とが知られているIL-17産生を中心に解析した。T細胞のNR4A2発現上昇はIL-17産生細胞に限局して認められ、IFN-γ産生能とは相関しなかった。一方、in vitroで分化させたTh17細胞のIL-17産生はNR4A2特異的siRNA処理により完全に消失したが、同時にTh17細胞分化に必要なIL-21産生と引き続くIL-23受容体発現も抑制が確認され、NR4A2の作用点がTh17細胞のIL-21産生あるいはその上流に位置することが示された。コラーゲンマトリクス封入siRNAの投与により、EAE病態とTh17細胞応答が有意に抑制されたことから、EAEにおける自己反応性Th17細胞の病原性に対して、NR4A2が本質的な制御因子であることが示された。以上より、NR4A2がTh17細胞機能とこれによるEAEおよびRRMS病態と密接に関わることが示された。一方、NR4A2cKOマウスのEAE誘導後期の病態においては、Th17細胞とは異なり特定のマーカー遺伝子を発現するユニークなT細胞がCNS中に認められた。このT細胞は強い病原性を有しており、このマーカー遺伝子を標的として設計したsiRNAのin vivo投与により後期の病態が有意に軽快したことから、後期EAE病態はこの特定のT細胞群に起因するものと考えられた。さらにSPMS患者末梢血では、このマーカー遺伝子を発現するT細胞の割合が選択的に増加していたことから、被験者末梢血T細胞のNR4A2、および今回同定した新たなマーカー遺伝子の発現を事前に測定することにより、客観的な根拠に基づいてRRMS患者とSPMS患者を鑑別できる方法が確立できる可能性が示された。

E. 結論

NR4A2は、自己応答反応に代表されるIL-17依存性のT細胞反応に極めて密接な関連を示す重要な免疫応答制御因子であり、EAEおよびRRMSに関連する自己免疫応答に密接に関わることが示された。一方、NR4A2cKOマウスのEAE解析から、後期EAE病態に関わる全く新しいマーカー遺伝子を発現するT細胞を同定し、さらに同T細胞がSPMS病態に関与する可能性を示した。今回、MSの動物モデルを用いて二つのMS病型を識別する新しい手がかりを得たことから、今後OCHの投与対象であるRRMS患者とSPMS患者を効率的に見分ける、根拠に基づいた客観的な鑑別法の確立が期待される。

F. 研究発表

1. 論文発表

Ben JE Raveney, Shinji Oki, Takashi Yamamura
Nuclear receptor NR4A2 orchestrates Th17 cell-mediated autoimmune inflammation via IL-21 signaling
PLoS ONE 7(7): e41114., 2013

Shinji Oki, Benjamin JE Raveney, Yoshimitsu Doi, Takashi Yamamura
Versatile Orphan Nuclear Receptor NR4A2 as a Promising Molecular Target for Multiple Sclerosis and Other Autoimmune Diseases. 193-200, 2012, In; Chembiomolecular Science - At the Frontier of Chemistry and Biology - Springer, Ed. Masakatsu Shibasaki et al.

Shinji Oki; Towards understanding the role of orphan nuclear receptor NR4A2 in Th17 cell- mediated CNS autoimmunity: An experimental approach employing an animal model of multiple sclerosis. Clin. Exp. Neuroimmunol. 5, 137-148 (2014) The Japanese Society for Neuroimmunology

大木　伸司、山村　隆；多発性硬化症の動物モデル - 横断的アプローチによる病態解明と治療標的の探索 - 日本臨床（日本臨床社）第72巻　第11号　1935-1940,(2014)

Ben Raveney, Shinji Oki, Takashi Yamamura; Nuclear Receptor NR4A2 Orchestrates Th17 Cell-Mediated Autoimmune Inflammation via IL-21 Signalling. PLOS ONE. 8(2):

e56595. (2013)

Norio Chihara, Toshimasa Aranami, Shinji Oki, Takako Matsuoka, Masakazu Nakamura, Hitaru Kishida, Kazumasa Yokoyama, Yoshiyuki Kuroiwa, Nobutaka Hattori, Tomoko Okamoto, Miho Murata, Tatsushi Toda, Sachiko Miyake, Takashi Yamamura; Plasmablasts as migratory IgG-producing cells in the pathogenesis of neuromyelitis optica. PLOS ONE. 8(12): e83036. (2013)

Shinji Oki, Ben Raveney, Takashi Yamamura; Versatile Orphan Nuclear Receptor NR4A2 as a Promising Molecular Target for Multiple Sclerosis and Other Autoimmune Diseases. In: Chembiomolecular Science: At the Frontier of Chemistry and Biology, 2012: Editor M. Shibasaki, M. Iino, H. Osada, Springer, Tokyo, Japan, p.193-200 (2012)

2. 学会発表

Ben Raveney, Shinji Oki, Takashi Yamamura: NR4A2 modulation during autoimmune inflammation of the central nervous system reduces pathogenic T cell responses and ameliorates clinical disease. The 12th International Congress of Neuroimmunology, Mainz Germany, Nov. 11th- 14th, 2014

Ben Raveney, Shinji Oki, Takashi Yamamura: NR4A2 controls pathogenic 'switched' Th17 cells in the CNS during autoimmune inflammation. 第43回 日本免疫学会総会・学術集会 京都 12月10日-12日 2014年

Raveney B., S. Oki, T. Yamamura: NR4A2 modulation during autoimmune inflammation of the central nervous system reduces pathogenic T cell responses and ameliorates clinical disease
6th Congress of the Pan-Asian Committee for Threatment and Research in Multiple Sclierosis (PACTRIMS), Kyoto, Nov. 6th-8th, 2013

Raveney B., S. Oki, T. Yamamura: NR4A2 controls distinct populations of Th17 13th Annual Conference of FOCIS (FOCIS2013), Vancouver, Jun. 20th-23rd, 2013

Ben Raveney, Shinji Oki, Takashi Yamamura: NR4A2 controls Th17-mediated autoimmunity 第42回 日本免疫学会総会・学術集会 幕張 12月11日-13日 2013年

大木 伸司、ベン・レイバニー、山村 隆：病原性IL-17産生細胞のNR4A2発現維持に関わる細胞間相互作用
第25回 日本神経免疫学会総会・学術集会 下関 11月27日-29日 2013年

大木 伸司、ベン・レイバニー、山村 隆：中枢神経系の炎症性自己免疫病態形成に関わる病原性T細胞群の解析
第41回 日本臨床免疫学会総会・学術集会 下関 11月27日-29日 2013年

Raveney B., S. Oki, T. Yamamura: NR4A2 controls Th17-mediated autoimmunity 12th Annual Conference of FOCIS (FOCIS2012), Vancouver, Jun. 20th--23rd, 2012

Chihara N., S. Oki, T. Matsuoka, W. Sato, Y. Lin, T. Okamoto, M. Ogawa, T. Toda, S. Miyake, T. Aranami, T. Yamamura: Activated Plasmablasts Migrate to the Central Nervous System
12th Annual Conference of FOCIS (FOCIS2012), Vancouver, Jun. 20th--23rd, 2012

Raveney B., S. Oki, T. Yamamura: Acute autoimmune inflammation of the central nervous system by IL-17-secreting

T cells is controlled by NR4A2
The 10th International Congress of Neuroimmunology, Boston, Nov. 4th- 8th, 2012

大木　伸司、ベン・レイバニー、山村　隆：中枢神経系の炎症性自己免疫病態形成に関わるIL-17産生性T細胞群の解析　第24回日本神経免疫学会学術集会、Sep, 20th-21st, 2012

S. Oki, Raveney B., T. Yamamura : Acute autoimmune inflammation of the central nervous system by IL-17-secreting T cells is controlled by NR4A2　International Symposium on Glyco-minded Biology of Diseases as a Basis of Pharmaceutical Sciences、Nov.30th-Dec.1st, 2012

Raveney B., S. Oki, T. Yamamura : NR4A2 controls Th17-mediated autoimmunity　第41回 日本免疫学会総会・学術集会、神戸　Dec. 5th-7th, 2012
（発表誌名巻号・頁・発行年等も記入）

G. 知的所有権の取得状況

1. 特許取得

発明の名称：siRNA、このsiRNAを発現する組換えベクター、NR4A2遺伝子発現抑制剤、IL-17遺伝子発現抑制剤、および、CD4陽性T細胞増殖抑制剤
出願番号：特願2009-501299
出願日：2008年2月28日
登録番号：特許第5098033号
登録日：2012年10月05日
発明・考案・創作者名：大木伸司、北條浩彦、三宅幸子、山村隆
権利者名：財団法人ヒューマンサイエンス振興財団

発明の名称：進行型免疫性脱髄疾患治療剤
出願人：（独）国立精神・神経医療研究センター
発明者：大木　伸司、北條　浩彦、山村　隆
出願番号：特願２０１４－１３５６３０
出願日：平成２６年７月１日

発明の名称：進行型免疫性脱髄疾患治療剤
出願人：（独（国立精神・神経医療研究センター
発明者：大木　伸司、山村　隆
出願番号：特願２０１５－６１４２
出願日：平成２７年１月１５日

2. 実用新案登録

とくになし

3. その他

とくになし

厚生労働科学研究費補助金難治性疾患等実用化研究事業(難治性疾患実用化研究事業)
分担研究報告書(総合)

多発性硬化症患者群の腸内細菌叢に関する研究

研究分担者　服部　正平　　東京大学大学院新領域創成科学研究科　教授

研究要旨
多発性硬化症(Multiple sclerosis, MS)は慢性的な中枢神経系の脱髄疾患であり、欧米では非常に多く近年日本及びアジア諸国においても患者数が急増している。MS の原因としては遺伝的な要因と環境的な要因が知られており、T 細胞に腸内菌叢が影響を及ぼし様々な自己免疫性疾患に重要な役割をしていることが示唆されている。本研究では、環境要因としての MS 患者の腸内(糞便)細菌叢を解析し、これら MS 群のデータと健常者腸内細菌叢データとの比較解析から、MS 腸内細菌叢の特徴解明を行った。

A. 研究目的

本研究は、MS 患者群の腸内細菌叢の菌種及び遺伝子組成を解析し、健常者群との比較による MS 腸内細菌叢の特徴及び本疾患におけるバイオマーカーあるいは発症に関わる細菌種・遺伝子・代謝系を解明し、MS 発症と腸内細菌叢の関係を明らかにすることを目的とする。

B. 研究方法

MS 患者群及び成人健常者群の糞便から調製した腸内細菌叢 DNA について、次世代シークエンサー(ロシュ社 454 やライフテクノロジー社 Ion PGM 等)を用いて、16S リボソーム RNA 遺伝子(16S)及びメタゲノムデータを収集する。得られた 16S データの OTU クラスタリング及びデータベースへの類似度検索により、各細菌叢の菌種と菌種組成比を解明し、また、系統樹を作成する。さらに、MS 群と健常者群間の上記 16S データの比較解析により、MS 群に有意に増減する菌種の特定等を行う。また、MS 群と健常者群の系統樹の比較解析(UniFrac 距離解析)により菌叢全体構造の両群間類似性を評価する。さらに、両群間のメタゲノムデータを比較することにより、両群間で有意に増減する遺伝子あるいは代謝系等の情報を得て、両群間の細菌叢機能の相違を評価する。

(倫理面への配慮)
本研究で使用した腸内細菌叢検体については、研究代表者の機関等における生命倫理審査会の承認を得ている。

C. 研究結果

20 名の二次進行型ではない再発性 MS 被験者群(MS20)の糞便及び腸内細菌叢を採取した。これらの検体から 454GS を用いて計 141,549 リード(7,080 ± 825 リード／被験者)の 16S データを収集した。この MS データから計 6 万リード(3,000 リード／MS 被験者)と 40 名の健常者群(HC40)から得た 12 万リード(3,000 リード／健常者)を用いて OTU(菌種)数、UniFrac 距離、菌種組成の比較解析を行った。その結果、OTU(菌種)数は MS20 と HC40 間において有意な差はみられなかった。一方、UniFrac 距離解析では両群の腸内細菌叢の全体構造が統計学的に有意に異なっていることを見いだした($P < 0.05$)。そこで、門、属、種レベルでの両群間に違いを生じる菌種の探索を行った。門レベルでは優占する Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria の 4 門の組成比は両群間に統計学的有意さはなかった。しかし、属レベルにおいて *Bacteroides*, *Faecalibacterium*, *Anaerostipes*, *Roseburia* の 4 属が MS20 で有意に減少していた。さらに、種レベルにおいて、MS20 で有意に減少している 12 菌種(*Faecalibacterium prausnitzii*, *Lachnospiraceae* bacterium 5_1_63FAA, *Anaerostipes hadrus*, *Clostridium* sp, *Eubacterium rectale*, *Lachnospira pectinoschiza*, *Lachnospiraceae* bacterium DJF_VP08k1, *Bacteroides stercoris*, *Coprococcus catus*, *Megamonas funiformis*, *Bacteroides coprophilus*)、有意に増加している 10 菌種(*Malikia spinosa*, Bacteroidetes 門に属する未知菌種, bacterium ic1311, *Subdoligranulum* sp., *Eggerthella lenta*, 2 種類の *Streptococcus* sp, *Streptococcus thermophiles*, *Streptococcus parasanguinis*, *Parabacteroides distasonis*)を検出した。MS20 で有意に減少していた 12 菌種の多くは減少していた 4 属に含まれていた。

さらに、これら 22 菌種について、新たな 18

名の健常者群（HC18）の経時サンプル（一人あたり2週間ごとの9サンプル）に対する増減を調べたところ、22菌種の大部分はHC40で観察された増減をHC18の経時サンプルにおいても同様に示した。すなわち、これら22菌種は時間を経てもMS群において安定的に増減していることが示唆された。

D. 考察

炎症性腸疾患（IBD）等の疾患患者の腸内細菌叢は、健常者との比較において細菌叢構造の異常（dysbiosis）を示すことが知られている。MS群の腸内細菌叢のOTU（菌種）数は健常者群との間で有意な違いはなかったが、UniFrac距離解析は明らかな両群間の違いを示した。また、両群間で特徴的に増減する菌種を検出した。これらの結果は、MS腸内細菌叢は中程度の細菌叢の構造異常（dysbiosis）をもつことが示唆された。とくに、検出されたMS群において有意にかつ安定的に増減していた22菌種の特性はMS発症の機構解明に有用であると考えられる。

E. 結論

大量の16S配列データを用いた細菌叢解析から、MS腸内細菌叢は健常者群との比較において、菌叢構造が有意に異なっていることを明らかにした。このことはMS発症に腸内細菌叢のdysbiosisが関与することを強く示唆するものである。

F. 研究発表

1. 論文発表

1. Kim SW 他：Robustness of gut microbiota of healthy adult in response to probiotic intervention revealed by high-throughput pyrosequencing. DNA Res., 20, 241-253 (2013).
2. Kawamoto S 他：Foxp3(+) T cells regulate immunoglobulin a selection and facilitate diversification of bacterial species responsible for immune homeostasis. Immunity 41: 152-65 (2014).
3. Narushima S 他: Characterization of the 17 strains of regulatory T cell-inducing human-derived Clostridia. Gut Microbes 5: 1-7 (2014).
4. Said HS 他: Dysbiosis of Salivary Microbiota in Inflammatory Bowel Disease and Its Association With Oral Immunological Biomarkers. DNA Res. 21: 15-25 (2014).

2. 学会発表

金相完他：高速シーケンシングによる多発性硬化症患者の腸内細菌叢解析. 日本分子生物学会 第36回年会 ポスター発表（2013年12月3日）

G. 知的所有権の取得状況

1. 特許取得

なし

2. 実用新案登録

なし

3. その他

なし

研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表（平成24年度～26年度）

書籍

	著者氏名	論文タイトル名	書籍全体の編集者名 書籍名	出版社名 出版地	出版年 ページ
1	Shinji Oki, Ben Raveney, Takashi Yamamura	Versatile Orphan Nuclear Receptor NR4A2 as a Promising Molecular Target for Multiple Sclerosis and Other Autoimmune Diseases	Masakatsu Shibasaki, Masamitsu Iino, Hiroyuki Osada Chembiomolecular Science At the Frontier of Chemistry and Biology	Springer Tokyo, Japan	2012 193-200

研究成果の刊行に関する一覧表（平成24年度～26年度）

雑誌

	発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
1	Sato W, Tomita A, Ichikawa D, Lin Y, Kishida H, Miyake S, Ogawa M, Okamoto T, Murata M, Kuroiwa Y, Aranami T, Yamamura T.	CCR2(+)CCR5(+) T cells produce matrix metalloproteinase-9 and osteopontin in the pathogenesis of multiple sclerosis.	J Immunol.	189	5057-5065	2012
2	Kawazoe T, Araki M, Lin Y, Ogawa M, Okamoto T, Yamamura T, Wakakura M, Murata M.	New-onset type 1 diabetes mellitus and anti-aquaporin-4 antibody positive optic neuritis associated with type 1 interferon therapy for chronic hepatitis C.	Intern Med.	51	2625-2629	2012
3	Yamamura T, Miyake S.	B-cell-directed therapy: which B cells should be targeted and how?	Immunotherapy.	4	455-457	2012
4	Toba T, Murata K, Futamura J, Nakanishi K, Takahashi B, Takemoto N, Tomino M, Nakatsuka T, Imajo S, Goto M, Yamamura T, Miyake S, Annoura H.	Synthesis and biological evaluation of truncated α-galactosylceramide derivatives focusing on cytokine induction profile.	Bioorg Med Chem.	20	2850-2859	2012
5	Chiba A, Mizuno M, Tomi C, Tajima R, Alloza I, di Penta A, Yamamura T, Vandenbroeck K, Miyake S.	A 4-trifluoromethyl analogue of celecoxib inhibits arthritis by suppressing innate immune cell activation.	Arthritis Res Ther.	14	R9	2012
6	Chiba A, Tajima R, Tomi C, Miyazaki Y, Yamamura T, Miyake S.	Mucosal-associated invariant T cells promote inflammation and exacerbate disease in murine models of arthritis.	Arthritis Rheum	64	153-161	2012
7	Satoh J.	Molecular network of microRNA targets in Alzheimer's disease brains.	Experimental Neurology	235 (2)	436-446	2012
8	Chihara N, Aranami T, Oki S, Matsuoka T, Nakamura M, Kishida H, Yokoyama K, Kuroiwa Y, Hattori N, Okamoto T, Murata M, Toda T, Miyake S, Yamamura T.	Plasmablasts as migratory IgG-producing cells in the pathogenesis of neuromyelitis optica.	PLoS One.	8	e83036	2013
9	Di Penta A, Chiba A, Alloza I, Wyssenbach A, Yamamura T, Villoslada P, Miyake S, Vandenbroeck K.	A trifluoromethyl analogue of celecoxib exerts beneficial effects in neuroinflammation.	PLoS One.	8	e83119	2013
10	Raveney BJ, Oki S, Yamamura T.	Nuclear receptor NR4A2 orchestrates Th17 cell-mediated autoimmune inflammation via IL-21 signalling.	PLoS One.	8	e56595	2013
11	Ota M, Sato N, Nakata Y, Ito K, Kamiya K, Maikusa N, Ogawa M, Okamoto T, Obu S, Noda T, Araki M, Yamamura T, Kunugi H	Abnormalities of cerebral blood flow in multiple sclerosis: a pseudocontinuous arterial spin labeling MRI study.	Magn Reson Imaging.	31	990-995	2013
12	Ayzenberg I, Kleiter I, Schröder A, Hellwig K, Chan A, Yamamura T, Gold R.	Interleukin 6 receptor blockade in patients with neuromyelitis optica nonresponsive to anti-CD20 therapy.	JAMA Neurol.	70	394-397	2013

13	Satoh J, Tabunoki H.	Molecular network of chromatin immuno-precipitaiton followed by deep sequecing-based vitamin D receptor target genes.	Multiple Sclerosis	19 (8)	1035-1045	2013
14	Satoh J, Tabunoki H, Ishida T, Saito Y, Konno H, Arima K.	Reactive astrocytes express the potassium channel Kir4.1 in active multiple sclerosis lesions.	Clinical and Experimental Neuroimmunology	4 (1)	19-28	2013
15	Satoh J, Kawana N, Yamamoto Y.	Molecular network of ChIP-Seq-based EBNA1-target cellular genes supports biological implications of EBV persistence in multiple sclerosis.	Clinical and Experimental Neuroimmunology	4 (2)	181-192	2013
16	Kawana N, Yamamoto Y, Ishida T, Saito Y, Konno H, Arima K, Satoh J.	Reactive astrocytes and perivascular macrophages express NLRP3 inflammasome in active demyelinating lesions of multiple sclerosis and necrotic lesions of neuromyelitis optica and cerebral infarction.	Clinical and Experimental Neuroimmunology	4 (3)	296-304	2013
17	Ota M, Sato N, Matsuo J, Kinoshita Y, Kawamoto Y, Hori H, Teraishi T, Sasayama D, Hattori K, Obu S, Nakata Y, Kunugi H	Multimodal image analysis of sensorimotor gating in healthy women	Brain Res	1499	61-68	2013
18	Ota M, Hori H, Sato N, Sasayama D, Hattori K, Teraishi T, Obu S, Nakata Y, Kunugi H	Hypothalamic-pituitary-adrenal axis hyperactivity and brain differences in healthy women	Neuropsycho-biology	68(4)	205-211	2013
19	Shigemoto Y, Matsuda H, Kamiya K, Maikusa N, Nakata Y, Ito K, Ota M, Matsunaga N, Sato N	In vivo evaluation of gray and white matter volume loss in the parkinsonian variant of multiple system atrophy using SPM8 plus DARTEL for VBM	Neuroimage Clin	2	491-496	2013
20	Ota M, Nakata Y, Ito K, Kamiya K, Ogawa M, Murata M, Obu S, Kunugi H, Sato N.	Differential diagnosis tool for parkinsonian syndrome using multiple structural brain measures	Comput Math Methods Med.		571289	2013
21	Kim SW, Suda W, Kim S, Oshima K, Fukuda S, Ohno H, Morita H, Hattori M	Robustness of gut microbiota of healthy adults in response to probiotic intervention revealed by high-throughput pyrosequencing	DNA Research	20	241-253	2013
22	Satoh J.	Molecular network of ChIP-Seq-based NF-κB p65 target genes involves diverse immune functions relevant to the immunopathogenesis of multiple sclerosis.	Multiple Sclerosis and Related Disorders	3 (1)	94-106	2014
23	Manabu Araki, Takako Matsuoka, Katsuichi Miyamoto, Susumu Kusunoki, Tomoko Okamoto, Miho Murata, Sachiko Miyake, Toshimasa Aranami, Takashi Yamamura.	Efficacy of the anti-IL-6 receptor antibody tocilizumab in neuromyelitis optica	Neurology	82 (15)	1302-1306	2014

24	Masakazu Nakamura, Takako Matsuoka, Norio Chihara, Sachiko Miyake, Wakiro Sato, Manabu Araki, Tomoko Okamoto, Youwei Lin, Masafumi Ogawa, Miho Murata, Toshimasa Aranami, Takashi Yamamura	Differential effects of fingolimod on B-cell populations in multiple sclerosis.	Multiple Sclerosis J	20 (10)	1371-1380	2014
25	Niino M, Mifune N, Kohriyama T, Mori M, Ohashi T, Kawachi I, Shimizu Y, Fukaura H, Nakashima I, Kusunoki S, Miyamoto K, Yoshida K, Kanda T, Nomura K, Yamamura T, Yoshii F, Kira J, Nakane S, Yokoyama K, Matsui M, Miyazaki Y, Kikuchi S	Apathy/depression, but not subjective fatigue, is related with cognitive dysfunction in patients with multiple sclerosis	BMC neurology	14	3	2014
26	Kamachi F, Harada N, Usui Y, Sakanishi T, Ishii N, Okumura K, Miyake S, Akiba H.	OX40 ligand regulates splenic CD8(-) dendritic cell-induced Th2 responses in vivo.	Biochem Biophys Res Commun.	444	235-240	2014
27	Wakiro Sato	Chemokine receptors on T cells in multiple sclerosis.	Clin. Exp. Neuroimmunol.	5(2)	162-174	2014
28	Satoh J, Motohashi N, Kino Y, Ishida T, Yagishita S, Jinnai K, Arai N, Nakamagoe K, Tamaoka A, Saito Y, Arima K.	LC3, an autophagosome marker, is expressed on oligodendrocytes in Nasu-Hakola disease brains.	Orphanet Journal of Rare Diseases	9	e68	2014
29	Choi SS, Lee HJ, Lim I, Satoh J, Kim SU.	Human astrocytes: Secretome profiles of cytokines and chemokines.	PLoS One	9 (4)	e92325	2014
30	Kawana N, Yamamoto Y, Kino Y, Satoh J.	Molecular network of NLRP3 inflammasome activation-responsive genes in a human monocyte cell line.	Austin Journal of Clinical Immunology	1 (4)	e1071	2014
31	Adachi Y, Sato N, Saito Y, Kimura Y, Nakata Y, Ito K, Kamiya K, Matsuda H, Tsukamoto T, Ogawa M	Usefulness of SWI for the Detection of Iron in the Motor Cortex in Amyotrophic Lateral Sclerosis	J Neuroimaging	44(4)	311-319	2014
32	Ota M, Sato N, Sakai K, Okazaki M, Maikusa N, Hattori K, Hori H, Teraishi T, Shimoji K, Yamada K, Kunugi H	Altered coupling of regional cerebral blood flow and brain temperature in schizophrenia compared with bipolar disorder and healthy subjects	J Cereb Blood Flow Metab	34 (12)	1868-1872	2014
33	Ota M, Noda T, Sato N, Hattori K, Teraishi T, Hori H, Nagashima A, Shimoji K, Higuchi T, Kunugi H.	Characteristic distributions of regional cerebral blood flow changes in major depressive disorder patients: a pseudo-continuous arterial spin labeling (pCASL) study	J Affect Disord.	165	59-63	2014

34	Shinji Oki	Towards understanding the role of orphan nuclear receptor NR4A2 in Th17 cell- mediated CNS autoimmunity: An experimental approach using an animal model of multiple sclerosis	Clin. Exp. Neuroimmunol.	5(2)	137-148	2014
35	Kawamoto S, Maruya M, Kato LM, Suda W, Atarashi K, Doi Y, Tsutsui Y, Qin H, Honda K, Okada T, Hattori M, Fagarasan S	Foxp3(+) T cells regulate immunoglobulin a selection and facilitate diversification of bacterial species responsible for immune homeostasis	Immunity	41	152-165	2014
36	Narushima S, Sugiura Y, Oshima K, Atarashi K, Hattori M, Suematsu M, Honda K	Characterization of the 17 strains of regulatory T cell-inducing human-derived Clostridia	Gut Microbes	5(3)	333-339	2014
37	Said HS, Suda W, Nakagome S, Chinen H, Oshima K, Kim S, Kimura R, Iraha A, Ishida H, Fujita J, Mano S, Morita H, Dohi T, Oota H, Hattori M	Dysbiosis of Salivary Microbiota in Inflammatory Bowel Disease and Its Association With Oral Immunological Biomarkers	DNA Research	21	15-25	2104
38	服部正平	ヒト腸内細菌研究の進歩	炎症と免疫	21(6)	472-479	2013
39	服部正平	「体内の細菌が作るもう一つの世界:マイクロバイオームの驚異」基礎の基礎	細胞工学	32(11)	1110-1114	2013
40	佐藤和貴郎	慢性炎症性脱髄性神経炎（CIDP)におけるT細胞ケモカイン受容体の解析	Periheral Nerve	25(2)	238-242	2014
41	佐藤和貴郎	多発性硬化症における脳脊髄液T細胞ケモカイン受容体解析	Jpn. J. Clin. Immunol.	37(2)	83-89	2014
42	佐藤和貴郎、山村 隆	免疫動態	Clinical Neurocsience	32(11)	1218-1221	2014
43	大木伸司、山村　隆	多発性硬化症の動物モデル - 横断的アプローチによる病態解明と治療標的の探索 -	日本臨床	72(11)	1935-1940	2014
44	服部正平	ヒト腸内細菌叢のMetagenomics	Bio Clinica	29(8)	728-733	2014
45	服部正平、西嶋　傑	ヒト腸内細菌マイクロバイオームの特徴	G. I. Research	22(2)	128-135	2014
46	服部正平	ヒト常在菌叢のメタゲノム解析	医学のあゆみ	249(5)	385	2014

研究成果の刊行物・別刷

Versatile Orphan Nuclear Receptor NR4A2 as a Promising Molecular Target for Multiple Sclerosis and Other Autoimmune Diseases

Shinji Oki, Benjamin J.E. Raveney, Yoshimitsu Doi, and Takashi Yamamura

Abbreviations

AF2	Activation-function 2
CNS	Central nervous system
DBD	DNA-binding domain
EAE	Experimental autoimmune encephalomyelitis
IFN	Interferon
IL	Interleukin
LBD	Ligand-binding domain
MBP	Myelin basic protein
MHC	Major histocompatibility complex
MOG	Myelin oligodendrocyte glycoprotein
MS	Multiple sclerosis
NBRE	NGFI-B response element
NurRE	Nur-responsive element
PLP	Proteolipid protein
RXR	Retinoid X receptor
siRNA	Small interfering RNA

S. Oki • B.J.E. Raveney • Y. Doi • T. Yamamura (✉)
Department of Immunology, National Institute of Neuroscience, National Center of Neurology and Psychiatry, 4-1-1 Ogawahigashi, Kodaira, Tokyo 187-8502, Japan
e-mail: yamamura@ncnp.go.jp

M. Shibasaki et al. (eds.), *Chembiomolecular Science: At the Frontier of Chemistry and Biology*, DOI 10.1007/978-4-431-54038-0_19, © Springer Japan 2013

Introduction

The nuclear receptor family is a large group of ligand-dependent or ligand-independent transcription factors with 48 genes identified in the human genome. There is accumulating evidence that nuclear receptors are very fascinating components in terms of biological relevance to human diseases such as cancer, heart diseases, diabetes, and other lifestyle-related diseases or regulatory functions by natural and synthetic ligands. However, because of the multifunctional properties of individual nuclear receptor, the precise molecular behavior of nuclear receptors under physiological circumstances is still far from being completely understood. In addition, nuclear receptors have long been attractive drug targets and provide an enormous body of knowledge about the medicinal chemistry of their small molecule modulators. Importantly, many of the nuclear receptors are druggable targets, which is why numerous natural and synthetic nuclear receptor ligands, mostly composed of the steroid structural class, are on the market. The huge economic impact of those ligands is represented by their estimated share of 10–15% of the global pharmaceutical market. Many nuclear receptors are known as intrinsic components of immune responses including glucocorticoid receptor (GR), retinoic acid receptors (RARs), vitamin D receptor (VDR), peroxisome proliferator-activated receptors (PPARs), and retinoid orphan receptors (RORs). Herein, we discuss our recent findings that orphan nuclear receptor NR4A2 is profoundly involved in the development of autoreactive T cells and to be added to the list of beneficial molecular targets for autoimmune diseases such as multiple sclerosis.

Functions of NR4A2 Orphan Nuclear Receptor

The members of the NR4A subfamily are expressed mostly at low levels in a wide variety of metabolically demanding and energy-dependent tissues such as skeletal muscle, adipose tissue, heart, kidney, liver, and brain [1]. On certain stimuli, however, they are induced to express at very high levels, reminiscent of immediate/early genes. The diversity of signals leading to their expression suggests that they function in a highly cell-type and context-dependent manner. NR4A2 is mainly expressed in the central nervous system (CNS), especially in the cortex, ventral midbrain, brainstem, and spinal cord.

In general, nuclear receptors are composed of several conserved functional domains including DNA-binding domain (DBD) with two zinc fingers in the N-terminal region of the molecule and ligand-binding domain (LBD) in the C-terminal region with less conserved structures. In the absence of specific ligands, most of the nuclear receptors are inactive through interacting with co-repressor protein. Upon ligand binding to a hydrophobic cleft in the LBD, a conformational repositioning occurs at the C-terminal amphipathic α-helix (H12) of the LBD that provide a well-defined surface (activation-function 2, AF-2) recognized by co-activator

Table 1 NR4A2 expressed in autoreactive T cells mediates production of inflammatory cytokines and autoimmune response

Intervention	Target	Readout	IL-17
Transfection of NR4A2 plasmid	EL4	Promoter activity (luciferase assay)	↑
Infection of NR4A2-encoding retrovirus	Murine CD4 T cell	Protein expression	↑
NR4A2-specific siRNA	Murine CD4 T cell	Protein expression	↓
NR4A2-specific siRNA	Human CD4 T cell	Protein expression	↓
NR4A2-specific siRNA	PLP-reactive T cell	Experimental autoimmune encephalomyelitis induction (passive EAE)	↓

proteins, leading to the formation of a multiprotein complex mediating gene activation such as histone acetylation and other chromatin modifications. However, NR4A2 would encode unusual and atypical LBDs that lack canonical ligand-binding properties. Therefore, NR4A2 is believed to be a ligand-independent and constitutively active receptor, and its activity is tightly controlled at the level of gene expression, posttranscriptional modification, and multivalent complex formation with other molecules. The DNA-binding motif for the NR4A family members is the octanucleotide 5′-A/TAAAGGTCA (NGFI-B response element, NBRE) where NR4A2 binds as monomers and homodimers. The pro-opiomelanocortin gene promoter contains another class of transcriptional target of homodimers (Nur-responsive element, NurRE) with an inverted repeat of the NBRE-related octanucleatide, AAAT(G/A)(C/T)CA. NR4A1 and NR4A2 also bind as heterodimers with the retinoid X receptor (RXR) and bind a motif called DR-5. In addition, multivalent complex formation of NR4A2 with other transcription factors enables it to show noncanonical DNA binding.

NR4A2-deficent neonates die at birth because of a severe defect in respiratory function even with having intact NR4A1/3 genes, suggesting the unique functional property of NR4A2. Because of the selective expression of NR4A2 in the CNS, most of the target genes of NR4A2 known to date are limited to this region. For instance, NR4A2 is shown to play a role in the transcriptional activation of tyrosine hydroxylase involved in the synthesis of dopamine. Another group of NR4A2 target genes resides in those relevant to bone formation, such as osteopontin and osteocalcin. NR4A1 (also known as Nur77) and NR4A3 (NOR-1) are suggested to be expressed in thymus and mediate T-cell receptor-mediated T-cell apoptosis; however, distribution and function of NR4A2 in immune cells is not well elucidated. Meanwhile, there is accumulating evidence suggesting the pivotal roles of NR4A family members on inflammatory responses and that they are aberrantly expressed in inflamed synovial tissue of rheumatoid arthritis, psoriatic skin, and atherosclerotic lesions. Therefore, NR4A receptors may contribute to the cellular processes that control inflammatory disorders including autoimmunity (Fig. 1).

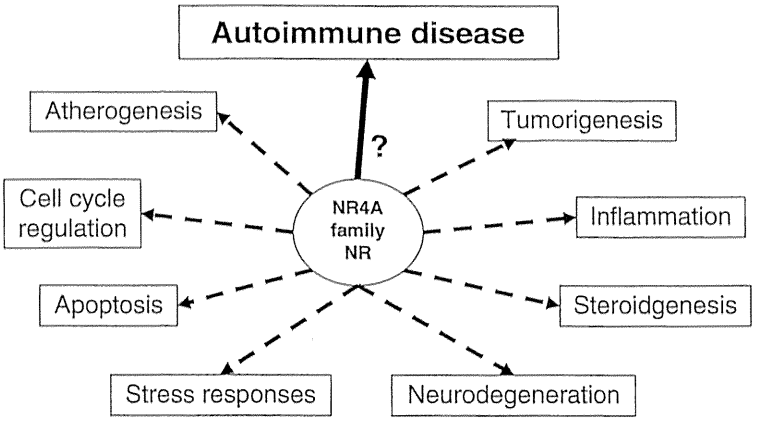


Fig. 1 Versatile function of NR4A2 on various biological responses. *NR* nuclear receptor

NR4A2 in Autoimmunity

Multiple sclerosis (MS) is a chronic inflammatory disease of the CNS, accompanying multiple foci of inflammatory demyelinating lesions. MS is thought to have an autoimmune pathogenesis, which is mediated by autoimmune T cells reactive to myelin antigens such as myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG), and proteolipid protein (PLP). Development of inflammatory processes within the CNS is triggered by proinflammatory cytokines and chemokines produced by the autoimmune T cells after their entry into the CNS. Notably, the encephalitogenic T cells need to be preactivated in the periphery before they penetrate into the CNS parenchyma. A large portion of the pathogenic Th cells that mediate autoimmunity secrete proinflammatory cytokines and chemokines after recognizing self-antigen in a major histocompatibility complex (MHC) class II-restricted manner. Previously it was thought that $CD4^+$ IFN-γ-secreting Th1 cells played a central role in causing autoimmune diseases, but the discovery of Th17 cells with a significant pathogenic activity in autoimmunity has opened the gate for new directions of research [2, 3]. Although interleukin (IL)-12 controls Th1 differentiation from naive $CD4^+$ T cells, transforming growth factor (TGF)-β in combination with IL-6 is appreciated as the classical Th17 cell-differentiating cytokine milieu. Th1 cells express the lineage marker transcription factor T-bet; Th17 cells express another transcription factor, ROR-γt. There is now a consensus that both Th1 and Th17 cells contribute to the development of autoimmune disease, including MS, and that the relative contributions of either of these different helper T-cell populations might explain diversity in clinical and pathological manifestations of autoimmune diseases as well as in their response to therapy.

Experimental autoimmune encephalomyelitis (EAE) is a prototype autoimmune disease model that has greatly contributed to elucidating the pathogenesis of MS. EAE can be induced in laboratory animals by active immunization with myelin antigens or by passive transfer of myelin antigen-specific T cells. Because Th1 cell clones reactive to MBP, PLP, or MOG are capable of inducing clinical and pathological

manifestations of EAE in naive mice, Th1 cells producing interferon (IFN)-γ have long been believed to play a central role in the pathogenesis of MS. However, the "Th1 disease" dogma has been challenged by contradicting results obtained from rodent models of MS. Namely, despite obvious lack of Th1 cells, gene-targeted mice deficient for IFN-γ or IFN-γ receptor are still susceptible to EAE. Furthermore, mice deficient for IL-12 signaling were found to develop EAE. Subsequent studies have clarified that IL-23 rather than IL-12 is essential for the development of EAE. Lately, it was revealed that the IL-23-dependent pathogenic T cells would represent Th17 cells, a novel helper T-cell subset characterized by production of IL-17. Currently, it is widely appreciated that Th17 cells play an important role in the development of inflammatory autoimmune diseases, either independently or collaboratively with Th1 cells.

DNA microarray analysis previously revealed upregulation of IL-17 as well as the downstream transcripts in the brain lesions of MS. More recently, a pathological study has demonstrated that IL-17-secreting T cells are present in active rather than chronic lesions of MS. These results indicate that Th17 cells actively participate in the autoimmune inflammation in the MS brain. Gene expression profiling for brain lesions of MS provided a number of potential candidate molecules that might be appropriate as a therapeutic target. Similarly, microarray analysis of peripheral blood could help characterize a disease signature of MS, leading to an identification of potential therapeutic targets. We have previously characterized gene expression profile of peripheral blood T cells derived from Japanese MS patients and found that expression of the nuclear orphan receptor NR4A2 is most significantly augmented in MS compared with healthy subjects [4]. Quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) analysis consolidated the overexpression of NR4A2 in the peripheral T cells of MS, and expression of NR4A2 in T cells from MS showed an approximately fivefold increase compared to those from healthy donors. NR4A2 mutations are well known to cause familial Parkinson's disease, reflecting its essential role in the development and survival of substantia nigra neurons. In contrast, much less attention has been paid to its functional role in T cells. More than a decade ago, NR4A1 and NR4A3 were shown to mediate apoptotic processes of mature as well as immature T cells. However, these studies do not give insights into the functional implication of upregulated expression of NR4A2 in T cells from MS. Therefore, we have begun functional analysis of NR4A2 as an important molecule regulating the Th1/Th17 cell function through expression of key cytokines including IL-17 and IFN-γ in the pathogenesis of MS. Although NR4A2 is a transcription factor of the steroid/thyroid receptor family and has been implicated in various cellular responses such as steroidogenesis, neuronal development, atherogenesis, and cell-cycle regulation, the physiological role of NR4A2 in the development or regulation of T-cell-mediated autoimmune diseases is unknown. Therefore, we have explored the functional involvement of NR4A2 in EAE, a representative rodent model of MS. To explore the possible involvement of NR4A2 in EAE induced in B6 mice by immunization with MOG peptide, T-cell expression of NR4A2 was measured by quantitative RT-PCR. NR4A2 expression in peripheral T cells gave a maximum value on day 21, and the entire expression pattern of NR4A2 in peripheral blood mononuclear cell (PBMC) T cells was well correlated with the clinical severity of EAE. Meanwhile, remarkable expression of NR4A2 was

observed in the CNS-infiltrating T cells on day 9, when an early sign of EAE becomes evident. NR4A2 expression was induced in EAE T cells, but the kinetics of expression significantly differs between PBMC T cells and CNS-infiltrating T cells. Recent studies have indicated that autoimmune Th17 rather than Th1 cells would play a central role in causing autoimmune inflammation, and a major proportion of the CNS-infiltrating cells were found to produce IL-17 or IFN-γ. Therefore, T cells accumulating in the CNS are characterized by massive production of inflammatory cytokines with an intensive expression of NR4A2. The concomitant expression of inflammatory cytokines and NR4A2 in the CNS-infiltrating T cells has guided us to investigate whether NR4A2 directly affects gene expression of inflammatory cytokines as a transcription factor in T cells. Luciferase assay for IL-17 promoter has revealed that transduction of NR4A2 gene would result in upregulation of promoter activity for IL-17 in the EL4 cell line. In addition, NR4A2 transduction by retroviral infection containing NR4A2 gene fragment with GFP into CD4 T cells showed an enhancement of IL-17 expression. Intriguingly, when the small interfering RNA (siRNA) specific for NR4A2 was introduced into encephalitogenic T cells induced after immunization with PLP peptide into SJL mice, progression of clinical disease and histological severity of EAE passively induced by the encephalitogenic T cells was significantly prevented in the siRNA-treated group as compared with control RNA-treated group. Furthermore, evaluation of cytokines in the supernatant has revealed that the siRNA treatment significantly reduced the production of IL-17 by T cells from both healthy donors and MS patients. These results support the important role of NR4A2 in the regulation of cytokine production in pathogenic T cells and modulation of NR4A2 expression by specific siRNAs or putative chemical compounds might be a promising treatment for intervention of active MS that are harboring more potent IL-17-producing T cells [5] (Table 1).

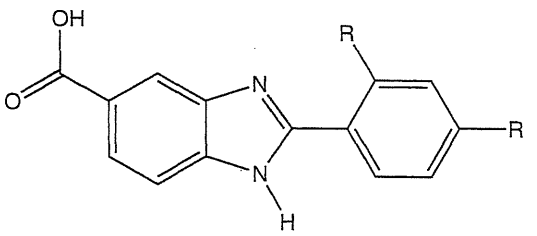
Direct and Indirect Modulators of NR4A2

As summarized in the previous section, we have identified NR4A2 as the most highly upregulated gene among circulating T cells in MS patients. In addition, we observed increased expression of NR4A2 transcripts in the blood and CNS of mice following EAE induction. Forced expression of NR4A2 led to enhanced Th1 and Th17 responses whereas reducing NR4A2 expression decreased the encephalitogenicity of autoimmune T cells in a model of passive EAE. Therefore, NR4A2 has been implicated as a promising molecular target for MS therapy. Intriguingly, the NR4A subfamily of nuclear receptors has been implicated not only in MS but also in rheumatoid arthritis, psoriasis, atherogenesis, Parkinson's disease, schizophrenia, manic depression, Alzheimer's disease, and cancer, which has led to great interest in the identification of selective low molecular weight modulators that is helpful for analyzing the mode of action of the NR4A subfamily [6].

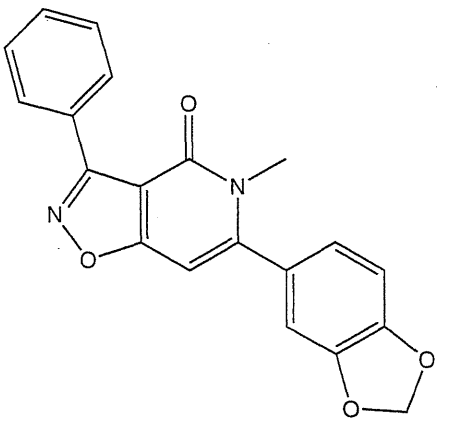
An antineoplastic and antiinflammatory drug, 6-mercaptopurine, has been shown to activate NR4A2, possibly by modulating the cellular content of purine nucleotides. NR4A2 expression is induced by forskolin through the activation of Erk1/2. A number of typical and atypical antipsychotic drugs such as haloperidol, chlorpromazine,

and clozapine induce the transcription of NR4A2. In contrast, methotrexate significantly suppresses expression of NR4A2 in patients with active psoriatic arthritis. Interestingly, the expression level of NR4A2 after treatment with methotrexate is well correlated with disease activity score. In addition, glucocorticoid has been shown to inhibit NR4A2 expression. Although growing numbers of modulators have been described, all these indirect modulators are selective enough to intervene NR4A2 activity. Therefore, the therapeutic utility of NR4A2 will depend on whether novel modulators directly interacting with NR4A2 can be developed.

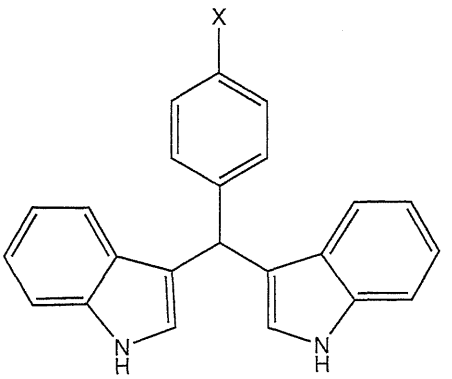
Using a NR4A2 luciferase reporter gene assay, a number of micromolar activators of NR4A2 has been identified in a combinatorial library of benzimidazole, which has a structural overlap with known nuclear receptor ligands [7]. After screening of substituents having a structurally different moiety, a couple of potent NR4A2 activators (EC_{50} = 8–70 nM) was developed based on this benzimidazole scaffold. Another approach using a similar reporter gene assay identified a novel class of NR4A2 activators, isoxazolo[4,5-*c*]pyridin-4-one [8]. After screening of substituents having a structurally different moiety, a couple of very potent NR4A2 activators (EC_{50} = 0.8–3.9 nM) were developed based on this scaffold. Through pharmacokinetic experiments, some of these compounds have been shown to have excellent oral bioavailability and rapid distribution in mice. Even though most of these compounds are NR4A2 agonists, there is another class of NR4A2 modulators, 1,1-bis(3′-indolyl)-1-(*p*-substituted phenyl) methanes, that provide both NR4A2 agonist and antagonist [9] (Fig. 2). Ligands for RXR are



Benzimidazole derivatives



Isoxazolo(4,5-*c*)pyridin-4-one



1,1-Bis(3'-indolyl)-1-(*p*-substitutedphenyl) methanes

Fig. 2 Known chemical structures directly modulating NR4A2 function. *R* represents alkyl side chain; *X* is either H, OH, OMe, or CF3

another class of potent NR4A2 modulators as RXR potentially forms a heterodimer with NR4A2. The weak RXR agonist HX600 was found to activate NR4A2-RXR heterodimers [10].

Conclusion

We have demonstrated that NR4A2 may represent a promising target for MS and other autoimmune diseases. There were some clinical trials for MS of neutralizing the relevant cytokines IL-17 and IL-23 by specific antibodies, neither of which revealed significant beneficial effects on MS symptoms. Our results demonstrated that NR4A2 acts as an early event in the differentiation of pathogenic T cells, and thus modulating NR4A2 activity may allow a more complete inhibition of effector responses of pathogenic T cells compared with cytokine neutralization.

References

1. Maxwell MA, Muscat GE (2006) The NR4A subgroup: immediate early response genes with pleiotropic physiological roles. Nucl Recept Signal 4:e002
2. Aranami T, Yamamura T (2008) Th17 cells and autoimmune encephalomyelitis (EAE/MS). Allergol Int 57(2):115–120
3. McFarland HF, Martin R (2007) Multiple sclerosis: a complicated picture of autoimmunity. Nat Immunol 8(9):913–919
4. Satoh J, Nakanishi M, Koike F, Miyake S, Yamamoto T, Kawai M et al (2005) Microarray analysis identifies an aberrant expression of apoptosis and DNA damage-regulatory genes in multiple sclerosis. Neurobiol Dis 18(3):537–550
5. Doi Y, Oki S, Ozawa T, Hohjoh H, Miyake S, Yamamura T (2008) Orphan nuclear receptor NR4A2 expressed in T cells from multiple sclerosis mediates production of inflammatory cytokines. Proc Natl Acad Sci USA 105(24):8381–8386
6. Mattes H (2008) NR4A Subfamily of receptors and thier modulators. In: Ottow E, Weinmann H (eds) Nuclear receptors as drug target. Methods and principles in medicinal chemistry, vol 39. Wiley-VCH, New York
7. Dubois C, Hengerer B, Mattes H (2006) Identification of a potent agonist of the orphan nuclear receptor Nurr1. ChemMedChem 1(9):955–958
8. Hintermann S, Chiesi M, von Krosigk U, Mathe D, Felber R, Hengerer B (2007) Identification of a series of highly potent activators of the Nurr1 signaling pathway. Bioorg Med Chem Lett 17(1):193–196
9. Chintharlapalli S, Burghardt R, Papineni S, Ramaiah S, Yoon K, Safe S (2005) Activation of Nur77 by selected 1,1-bis(3′-indolyl)-1-(*p*-substituted phenyl) methanes induces apoptosis through nuclear pathways. J Biol Chem 280(26):24903–24904
10. Morita K, Kawana K, Sodeyama M, Shimomura I, Kagechika H, Makishima M (2005) Selective allosteric ligand activation of the retinoid X receptor heterodimers of NGFI-B and Nurr1. Biochem Pharmacol 71(1–2):98–107