Natriuretic peptides in embryonic stem cell-derived cardiomyocytes and their receptors in the central nervous system. 1) In vitro expression of natriuretic peptides in cardiomyocytes differentiated from monkey embryonic stem cells. 2) Immunohistochemical mapping of NPR-A in the brainstem of macaca fascicularis

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様式３

論　文　内　容　要　旨

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学位論文題目	Natiruretic Peptides in Embryonic Stem Cell-Derived Cardiomyocytes and Their Receptors in the Central Nervous System.（胎性胚細胞由来の心筋細胞におけるナトリウム利尿ペプチドと中枢神経系におけるナトリウム利尿ペプチド受容体について）		

Background and purpose:

The natriuretic peptides (NPs) are a family of three peptide hormones: atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and C-type natriuretic peptide (CNP). NPs are released into the circulation from cardiac cells to act as hormones in the control of fluid volume homeostasis and blood pressure by causing natriuresis, diuresis, vasorelaxation and inhibition of the renin–angiotensin–aldosterone system. In addition, cell-based studies have shown that NPs exhibit important autocrine and paracrine functions such as modulating myocyte growth, apoptosis and proliferation in smooth muscle cells and cardiac myocytes, and suppress cardiac fibroblast proliferation and extracellular matrix secretion. The physiological effects of natriuretic peptides are initiated by binding to cell surface receptors. These include natriuretic peptide receptor type A (NPR-A), which is sensitive to ANP and BNP, natriuretic peptide receptor type B (NPR-B), which is highly specific for CNP, and natriuretic peptide receptor type C which may comprise up to 95% of the total NPR population and is known to bind all the natriuretic peptides with similar affinity.

Physiological functions of embryonic stem (ES) cell-derived cardiomyocytes are important for transplantation therapy and several questions need to be addressed before ES cell-derived cardiomyocytes can find their way into cardiac cell therapy in human. One of these questions is their abilities to function as normal cardiac cells, which is not investigated in detail. Therefore, in the our study, we investigated the functional properties especially expression of ANP and BNP in the cardiomyocytes differentiated from monkey ES cells *in vitro*.

On the other hand, although natriuretic peptide receptors have been shown to modulate a variety of natriuretic peptide activities in the brain, little information is available about their protein distribution in the brain. Therefore, in our study we examined the distribution of natriuretic peptide receptors in the brainstem.

Design and Methods:

(A) Natiruretic Peptides in Embryonic Stem Cell-Derived Cardiomyocytes

- ***Monkey ES Cell Culture and differentiation:*** Monkey undifferentiated ES cells of CMS-A-2-G1 and CMS-A-2 lines were cultured on the mitotically inactivated embryonic fibroblast feeder layer. Hanging drop technique was used for differentiation of ES cells.
- ***Electrophysiology:*** Membrane potentials were recorded from beating cells in the current-clamp configuration with an EPC-8 patch clamp amplifier.
- ***Reverse Transcription-Polymerase Chain Reaction (RT-PCR):*** We examined mRNA levels of cardiac natriuretic peptides (ANP and BNP) in monkey ES cell-derived beating cells, as well as, GATA-4 (cardiac transcription factor).
- ***Immunocytochemistry:*** Immunostaining of beating cells with cardiac specific proteins (cardiac actin, cardiac troponin I, and GATA-4). Examination of ANP and BNP in beating cells as well as their co-localization with trans-Golgi network (p230 trans Golgi antibody).

(B) Natiruretic Peptide Receptors in the Central Nervous System

- ***Immunohistochemistry and double immunohistochemistry:*** brainstem serial cryosections (20 μm) were processed in a free-floating state, and immunostained with NPR-A antibody alone or in combination with goat anti- choline acetyltransferase (ChAT) or mouse monoclonal anti-tyrosine hydroxylase (TH) antibodies.
- ***Mapping:*** Mapping was carried out using a camera lucida, with diagrams being prepared for the brainstem showing NPR-A distribution.
- ***Western blotting:*** Western blot analysis was performed to confirm the specificity of the NPR-C antibody and to detect its expression in the rat brainstem.
- ***Immunofluorescence and double Immunofluorescence:*** sections of rat brainstem were immunostained with NPR-C antibody alone or in combination with ChAT or TH antibodies.

Results

(A) Natiruretic Peptides in Embryonic Stem Cell-Derived Cardiomyocytes

Spontaneously contracting cells appeared as clusters of beating cells in the differentiated embryoid bodies (EB) outgrowth at differentiation day 20 to 26 (3 to 9 days after plating). Spontaneously contracting cells showed nodal-like action potentials, and expression of ANP and BNP by RT-PCR and immunocytochemistry. The immunostaining of beating cells showed the presence of cardiac specific proteins; the beating cells were stained positive for cardiac actin and cardiac troponin I (cTnI).

cTnI-positive cells showed well–organized parallel myofilament and some cells showed a typical cross striation which is specific for striated muscles. Also, cells showed positive staining for GATA-4 (a transcription factor) and it was localized in the nucleus of contracted cells. Interestingly, ANP and BNP expressions were detected as immunoreactive granules in the perinuclear area and these signals appeared to co-localize with trans-Golgi network. These findings suggest that monkey ES cells were able to differentiate into cardiomyocytes producing natriuretic peptides in vitro.

(B) Natiruretic Peptide Receptors in the Central Nervous System

- In monkey brainstem, NPR-A immunoreactivity was localized to neurons in specific brainstem regions. NPR-A–immunoreactive perikarya were found in the red nucleus and the oculomotor nucleus in the midbrain, the parabrachial nucleus and the locus coeruleus in the pons, and the dorsal motor nucleus of the vagus, the hypoglossal nucleus, the cuneate nucleus, the gracile nucleus, the nucleus ambiguus, the lateral reticular nucleus, the reticular formation, and the inferior olivary nucleus in the medulla oblongata. Extensive networks of immunoreactive fibers were apparent in the red nucleus, the oculomotor nucleus, the principal sensory trigeminal nucleus, and the parabrachial nucleus. Double immunostaining revealed NPR-A immunoreactivity in cholinergic neurons of the parabrachial nucleus, the dorsal motor nucleus of vagus, the hypoglossal nucleus, and the nucleus ambiguus. However, there was no colocalization of NPR-A and tyrosine hydroxylase in the locus coeruleus.

- In rat brainstem, NPR-C immunoreactivity was detected in several regions, including the periaqueductal gray, oculomotor nucleus, red nucleus and trochlear nucleus of the midbrain; the pontine nucleus, dorsal tegmental nucleus, vestibular nucleus, locus coeruleus, trigeminal motor nucleus, nucleus of the trapezoid body, abducens nucleus and facial nucleus of the pons; and the dorsal motor nucleus of the vagus, hypoglossal nucleus, lateral reticular nucleus, nucleus ambiguus and inferior olivary nucleus of the medulla oblongata. Interestingly, NPR-C immunoreactivity was detected in the cholinergic neurons of the oculomotor nucleus, trochlear nucleus, dorsal tegmental nucleus, motor trigeminal nucleus, facial nucleus, dorsal motor nucleus of the vagus, nucleus ambiguus and hypoglossal nucleus. Furthermore, NPR-C immunoreactivity was detected in several catecholaminergic neuronal groups including the A6, A5, A1, C3 and C1 cell groups.

Conclusion:

The results presented here indicate that the monkey ES cells have the ability to differentiate into functional cardiomyocytes, which have the ability to produce natriuretic peptides *in vitro*. These results suggest a possible role of cardiac natriuretic peptides (ANP and BNP) in the regulation of cardiomyocytes development *in vitro* through paracrine and/or autocrine functions. In addition, these findings suggest that ES cell-derived cardiomyocytes are functional cardiac cells and can be used as a model to study mechanisms and functions in early stages of cardiogenesis as well as for their application in cell therapy.

On the other hand, NPR-A and NPR-C are widely distributed throughout the brainstem in regions related to cardiovascular control and in other regions related to other functions, suggesting the involvement of natriuretic peptide system in broad range of functions in the brain. The wide distribution of NPR-A and NPR-C suggests that natriuretic peptides play roles not only in the central regulation of endocrine and cardiovascular homeostasis but also act in neurotransmitter/neuromodulator pathways that mediate diverse physiological functions, particularly via cholinergic and catecholaminergic neurons.

別紙様式８（課程・論文博士共用）

学位論文審査の結果の要旨

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（学位論文審査の結果の要旨）

ES 細胞は、再生医療や細胞治療に重要な役割を果たすと期待されているが、これまでサル ES 細胞から心筋細胞を分化・誘導した報告はなかった。本研究では、サル ES 細胞からＡおよびＢ型ナトリウム利尿ペプチド（ANP と BNP）を産生する機能をもった心筋細胞への分化を試みるとともに、サル脳幹部における ANP と BNP の受容体、NPR-A の分布について免疫組織化学法で検討した。

その結果、分化・誘導細胞は、心筋マーカーである心筋アクチンと心筋トロポニンＩ陽性で、ANP と BNP を発現していた。生理学的検査でも自律拍動能を確認した。以上の結果は、機能性をもつ心筋細胞が分化したことを示している。また脳幹部の NPR-A 受容体は、赤核、動眼神経核、青斑核、迷走神経背側核、擬核、舌下神経核など、特定の神経核の神経細胞に局在していた。

本研究は、サル ES 細胞から心筋細胞への分化・誘導法を確立するとともに、サル脳幹部における NPR-A の分布を明らかにしたものであり、博士（医学）の学位を授与するに値するものと認められる。

なお、本学位授与申請者は、平成 21 年２月 ４日実施の論文内容とそれに関連した試問を受け、合格と認められた。

（平成２１年２月４日）