle. The challenge still remains to determine why a neural adhesion molecule such as NCAM would be needed in such abundance and with such a widespread expression in the developing, adult, and diseased heart.

Key words: cardiogenesis, cardiomyocytes, polysialic acid, cell-cell interactions, N-CAM.

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The neural cell adhesion molecule (NCAM), as the name implies, is an important component of the developing and adult nervous system of vertebrate species (recently reviewed in [102, 118]). It is a cell membrane-associated glycoprotein which serves as a homophilic [100] and a heterophilic ligand [19-21]. It is also present in many if not all other tissues at least some time during development [24, 70, 113]. The expression of NCAM in the developing and adult heart tissue was reported many years ago [113]. The high levels of staining for NCAM in the myocardium, which at times appears to be comparable to that found in the nervous system, suggest a role for NCAM in the heart. Its function in the nervous system has been extensively studied, but the functional significance of NCAM in the heart is still unknown. Nonetheless, the regulated and specific patterns of NCAM expression suggest intriguing functions for this molecule in all three layers of the developing heart, the epicardium, the myocardium, and the endocardium [12, 26, 49, 69]. It is the purpose of this review to briefly describe what is

known about NCAM and its expression in the heart and discuss hypotheses to test in the future.

The Many Forms of NCAM

NCAM is a member of a large family of proteins with immunoglobulin (Ig)-like domains. Some of NCAM's aliases are N-CAM, BSP-2, D2, CD56, NKH1, and Leu-19. The NCAM protein has five repeated Ig-like domains exposed on the extracellular surface (Figure 1, labelled with Roman numerals). The heterophilic heparin-binding site and the homophilic NCAM-binding site are within the second and third Ig-like domains [20, 27, 46, 94]. The function-modulating polysialic acid (PSA) moieties attach to the fifth domain [25, 83]. Multiple NCAM isoforms occur by alternative splicing of the RNA from a single gene [80] which is located on chromosome 9 in chickens [89], chromosome 9 in mice [28], and chromosome 11 (11q23-q24) in humans [84]. In frog (*Xenopus*), two genes for NCAM have been discovered, NCAM gene 1 and NCAM gene 2 [116], which differ in amino acid sequence by 8%. The expression patterns of these two frog NCAMs suggest

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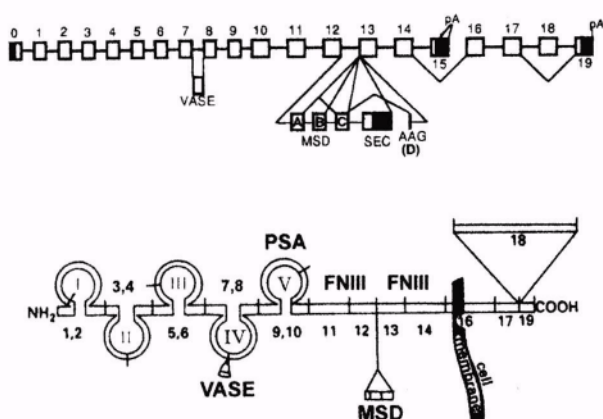


Figure 1. There are many forms of NCAM. The diagram at the top represents the NCAM gene exons (numbered) with lines indicating potential splicing choices. The diagram at the bottom illustrates the NCAM transmembrane protein isoforms with splicing sites. The Ig-like repeats are indicated by loops numbered using Roman numerals. The exons in the MSD are indicated by A.B.C. and D. FN = region of fibronectin type III repeats, MSD = muscle specific domain, SEC -the exon added to the secretory form, VASE = variable alternatively spliced exon, PSA = polysialic acid. Adapted from [7, 45, 81, 117].

that they are similarly regulated and serve similar functions to each other.

NCAM, like other cell adhesion molecules LI and N-cadherin, has been linked to signal transduction events (recently reviewed in [118, 119]). Stimulation of NCAM using antibodies or NCAM peptides initiates signal transduction events involving G-proteins, Ca^{++} fluxes, and the basic FGF receptor which are linked to pathways of other adhesion molecules [32, 37-39, 105, 118, 119]. The study of the function of this molecule and other adhesion molecules requires a consideration of what is happening outside and inside the cell as well as at the cell surface.

A detailed analysis of where and when NCAM is expressed is obviously important in crystallizing hypotheses for NCAM function in the heart. Deduction of functional state based on detecting the presence of the NCAM protein by a polyclonal antibody to the whole molecule is not enough. The levels of NCAM on the surface may have a nonlinear relationship to its function [35] and there are a number of NCAM isoforms arising from alternative splicing which can vary in functional capacity. In addition to differences in polypeptide sequence, NCAM varies in glycosylation state. The most studied of the carbohydrate moieties which attach to NCAM are the function-modulating carbohydrate polysialic acid (PSA) and the HNK-1 antigen. Another point for consideration is that the post-translational modification of phosphorylation and

sulfation sites could also be of significance to the function of NCAM [77, 110]. Thus, a thorough analysis of NCAM expression would necessitate identification of isoforms, glycosylation status, phosphorylation and sulfation state, and the amount of NCAM present on cells. Should all this information be collected, an important component would still be missing, functional significance of the different forms in vivo. At present, strong experimental evidence exists for the significance of PSA-NCAM function in biological systems while the significance in vivo of the isoforms and other post-translational modifications are less well supported.

NCAM Polypeptide Isoforms (Figure 1)

As many as 29 NCAM isoforms have been detected in the rat heart by RT-PCR [95]. While it is unlikely that all these mRNAs are made, stable, and translated to make substantial amounts of protein, this finding illustrates the great potential for alternative RNA splicing of NCAM. Analysis of the genomic structure of NCAM reveals that theoretically 100 isoforms are possible with alternative splicing [117]. The major NCAM isoforms detected in the tissues of the nervous system are referred to by their apparent desialylated molecular weights as detected by gel electrophoresis, 180 kDa, 140 kDa, 120 kDa, and 105 kDa. These four major brain isoforms differ in their carboxy terminal domains. The NCAM-180 and NCAM-140 both cross the plasma membrane once and extend into the cytoplasm with the NCAM-180 carrying an extra 261 amino acid sequence in the cytoplasmic region derived from a single exon. The NCAM-120 is linked to the membrane by glycosylphosphatidylinositol [55] and the NCAM-105 is the secreted isoform [53].

Muscle cells, including cardiac myocytes, express NCAM isoforms containing a Muscle Specific Domain (MSD) that can be inserted among the fibronectin repeats found between the 5th Ig-like domain and the transmembrane domain. The MSD can be inserted into any of the major forms to produce NCAMs of slightly higher molecular weight isoforms, for example NCAM-125, NCAM-145, or NCAM-185 [9, 13, 30]. An additional complication is that the MSD is encoded by 4 exons which can be spliced in various combinations [92, 95, 106, 108, 114, 128]. With the small size of these exons (as small as three nucleotides), some of the splice variations would not be detectable by changes in molecular weight on protein separation gels.

The MSD domain has an O-glycosylation site at a serine or threonine [117] with the attached carbohydrates (GalBI-3GalNAc) recognized by peanut lectin and jacalin lectin but only after deglycosylation (by exoneuraminidase treatment) of the NCAM. Peanut lectin has been used to identify O-linked carbohydrates on other membrane bound molecules [98] and appears to recognize no other site of glycosylation on NCAM.

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This lectin has therefore been used to identify or enrich for NCAM isoforms or fragments containing MSD [13, 120]. Analysis of NCAM sequences within the MSD region from different species suggests that the small 3 base exon AAG is important because it is conserved across *Xenopus*, mammals, and chickens [128]. The addition of the AAG exon could result in substitution of a Gln-Gly for an Arg residue which may cause conformational changes in the polypeptide. The rest of the MSD region varies in size and amino acid sequence across species. In fact, the MSD nucleotide and protein sequence excluding the AAG exon diverges the most across species when compared to other regions of NCAM. It has been proposed [128] that only a few residues are important to carry the O-glycosylation. In support of this notion, all species studied so far have MSD sequences with serine and threonine residues that could serve as sites for O-glycosylation. The stability of NCAM may be affected by O-glycosylation as demonstrated for other proteins. For example, there is an increased turnover of the LDL receptor when the O-glycosylation is absent [65]. The MSD is rich in proline, serine, and threonine and has homology with the hinge region of Igs [30, 120]. It has been hypothesized that this hinge allows NCAM isoforms with MSD to bend [92, 114] and that this conformational change may have functional significance. The MSD-containing NCAM isoforms appear to be well-represented in muscle cells including skeletal and cardiac myocytes but not readily detectable in brain tissue [92].

A large proportion of the NCAM mRNAs expressed in the developing and adult heart contain the small VASE (variable alternatively spliced exon) [109] or n [106] sequence, a highly conserved 30-base sequence which inserts into the fourth extracellular domain of NCAM. Its insertion into NCAM is difficult to detect by analysis of differences in molecular weight of NCAM by the immunoblot technique. The encoded protein sequence is identical in 9 out of 10 amino acids between *Xenopus* and mammals [128] and its insertion changes the fourth domain so that it becomes more similar in structure to the variable region rather than the constant region of immunoglobulins [109]. It has been associated with a loss in neural plasticity based on expression pattern and findings from *in vitro* studies. The VASE-inserted NCAM mRNAs increase in proportion compared to total NCAM mRNAs at later stages of neurogenesis and remain high in the adult except in the olfactory bulb and epithelium which retain a measure of synaptic plasticity in the adult [108, 109]. Insertion of this small domain reduces the ability of NCAM-140 to support neurite outgrowth *in vitro* [36, 39].

The complex expression pattern of the various NCAM forms suggests a complex regulation system at various levels, transcription, splicing, translation, and post-

translation. Some progress has been made in analyzing the transcriptional regulation of NCAM [40, 41]. A small region (70 bases) of NCAM promoter is conserved across species (mouse, *Xenopus*, human) [66] which contains the OZ enhancer element and N-box silencer element. The B-box can silence OZ and OZ is bound by OZ-1 [88]. NCAM expression is also correlated with Hox gene expression [16, 18, 72] and can be regulated by Hox genes *in vitro* [59].

Functional Significance of the Polypeptide Isoforms?

The experimental evidence to support the functional roles for particular NCAM isoforms is limited to those obtained from *in vitro* models. The major NCAM isoforms present in the nervous system, NCAM-180, NCAM-140, and NCAM-120, appear to be equivalent in their homophilic binding capacity *in vitro*. When each are individually transfected into cell lines, they all initiated cell aggregation [90]. Such transfected cell lines also served as a substrate for neurite outgrowth from dorsal root ganglion cells [33-35, 39]. Transmembrane forms NCAM-140 and NCAM-180 enhanced neurite outgrowth as did the Pi-linked forms with and without the MSD region (NCAM-125 and NCAM-120), while the secreted form (NCAM-105) did not. The VASE-inserted NCAM forms were reduced in their ability to promote neurite outgrowth compared to VASE-minus NCAM [39].

PSA-NCAM

As if alternative splicing of the protein portion of NCAM is not enough, different glycosylation flavors of NCAM are available as well. NCAM can sport the HNK-1 epitope, O-linked glycosylation when it has the MSD, and various levels of polysialic acid.

NCAM amino acid sequence reveals a number of potential asparagine-linked (N-linked) glycosylation sites [8, 9, 27]. Much attention has been focussed on the polysialic acid (PSA) moiety which specifically binds to the fifth Ig-like domain and has a profound effect on NCAM homophilic binding [56, 103]. PSA also has effects even in the absence of NCAM homophilic binding on the function of other cell-surface ligands including laminin-binding receptors [3] and even artificially induced cell-cell contact mediated by the lectin WGA (wheat germ agglutinin) [2]. These findings and others support a model for PSA-NCAM (polysialylated neural cell adhesion molecule) function in which it serves to reduce rather than enhance adhesion [99, 102]. The negatively-charged, highly hydrated PSA-NCAM has properties consistent with it occupying a large volume [126]. These properties of PSA combined with the abundance of its carrier NCAM on plasma membranes could account for its effectiveness as a barrier hindering the ability of cell

plasma membranes to appose and reducing cell-cell interactions of many types. PSA can have its effect even in the absence of NCAM homophilic binding and may hinder heterophilic binding across two membranes or between the membrane and the extracellular matrix (trans-interactions). Another possibility is that PSA-NCAM may also alter the ability of molecules within the same plasma membrane to interact (cis-interactions).

The studies of the PSA on NCAM has been advanced by the use of two sets of reagents, antibodies that bind specifically to the polysialic acid moiety [e.g., 735 [47], 5A5 [31]] and endoneuraminidases [44, 54, 103] which cleave long linear chains of polysialic acid on NCAM under physiological conditions. This has allowed study of the effect of PSA removal in complex *in vivo* as well as *in vitro* systems. Removal of PSA by mutation or by the specific enzyme endo-N results in abnormalities of innervation patterns within selected PSA-positive regions of the nervous system such as the hindlimb [71, 101] and tectum [127]. Defects in olfactory bulb development in the NCAM-180 null transgenic mouse line were found to be the result of the failure of olfactory neurons to migrate in the absence of the normal amount of PSA-NCAM [58, 87, 115]. Abnormalities in behavior, such as circadian activity rhythms, have also been detected after PSA removal by mutation or enzyme injection [107].

Polysialyltransferases have recently been cloned which have been shown to add polysialic acid to NCAM transfected into cultured cells [6, 82]. This breakthrough would be important in understanding the regulation of PSA addition to NCAM and may also allow manipulation of the polysialylation state *in vivo*.

HNK-1

The HNK-1 carbohydrate moiety is found on a number of adhesion and extracellular matrix molecules [68]. In heart extracts, a number of protein bands stain with HNK-1 and have molecular weights in the range of NCAM [75]. Although the HNK-1 epitope has not yet been shown to be present on heart NCAM, it is considered here because its expression patterns suggest intriguing roles for the HNK-1 epitope in heart development. Subsets of NCAM can carry the sulfated carbohydrate moiety [68] recognized by HNK-1 (a monoclonal antibody against human natural killer cell antigen 1 [1]). HNK-1 is well known in developmental biology circles for marking chick neural crest cells as they distinguish themselves and migrate away from the neural tube [10, 11]. Furthermore, addition of the HNK-1 antibody during embryogenesis causes aberrant migration of neural crest cells [10], suggesting a role for this epitope in cell-extracellular matrix interactions. In heart, the HNK-1 carbohydrate has an interesting distribution, to be discussed later, which is not restricted to neural crest cells or developing nervous tissue. The

transferases responsible for HNK-1 epitope addition have been cloned [61, 85, 112] and this development may finally allow manipulation of its expression and determination of its function.

Expression Patterns in the Heart

In the first immunohistological studies, polyclonal antibodies that detect all isoforms and probably all glycosylation forms of NCAM were used. NCAM was detected on cells of the precardiac mesenchyme at the end of gastrulation, in the cardiac splanchnopleure, and in cardiomyocytes at stages 11+, 18, and 23 chicken embryos using polyclonal antibodies to NCAM [113]. Subsequent studies have documented its presence throughout heart development as early as precardiac stages in chicken [73] and in embryonic, fetal, and adult stages in frog, chicken, rat [48], mouse [93], and human [5, 51]. In addition to cardiomyocytes, endocardial cells [26, 49] and epicardial cells [69] also express NCAM. As expected, neuronal tissues that invade and innervate the heart also express NCAM [51, 69, 74, 123].

NCAM is often co-localized with the calcium dependent neural cell adhesion molecule N-cadherin. This relationship exists in heart tissue during development. The subcellular localization of these two adhesion molecules however differs. At stages 5-8 in chicken heart development, the somatic and splanchnic mesoderm separate and form a coelom and the cardiac mesoderm forms an epithelium. NCAM is detected by the polyclonal antibodies all over the surfaces of anterior bilateral mesoderm cells and becomes restricted by stages 6 and 7 to the basolateral surfaces with Na⁺,K⁺-ATPase during epithelialization. NCAM is not present on apical surfaces exposed to the coelom nor is it localized to the apical-lateral junctional complexes where N-cadherin and catenins concentrate [73]. At stage 8 when the epithelialization has progressed, the mesenchyme from which the endocardium arises is still NCAM-positive. This expression pattern suggests that there is a potential for NCAM to play a role on the basolateral surfaces of the myocardial epithelium and in mesenchymal-epithelial transformation of the endocardium. The NCAM isoform and PSA-NCAM expression have not been studied in detail at these early stages.

In chicken hearts of later stages [12], heart NCAM expression was studied using immunoperoxidase staining using a monoclonal antibody 5E that binds to an extracellular epitope of the NCAM polypeptide common to all forms [46, 121]. NCAM was detected in the myocardium at all embryonic stages between 12-46 with a gradual decrease in intensity of staining at later stages of development. The trabeculae were more intensely stained than the mural myocardium in the ventricles from the time that trabeculae could be detected at stage 16 to post-septation stages.

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Similar patterns have been observed in the rat heart [124] and human heart [51], with a decline in NCAM protein expression noted postnatally.

In contrast to the fairly homogeneous and ubiquitous presence of NCAM throughout the heart myocardium during cardiogenesis, PSA-NCAM has a very restricted expression pattern after tubular heart stages [14, 15, 69, 123]. In the looped heart, PSA-NCAM is expressed only on at the myocardium of the atrioventricular junction (AVJ, Figure 2), the OFT (outflow tract), and the lumen of the primitive ventricle on the edges of the primitive trabeculae where the His-Purkinje system (HPS) tissue is thought to be located. The AVJ and the OFT are both sites of slow conduction in the early embryo [29] and undergo major changes in morphogenesis during septation. Whether PSA-NCAM is involved in either property of the tissues is speculative. At late septation stages, when the heart achieves its four-chambered structure, the PSA-NCAM is located on portions of the HPS (See Figure 3) [15, 123].

NCAM is present in the endocardium at low levels even in the tubular heart [12] and at higher levels in discrete sites during cardiac septation where the endocardium swells into the lumen. These regions include the outflow tract, atrioventricular junction, and leading edge of the atrial septum [12, 49]. This swelling of tissue occurs primarily by the enlargement of the extracellular matrix region called the cardiac jelly followed by the epithelial-mesenchymal transformation of selected endocardial cells which invade and seed this matrix [78]. During this process, changes in NCAM level, distribution, and form occur. PSA-NCAM, the embryonic or "plastic" form of NCAM, appears all over the surface of endocardial cells as they begin

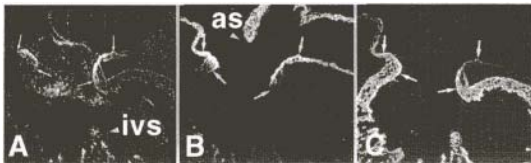


Figure 2. The myocardium of the atrioventricular junction immunostains for PSA-NCAM. Alternate 20- μ m thick frozen frontal sections of stage 26 chicken embryo hearts were immuno-fluorescently double-stained using the indirect antibody technique for PSA (5A5; Panel A) and myosin isoforms [anti-atrial (in Panel B) and anti-ventricular (in Panel C) myosins, [29, 104]]. The myocardium at the AVJ (between arrows) was specifically positive for both PSA and co-expression of myosins while the flanking ventricular and atrial myocardium was not.

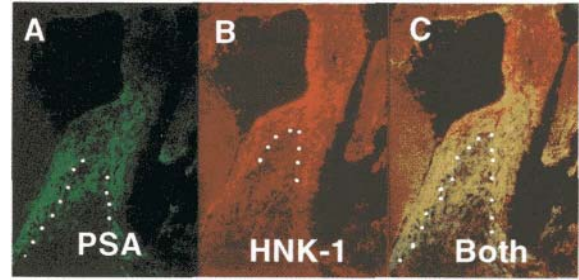


Figure 3. PSA-NCAM and HNK-1 epitope are expressed in a complementary pattern in the His Purkinje System (HPS) of the embryonic chicken heart. The location of the carbohydrate markers for the HPS, PSA-NCAM and HNK-1 epitope, were immunofluorescently detected in the same frozen section using a modification of the indirect antibody double-labelling technique. The frozen sections were incubated sequentially with the first antibody (5A5, anti-PSA), and the Fab fragment of anti-mouse IgM conjugated to FITC, the second antibody (HNK-1), followed by the anti-mouse IgM-RITC. Sections exhibited no staining under negative-control conditions including no first antibody or no second antibody. Sections of the stage 31 central HPS (frontal sections through the interventricular septum) were immunostained for both PSA-NCAM (FITC) and HNK-1 epitope (RITC). Panels A,B, C were taken from the same frontal section with different filters. Panel C is a double exposure to show PSA (yellow, FITC) and HNK-1 epitope (red, RITC) simultaneously. The HNK-1 is expressed in the central HPS in the common His bundle and the upper bundle branches, while PSA is found in the distal bundle branches and the Purkinje fibers. The dotted line indicates interface between immunostained HPS myocardium and the rest of the myocardium.

mesenchymal transformation at the leading edge of the atrial septum [49]. The *in vitro* and *in situ* data from studies of the atrioventricular cushion demonstrate a marked down-regulation in the levels of NCAM during the detachment of cells from the epithelium during the later stages of mesenchymal transformation [12, 26, 79]. At later stages, up-regulation has been noted in regions of mesenchymal cell coalescence [69]. Expression of PSA-NCAM may be part of the reparation of these cells for release and migration into the matrix. Once the cells have released, all forms of NCAM may be down-regulated. This may be a pattern reiterated in other places where epithelial-mesenchymal transformation

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occurs as during neural crest cell migration away from the neural tube [4, 11].

Expression in the Normal and Diseased Adult Heart

A down-regulation of myocardial NCAM expression occurs with maturation to a low level that persists in the myocardium of adult rats [124], chickens [12], and humans [51]. In the adult rat, the atrial myocardium and much of ventricular myocardium loses staining for NCAM, but persists in the myocardium around coronary artery branches and at the top of the IVS, which are regions where cardiac conduction system cells could be located. NCAM undergoes a modest increase in expression in the aging rat [74]. Higher than normal levels of NCAM in the human myocardium has also been associated with myocarditis and transplanted hearts [51] and in the rat myocardium in a hypoxia-induced model of myocardial hypertrophy [50]. The increase in NCAM above normal in all these cases have been proposed to be a mark of regeneration or degeneration.

Immunotherapies which may involve production of antibodies against NCAM and PSA-NCAM has been of concern because both continue to be expressed in the heart, nervous system, and other tissues of infants and adults [43]. Anti-meningitis inoculation for Group B meningococci and *E. coli* KI [43] may cause production of anti-PSAs which could attack the young nervous system. Anti-NCAM monoclonal antibody is being tested against small lung cell carcinoma which express NCAM [76]. The results suggest that the therapy appears not to adversely affect nerve and heart function in the short term.

Multiple NCAM Isoforms in the Heart

Multiple NCAM isoforms are expressed in the heart and vary in size, glycosylation, and relative amount with

developmental state, species, and disease. The table is a partial list of studies in which NCAM isoform expression has been documented by immunoblot or Northern blot analyses. The overall pattern in the myocardium is that the MSD-containing isoforms plus and minus VASE are highly represented and the PI-linked forms with MSD are expressed at later stages of development and in the adult. Low levels of PSA-NCAM are prevalent even during development with transient high level expression in restricted areas. The PST and STX mRNAs are present in heart tissue extracts [6] but it is not yet clear which tissues or cells are expressing which transferase. The studies of NCAM isoform expression have primarily relied on Northern blot or immunoblot analysis of extracts from the whole heart. These analyses probably detect forms primarily from the ventricular myocardium rather than representing the forms in other regions or tissues which express NCAM. Thus, conclusions about which isoform is produced by which region, tissue, or cell types can only be made in limited areas and stages. There is still a need for detailed study of isoform expression by *in situ* hybridization combined with immunohistological analysis using antibodies, lectins, or other markers which will be able to distinguish between various NCAM forms. These studies however would be more informative when coupled with experimental manipulation to determine the functional significance of these different isoforms and differentially glycosylated forms.

Possible Functions for NCAM in the Heart

There are a number of lines of evidence that innervation of the skeletal muscle requires NCAM and can regulate NCAM expression [22, 71]. It has been proposed that NCAM may serve a similar purpose on the surface of cardiac muscle cells based on the evidence that NCAM levels in the heart decrease

Table 1. Heart NCAM is of arm references.

	Comments	Reference
frog	VASE	Zorn and Krieg, 1992
rat	hypoxia-induced hypertrophy	Gordon et al., 1990(49)
rat	embryonic to adult stages	Whartonet al., 1989
rat	postnatal - aging	Gaardsvoll et al., 1993
rat	PCR	Reyesetal., 1991
rat	VASE	Small and Akeson, 1990
rat	embryonic to adult	Eackieetal, 1991
mouse	embryonic days 7.5-10	Probstmeier et al., 1994
chicken	embryonic days	Burroughs et al., 1991; Watanabe et al., 1992
chicken	embryonic days, Pabs to NCAM, Pabs to MSD, Mab 4D to long transmembrane form	Byeonetal., 1995
human	fetal-adult and transplanted hearts	Gordon et al., 1990(50)
h		Al-Mahdawi et al., 1990

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postnatally after innervation is complete, arc high in the sinus and atrioventricular node of fetal hearts which eventually receive a high degree of innervation in the mature heart, and increase in transplanted heart tissues which have no innervation [51]. However, NCAM on heart myocytes is present well before innervation begins and transplanted tissues are exposed to such abnormal conditions (e.g., bathed in drugs for immune suppression) in addition to being denervated. NCAM homophilic interactions do appear to be involved in nerve-cardiac muscle cell interactions *in vitro* [57]. However, the hypothesis that NCAM is involved in nerve-cardiomyocyte interactions *in vivo* has yet to be supported by close correlative or experimental evidence. Heart innervation is different from skeletal muscle innervation in that it does not require direct contact between nerve and muscle cells. Therefore, NCAMs influence on cell-matrix associations may be an important focus when considering its role in cardiac innervation.

Another role related to innervation involves the study of neural crest cell migration into the heart. Neural crest cells are required for normal heart development [63]. They contribute to cardiac ganglia formation within the epicardium and also invade the heart via the aortic arch mesenchyme and the OFT endocardium. These cells may use NCAM for their migration as do olfactory bulb neurons [58, 87]. An anti-mouse NCAM monoclonal antibody [86] stains the embryonic rat heart with a much more restricted NCAM staining pattern than reported in other studies. This restricted pattern may reflect antibody specificity for a subset of NCAM forms not yet characterized. The staining extended from the recurrent nerve (derived from neural crest) along the outflow tract, before OFT septation and valve formation, and also around the sinus venosus. This pattern suggested to the authors that NCAM may serve to mark the pathway for neural crest cells as they migrate into the heart.

NCAM is present on the embryonic cardiomyocyte plasma membrane interfaces as detected by electron microscopy (see Figure 4A and [123]) and *in vitro* evidence suggests that NCAM functions homophilically at this surface [57]. Two important cardiomyocyte cell surface components, the cadherins and gap junctions, appear to be affected by anti-NCAM perturbation of NCAM function as tested in various *in vitro* models [62, 64, 122]. Our preliminary evidence supports NCAM's role in gap junction function (Figure 4B). Embryonic chicken cardiac cells cultured for 1 day express polysialylated NCAM with the ultrastructural distribution and electrophoretic profile similar to NCAM of embryonic cardiac tissues (data not shown). The function of gap junctions in these cultures was analyzed by the scrape-loading method [42]. Cardiocyte

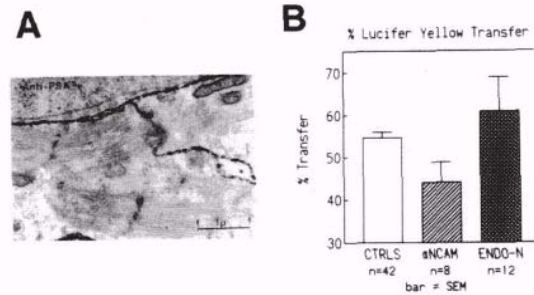


Figure 4. NCAM perturbation affects gap-junction-mediated dye coupling between embryonic cardiocytes. Primary myocardial cell cultures obtained from stages 24-29 (day 4-6) chicken embryo hearts were incubated with Fab fragments of polyclonal antibodies against chicken NCAM or with endo-N for 18 h and assayed for transfer of Lucifer Yellow (443D) using the scrape-loading method [42]. These cells express PSA-NCAM on their surfaces as detected by immunoblots (not shown) and by immunoelectron microscopy (A). For the assay of gap junction function (B), cells were scrape-loaded with fluorescent Lucifer Yellow and rhodamine-conjugated dextran. Cells with both fluorescent markers (scrape-loaded cells) were counted and that number compared to the number of cells with only Lucifer yellow (cells that received dye by transfer through gap junctions). After 28 h of incubation in anti-NCAM Fab fragments, there was a reduction in the percent of cells with dye transferred than when incubated in Fab prepared from control (non-immune) rabbit serum. Incubation of the cultures with endo-N resulted in a higher percent of cells with dye transfer than with vehicle alone. ANOVA ($P < 0.05$). These results support the hypothesis that altering NCAM function up or down affects gap junctional communication between embryonic myocardial cells.

cell membranes were disrupted by localized scraping to permit both the small fluorescent dye Lucifer Yellow and the large dextran molecule bound to rhodamine to enter a subset of cells. The extent of dye transfer via gap junctions was assayed by immunofluorescence microscopy. In the presence of anti-NCAM antibody fragments, the proportion of cells receiving dye from scrape-loaded cells decreased by 10.6%. In contrast, enzymatic removal of the PSA from NCAM increased dye transfer by 6.2%. Thus the difference between cells with inhibited NCAM function and enhanced NCAM

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function was 16.8%. While not dramatic, these differences were statistically significant. It is our hypothesis that these small alterations in NCAM function may finely regulate cardiomyocyte cell-cell communication without compromising the integrity of the dynamically functioning myocardium during cardiogenesis.

The cultures that we analyzed were probably predominantly ventricular cardiomyocytes which express low levels of PSA in the septating embryo. Yet even between these cells, gap junction function was detectably up-regulated with PSA removal. Developing HPS cells, which have high levels of PSA on their surfaces as detected by immunohistology, are likely to have even more reduced gap junction function compared to their myocardial neighbors and also reduced cell-cell interactions in general.

A detailed analysis of NCAM isoform expression revealed interesting patterns on developing cardiomyocytes. Antibodies to two regions of the chicken MSD sequence, 12 AB and 12 CD, were used to study the NCAM proteins in embryonic chicken heart. The MSD appeared to be spliced in or out in its entirety with no evidence for expression of mRNAs with differential splicing among the MSD exons 12A, B, C, and D. These antibodies to the MSD also revealed a different subcellular localization pattern for NCAM with MSD compared to the homogeneous distribution of total NCAM [13]. The MSD-containing NCAM immunostaining appeared in stripes on the surface of myocytes in register with the Z-bands. This pattern was present before N-cadherin or integrin relocates into this striped pattern. The MSD-inserted NCAM may be important in aligning adjacent myocytes to keep the Z-bands in register from one cell to the next. Because this pattern was present in cell-matrix interfaces as well as at cell-cell interfaces, it has been proposed that the NCAM with the MSD binds to surrounding extracellular matrix components.

The adhesive function of NCAM appears to be easily overridden or compensated by the presence of other more robust adhesion molecules, but there is *in vitro* evidence that NCAM can influence the function of cadherins and gap junctions (see above) which are key components in cardiomyocyte interactions at the intercalated discs. The transient appearance of PSA-NCAM specifically on the components of the HPS during development (Figure 4) combined with the understanding of how NCAM functions in other systems and the few pieces of evidence on developing cardiomyocytes *in vitro* suggests that PSA-NCAM may be important as the HPS differentiates in the following ways: it acts as an insulator in reducing the ability of HPS cells to interact with surrounding myocytes before the connective tissue sheath appears; it reduces lateral

myocyte interactions among the HPS cells; it serves to promote the physical separation of developing Purkinje fibers from their clonally related [52] neighbors, the working myocytes.

Endocardial Cell Mesenchymal Transformation

NCAM is regulated in expression during mesenchymal transformation of endocardial cells. The transient expression of PSA-NCAM on endocardial cells starts early during mesenchymal transformation while they are still within the epithelium [49]. A reduction in cell-cell interactions promoted by PSA-NCAM may allow cells to release themselves from each other in the epithelium and migrate into the matrix. The levels of NCAM is down-regulated during epithelial-mesenchymal transformation and migration of endocardial cells into the cardiac jelly [12, 26, 49, 79]. Advances in understanding the molecular mechanisms involved in this transformation have been due to the use of the *in vitro* collagen gel system (e.g., [67, 91, 97]). It would be of interest to see where in this cascade of molecular and cellular events the regulation of PSA-NCAM expression lies, and whether removal of the PSA could affect mesenchymal transformation.

Transgenic Mice Deficient in NCAM

Transgenic mice lacking NCAM have abnormalities in brain structures and behaviour [23], but survive and breed. While detailed analysis of the heart morphology and physiology has not yet been published, the fact that

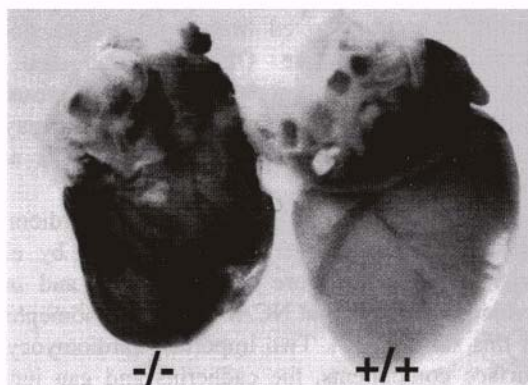


Figure 5. NCAM-null transgenic mice display abnormal cardiac morphology. Frontal view of a formaldehyde-fixed NCAM-null mutant mouse heart (-/-) and a wild-type mouse heart (+/+). Note the difference in the coronary vasculature and the shape and size of the ventricles. The knockout mouse heart appears darker than the control mouse heart because blood is visible through the thinner-walled ventricular-myocardium.

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these animals live to adulthood and are healthy enough to mate suggests that they have developed a functional heart that allows them to survive at least in the unchallenging environment of a cage in an animal facility. Preliminary observations comparing 5 knockout mice to control mice consistently reveal that these mice have smaller hearts with rounded ventricular apices, thin-walled ventricles, and abnormal-looking coronary vasculature (Figure 5, personal communication, Dr. Thomas Rosenquist, Dept. Anatomy, University of Nebraska Medical Center). Further analysis of the adult heart and the embryonic heart of this transgenic mouse line may reveal more defects. There is also another transgenic mouse line that was designed to have the NCAM-180 deleted [115]. NCAM-180 is abundant in the nervous system [17] so it is not surprising that defects have been noted in brain structures and functions [87, 107, 115]. The hearts of these mice have not been closely studied, but could be worth analysis because innervation of the heart may be compromised in these animals. In addition, the NCAM-180 and NCAM 185 (NCAM-180 with and without the MSD) have also been detected in heart extracts [13]. Analysis of these mouse hearts compared to the total NCAM knockout mouse heart could clarify which defects are likely to be directly linked to NCAM deficits in the myocytes and which may be the result of more indirect or extrinsic effects.

The viability of the NCAM-null transgenic mouse implies that human beings with NCAM mutations may be among us. The further analysis of phenotypes of the NCAM-null or other transgenic mice with alterations in NCAM expression could aid in the identification of a human clinical syndromes that could be linked to NCAM abnormalities. This kind of breakthrough has been made for another Ig-gene superfamily member, the adhesion molecule LI, which has been linked to neurological defects including X-linked hydrocephaly and MASA syndrome [60, 96, 125]. The defects that could influence NCAM might be found in the NCAM gene itself (1 q23-24), on regulatory regions, or in the polysialyltransferase genes (PST is on 5p21; STX is on 15q26). By extrapolation from the phenotype of the NCAM-mutant transgenic mice [Figure 5 and [23, 107, 111, 115]], the syndrome could include anosmia (deficits in the sense of smell), learning disorders, mood disorders (crankiness, violence), sleep-wake cycle disorders, and heart abnormalities.

Summary

There are a number of areas for further research on NCAM in the heart that are accessible for study at this time. The preferential expression of the polysialylated form of NCAM on cardiomyocytes within portions of the ventricular cardiac conduction system suggests that

interactions at a critical time during their development. The same is true for endocardial cells during their mesenchymal transformation. Testing these hypotheses may now be possible with the availability of reagents, advances in technology, and with an increase in a general interest in and understanding of heart development. The role of NCAM in heart development could also be advanced by analysis of the currently available NCAM-null transgenic mice which, preliminary studies have shown, have abnormal cardiac morphology. Support for the hypotheses that NCAM is important for side-by-side alignment of cardiomyocytes, endocardial cushion mesenchymal transformation, and neural crest migration into the heart may be found in such an analysis of the NCAM knockout mice. Currently the hypotheses about the function of NCAM in the heart are dependent on extrapolations from what has been found in the nervous system. Testing these hypotheses in the heart may not only reveal what NCAM is doing in the heart, but could elucidate some still puzzling aspects of NCAM function in the nervous system and skeletal muscle. These studies may eventually lead to an understanding of the role of NCAM in clinical syndromes and heart disease.

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