Contribution of Retinoid X Receptor Signaling to the Specification of Skeletal Muscle Lineage*

Received for publication, January 31, 2011, and in revised form, June 6, 2011 Published, JBC Papers in Press, June 8, 2011, DOI 10.1074/jbc.M111.227058

Melanie Le May‡, Hymn Mach§, Natascha Lacroix§, Chencheng Hou§, Jihong Chen§, and Qiao Li‡§

From the Department of Pathology and Laboratory Medicine and Department of Cellular and Molecular Medicine, Faculty of Medicine, University of Ottawa, Ottawa, Ontario K1H 8M5, Canada

Pluripotent stem cells possess a tremendous potential for the treatment of many diseases because of their capacity to differentiate into a variety of cell lineages. However, they provide little promise for muscle-related diseases, mainly because of the lack of small molecule inducers to efficiently direct myogenic conversion. Retinoic acid, acting through the retinoic acid receptor (RAR) and retinoid X receptor (RXR), affects stem cell fate determination in a concentration-dependent manner, but it only has a modest efficacy on the commitment of ES cells into skeletal muscle lineage. The RXR is very important for embryonic development but is generally considered as a silent partner of RAR in a non-permissive mode. In this study, we have examined whether activation of the RXR by rexinoid or RXR-specific signaling play a role in the specification of stem cells into muscle lineage. Our findings demonstrate that mouse ES cells generate skeletal myocytes effectively upon treatment with rexinoid at the early stage of differentiation and that on a molecular level, rexinoid-enhanced myogenesis simulates the sequential events observed in vivo. Moreover, RXR-mediated myogenic conversion requires the function of β-catenin but not RAR. Our studies establish the feasibility of applying RXR as a heterodimer within the genes they govern and, permissive heterodimers or homodimers (21, 22).

The temporal expression pattern of MRFs in ES cells reflects the sequential events observed during skeletal myogenesis in vivo (8). Similar to the ES cell system, pluripotent embryonic carcinoma cells respond well to developmental cues in vitro to differentiate into the cell types of all three germ layers (9). The differentiation of embryonic carcinoma stem cells simulates the molecular and cellular processes that occur during ES cell differentiation and early embryonic development (10). The pluripotent stem cells are promising resources for cell-based therapies but have proved difficult to apply in muscle-related diseases, mainly because of the lack of small molecule inducers to effectively direct skeletal myogenic conversion (11).

Retinoic acid (RA) is essential for a broad array of biological processes, including vertebrate body shaping, tissue homeostasis, apoptosis, and cell differentiation (12, 13). High concentrations of RA (>10−7 M) enhance neuronal differentiation but inhibit myogenesis, whereas low concentrations (<10−7 M) enhance myogenic conversion in ES and embryonic carcinoma cells (14–16). The diverse effects of RA are primarily mediated through retinoic acid receptors (RAR), which act as ligand-inducible transcription factors to regulate RA-responsive genes (17). The function of RAR depends on retinoid X receptors (RXR). RAR bind to specific DNA constitutively with the RXR as a heterodimer within the genes they govern and, upon ligand induction, recruit the p300 coactivator complex to activate gene transcription (18, 19). The RXR-RXR dimer binds to consensus sequences, including a DR5 motif, in which ligand induction is through RAR, whereas RXR is generally considered a silent partner (20). In addition to RAR, RXR can dimerize with one-third of the known nuclear receptors, and RXR is amenable to ligand activation in the permissive heterodimers or homodimers (21, 22).

Although RA is the best characterized inducer for myogenic conversion, it only has a modest efficacy on ES cells. Thus, it is imperative to comprehend on a molecular level how different signaling pathways converge to regulate the specification of muscle lineage to find efficient inducers that can produce large quantities of skeletal myocytes. In this study, we have examined the mechanisms of signaling-dependent events during myogenic conversion. Our studies have determined a role for RXR-specific signaling in this process and identified the RXR agonist as an effective inducer for the differentiation of ES cells into skeletal myocytes.
Retinoid X Receptor and Skeletal Myogenesis

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—P19 cells (ATCC) were grown in minimal essential medium α (Invitrogen) supplemented with 5% of fetal bovine serum, 5% of bovine calf serum (PAA), and 1% penicillin/streptomycin. After 4 days of aggregation in Petri dishes, the cells were transferred to tissue culture dishes, and coverslips were coated with 0.1% gelatin and grown for a further 5 days. D3 ES cells (ATCC) were maintained in DMEM (Invitrogen) supplemented with 15% of fetal bovine serum (PAA), 1% of penicillin/streptomycin, 1% of non-essential amino acids (Invitrogen), and 1.18 mM β-mercaptoethanol. Maintenance cultures were supplemented with 1000 units/ml of leukemia inhibitory factor (Chemicon). For differentiation, cells were grown in hanging drops for 48 h after which they were washed into Petri dishes and maintained for a further 5 days in suspension. Cells were then transferred to tissue culture dishes and coverslips or harvested for real-time RT-PCR and Western blotting analysis. D3 terminal differentiation medium was DMEM F12 supplemented with 1% N2 (Invitrogen) and 1% penicillin/streptomycin. RA was from Sigma-Aldrich, bexarotene from LC Laboratories, and Ro 41-5253 from Biomol International.

Immunofluorescence Microscopy—The cells were fixed on coverslips as described previously (23) and incubated with indicated primary antibodies overnight at 4 °C after which they were incubated with fluorescent secondary antibodies followed by incubation with 25 ng/ml of Hoechst (Molecular Probes) for quantification. Cells were then transferred to tissue culture dishes and coverslips were coated with 0.1% gelatin and grown for a further 5 days in suspension. Cell aggregates were sonicated, and DNA was quantified by the Bradford Method (Bio-Rad) using a protein assay (Bio-Rad). Membranes were incubated overnight in primary antibody followed by incubation with secondary antibody and visualized using Western Lightning chemiluminescence reagents (PerkinElmer Life Sciences). Quantification was performed using Scion Image (Scion Corp.). Primary antibodies used were anti-p300, RAR-α, RAR-β (Santa Cruz Biotechnology), anti-myogenin (F5D hybridoma), and anti-β-tubulin (E7 hybridoma).

Real-time RT-PCR Analysis—Total RNA was isolated using a Total RNA Kit I according to the manufacturer’s recommendations (Omega), and reverse-transcribed using a high-capacity cDNA reverse transcription kit (Applied Biosystems) as described previously (28). Real-time PCR was performed using a SYBR Green and ROX PCR Master Mix HotStarTaq DNA polymerase kit (Qiagen) and conducted on the Applied Biosystems 7500 Fast real-time PCR system. The amount of targets, normalized to the GAPDH endogenous reference and relative to calibrator control, was calculated using the arithmetic formula 2−ΔΔCT.

Transfection and Luciferase Assay—Transient transfection was performed with a reporter plasmid by using ExGen 500 as described previously (29). Briefly, P19 aggregates were transfected with a RAR luciferase and a RSV-β-gal reporter and then induced with bexarotene or RA. A luciferase assay was performed according to the manufacturer’s recommendations (Promega). Luciferase activities are expressed as fold induction relative to the untreated controls after being normalized to the β-galactosidase activity.

RESULTS

Effects of Rexinoid on Myogenic Conversion of Pluripotent P19 Cells—Pluripotent P19 cells are an excellent stem cell system to study cellular differentiation and to identify small molecule inducers for the specification of muscle lineage (9). They can be induced into differentiation with aggregated cultures. However, in the absence of exogenous stimuli or small molecule inducers, aggregation leads only to the expression of markers of the mesoderm but not myoblasts (30). Treatment of aggregates with DMSO induces small percentages of skeletal myocytes, whereas addition of all-trans RA enhances the commitment of muscle lineage (26). As shown in Fig. 1A, DMSO treatment induced about 5% of skeletal myocytes by day 9 of differentiation. In contrast, cotreatment of cells with RA enhanced myogenic conversion to about 15%, as determined by quantitative microscopy of myosin heavy chain and MyoD staining (Fig. 1A). Moreover, MyoD and myogenin heavy chain were also detected by day 9, which is indicative of skeletal myocyte identity (Fig. 1, B and C). Thus, RA signaling is important for the specification and development of skeletal muscle lineage.

Because RXR is very important for the early stages of embryonic development (31–33), we next used bexarotene, an RXR-selective agonist, to determine the impact of RXR signaling on the specification of muscle lineage. In the presence of DMSO, bexarotene enhanced myogenic conversion in a concentration-dependent manner and achieved an efficacy similar to RA (Fig. 2, A and B). MyoD and myogenin protein were also detected by

noblott PVDF membrane (Bio-Rad). Membranes were incubated overnight in primary antibody followed by incubation with secondary antibody and visualized using Western Lightning chemiluminescence reagents (PerkinElmer Life Sciences). Quantification was performed using Scion Image (Scion Corp.). Primary antibodies used were anti-p300, RAR-α, RAR-β (Santa Cruz Biotechnology), anti-myogenin (F5D hybridoma), and anti-β-tubulin (E7 hybridoma).
day 9 of differentiation and expressed at comparable levels to RA treatment (Fig. 2, C and D). In consistence with literature (26), the transcripts of Meox1 and Pax3 were increased by RA on day 4, whereas MyoD increased on day 9 of differentiation (Fig. 2E). Intriguingly, bexarotene caused a greater increase in the Meox1 transcript level than RA, whereas RA caused a larger increase in the Pax3 transcript level than bexarotene (Fig. 2E). Thus, the temporal expression pattern of myogenic-specific regulators induced by
Bexarotene in the P19 model is similar to that seen in vivo, and the RXR agonist is an effective inducer for the commitment of skeletal muscle lineage.

Effects of Rexinoid on the Differentiation of ES Cells into Skeletal Myocytes—Because ES cells have proved largely unresponsive to RA in the context of myogenic conversion, we next tested rexinoid in this system. A hanging-drop method was used to form embryoid bodies (EB) to induce ES cell differentiation, whereas DMSO was omitted from the protocol because of toxicity to the cells. Different concentrations of bexarotene were used to treat the embryoid bodies, and the development of myoblasts was examined by immunofluorescence microscopy. Consistent with previous reports, RA had a modest efficacy, about 3%, at converting the ES cells into skeletal myocytes (Fig. 3, A and B). In contrast, bexarotene was 5-fold more potent than RA and significantly increased the conversion of myogenic lineage to about 16% (Fig. 3, A and B). Moreover, bexarotene was also more efficient at inducing the expression of myogenin protein (Fig. 3C). As with the P19 cell system, bexarotene increased Meox1 transcripts about 11-fold more than RA, whereas RA was significantly more effective at augmenting Pax3 transcripts (Fig. 3D). Again the levels of MyoD transcripts appeared to reflect the efficacies of myogenic conversion (Fig. 3D). Together these findings demonstrate that the RXR agonist is a more effective inducer than RA to specify ES cells into skeletal muscle lineage.

Roles of β-Catenin, Meox1, and Pax3 in Rexinoid-enhanced Myogenic Conversion—To delineate the molecular pathway of rexinoid action in myogenic differentiation, we next used the P19 cell system to take advantage of several established stable cell lines. RA-enhanced skeletal myogenesis depends on the function of β-catenin (26). To determine the requirement of β-catenin for rexinoid-enhanced myogenesis, we employed a clone of cells stably expressing a dominant negative β-catenin in which the transcriptional activation domain is replaced by an engrailed repressor domain (26). The engrailed repression domain (EN-2) silences gene transcription by interacting with the members of the Groucho/transducin-like enhancer family of transcriptional repressors (4). Cells harboring the empty vector were used as a control. As shown in Fig. 4, A and B, the control cells were differentiated into skeletal myocytes by bexarotene and RA with similar efficacies as the parental cells (compare with Fig. 2A). However, the dominant negative β-catenin cells failed to differentiate into skeletal myocytes regardless of treatments, and myogenin was not detected (Fig. 4A). Therefore, bexarotene, just as RA, cannot bypass the β-catenin pathway to direct skeletal myogenesis, and the function of β-catenin is very important for RXR-mediated myogenic conversion.

Next, we employed Meox1 and Pax3 dominant negative cells (34, 35) to study the roles of Meox1 and Pax3 in rexinoid-enhanced myogenesis. As shown in figure 4C, DMSO did not convert these cells into myoblasts, in consistence with previous reports (34, 35). However, both bexarotene and RA induced the differentiation of the Meox1 and Pax3 dominant negative cells into skeletal myocytes, albeit with lower efficacies (Fig. 4A). Intriguingly, although the levels of engrailed Pax3 transcripts were comparable in the bexarotene- and RA-treated cells, the dominant negative Pax3 appeared to be less detrimental to the RA-induced myogenic conversion (Fig. 4, C and D). This may
be due to RA augmenting the endogenous Pax3 expression more effectively than bexarotene (Fig. 2E) to titrate out the interference of dominant negative Pax3. Taken together, these data suggest that rexinoid directs skeletal myogenesis through molecular pathways distinct from RA.

RAR-independent RXR Signaling in Myogenic Conversion—A RAR binding region has been identified previously at the Pax3 locus (26). This region contains a DR5 motif, two direct repeats of the consensus sequence (5'PuGGTCA) separated by five nucleotides, for occupancy by a RAR-RXR heterodimer (36, 37). We therefore examined the binding of RAR and RXR to this Pax3 locus. ChIP analysis showed that both RAR and RXR occupy this region constitutively (Fig. 5, A and B), consistent with the binding model of the DR5 motif (25). The recruitment of transcriptional coactivator p300 to this region, however, was only augmented by the addition of RA but not bexarotene, although the levels of p300 protein in these cells were constant (Fig. 5, C and D). Thus, RXR possibly acts as a silent partner of RAR at this locus to augment Pax3 gene expression. We also performed a ChIP analysis with a β-catenin antibody to examine the specificity of the ChIP protocol and did not detect apparent β-catenin binding to this locus, regardless of treatment in comparison to the IgG-negative ChIP control (n = 4).

We next used a reporter approach to examine the effects of rexinoid on transactivation requiring RXR acting as a silent partner of RAR. The cells were transfected with a well characterized DR5 RA-responsive reporter (38) during aggregation and treated with bexarotene or RA. As shown in Fig. 5E, RA, but not bexarotene, was able to transactivate the reporter. These data demonstrate that rexinoid does not affect the binding motif for a RAR-RXR heterodimer in the P19 differentiation system, suggesting an additional role for RXR in the specification of skeletal muscle lineage, acting through an activated or permissive mode.

To delineate whether RAR is required for rexinoid-enhanced skeletal myogenesis, we used RAC65 cells, which contain a dominant negative RAR-α that effectively blocks the DNA binding of wild-type receptors (39, 40). These cells are non-responsive to the RAR agonist but respond to the RXR agonist for neuronal differentiation (41, 42). As shown in figure 5F, bexarotene, but not RA, enhanced the specification of skeletal muscle lineage in the RAC65 cells. To further examine the role of RAR in rexinoid-enhanced myogenic conversion, we also used Ro 41-5253, a RAR-specific antagonist (43). As shown in Fig. 5G, Ro 41-5253 at 10 nM concentration selectively inhibited RA-enhanced skeletal muscle development, whereas bexarotene-enhanced myogenesis was not compromised. Thus, RXR directs the specification of skeletal muscle lineage through mechanisms independent of RAR.
DISCUSSION

We have examined whether the activation of RXR by rexinoid or RXR-specific signaling plays a role in myogenic differentiation. Our findings show that mouse ES cells can effectively generate skeletal myocytes following induction with the RXR agonist at the early stage of differentiation and that the molecular pathways involved in rexinoid-directed skeletal myogenesis recapitulate the sequential events observed in vivo.

Furthermore, rexinoid-enhanced myogenic conversion is mediated in a RAR-independent manner. Our studies establish the feasibility of applying rexinoid in stem cell therapies, particularly exploring RXR-specific signaling to convert ES cells into skeletal muscle lineage. The aptitude of ES cells to generate myocytes in response to rexinoid also offers a model system to decipher the complex signaling cascade implicated in skeletal muscle development and to develop non-toxic protocols for generating large quantities of skeletal myocytes.

Pluripotent stem cells, regardless of their origin, possess the potential of developing into skeletal myocytes, among many other cell lineages. The central issue is how to control a specific signaling pathway to preferentially enhance myogenic conversion in an efficacy suitable for clinical therapies. RA is able to enhance skeletal myogenesis in pluripotent EC stem cells if used in combination with other small molecular inducers such as DMSO (Fig. 1). However, DMSO is toxic to ES cells, and RA alone has only a modest effect on the differentiation of ES cells into myogenic lineage (Fig. 3). We found that rexinoid is a more effective inducer than RA for skeletal myogenesis in the ES cell system (Fig. 3). This is significant because to date there has been very little success at directing ES cells into skeletal muscle lineage, and thus, no methods are currently available to generate a sufficient population of skeletal myocytes for potential cell-based therapies.

In addition, our studies also shed new light on the role of RXR in skeletal myogenesis. It is well known that RXR is important for development, and RXR is generally considered to act as the silent partner of RAR in a non-permissive mode. Our studies establish a role of RXR-specific signaling in the differentiation of stem cells into skeletal muscle lineage, which is independent of RAR and distinct from the action mode of the RAR-RXR heterodimer (Fig. 5). Although the early specification events differ between rexinoid- and RA-enhanced myogenesis, both inducers critically depend on the function of β-catenin, which is required for MyoD expression (Fig. 4).

It appears that rexinoid has a significant impact on an early differentiation marker, Meso1, whereas RA may require DMSO cotreatment to activate Meso1 (Figs. 2 and 3). Our studies have not investigated other genomic targets activated by RAR-specific signaling besides the known MRFs. Additional systems studies are needed to uncover novel signaling pathways involved in rexinoid-enhanced skeletal myogenesis and to determine why the RXR agonist is a more suitable inducer for the ES cell system. Understanding the molecular mechanisms of myogenic specification is very important for manipulating stem cell fate determinations in cell-based therapies. We have...
Retinoid X Receptor and Skeletal Myogenesis

identified a potent inducer for a non-toxic protocol to direct the specification of muscle lineage. The efficacy with which retinoid is able to convert ES cells into skeletal myocytes suggests a potential to extend this strategy to human ES cells and to other types of pluripotent stem cells in view of generating functional skeletal myocytes.

Acknowledgements—We thank Dr. L. Wang for the support on ES cultures; Dr. I. S. Skerjanc for the dominant negative β-catenin, Meox1, and Pax3 stable cell lines; Dr. A. Blais for the MF20 and E7 hybridoma lines; and Dr. B. Jasmin for the FSD line.

REFERENCES

1. Tapscott, S. J. (2005) Development 132, 2685–2695
2. Weintraub, H., Tapscott, S. J., Davis, R. L., Thayer, M. J., Adam, M. A., Lassar, A. B., and Miller, A. D. (1989) Proc. Natl. Acad. Sci. U.S.A. 86, 5434–5438
3. Francetic, T., and Li, Q. (2011) Transcription 2, 109–114
4. Chen, G., and Courey, A. J. (2000) J. Biol. Chem. 275, 21999–22005
5. Williams, B. A., and Ordahl, C. P. (1994) Development 120, 785–796
6. Petropoulos, H., and Skerjanc, I. S. (2002) Dev. Biol. 277, 15393–15399
7. Djakovich, S., Rocancourt, D., Cosu, G., and Buckingham, M. (1997) Cell 89, 127–138
8. Rohwedel, J., Maltsev, V., Bober, E., Arnold, H. H., Hescheler, J., and Wobus, A. M. (1994) Dev. Biol. 164, 87–101
9. Yu, J., and Thomson, J. A. (2008) Genes Dev. 22, 1987–1997
10. Rudnicki, M. A., Reuhl, K. R., and McBurney, M. W. (1989) Development 107, 361–372
11. Allsopp, T. E., Bunnage, M. E., and Fish, P. V. (2010) J. Biol. Chem. 285, 1675–1683
12. Berbis, P. (2010) Ann. Dermatol. Venereol. 137, S97–S103
13. Mark, M., Ghyselinck, N. B., and Chambon, P. (2009) Nucl. Recept. Signal 7, 6002
14. Jones-Villeneuve, E. M., McBurney, M. W., Rogers, K. A., and Kalnins, V. I. (1982) J. Cell Biol. 94, 253–262
15. Sato, Y., and Heuckeroth, R. O. (2008) Dev. Biol. 320, 185–198
16. Hescheler, J., Fleischmann, B. K., Lentini, S., Maltsev, V. A., Rohwedel, J., Wobus, A. M., and Addicks, K. (1997) Cardiovasc. Res. 36, 149–162
17. Niedereither, K., and Dollé, P. (2008) Nat. Rev. Genet. 9, 541–553
18. Lonard, D. M., and O’Malley, B. W. (2007) Mol. Cell 27, 691–700
19. Hermanson, O., Glass, C. K., and Rosenfeld, M. G. (2002) Trends Endocrinol. Metab. 13, 55–60
20. Kurokawa, R., DiRenzo, J., Boehm, M., Sugarman, J., Gloss, B., Rosenfeld, M. G., Heyman, R. A., and Glass, C. K. (1994) Nature 371, 528–531
21. Mangelsdorf, D. J., Thummel, C., Beato, M., Herrlich, P., Schütz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P., and Evans, R. M. (1995) Cell 83, 835–839
22. Gampe, R. T., Jr., Montana, V. G., Lambert, M. H., Wisely, G. B., Milburn, M. V., and Xu, H. E. (2000) Genes Dev. 14, 2229–2241
23. Chen, J., Halappanavar, S., Thi’ng, J. P., and Li, Q. (2007) Epigenetics 2, 92–99
24. St-Germain, J. R., Chen, J., and Li, Q. (2008) Epigenetics 3, 342–349
25. Higazi, A., Abed, M., Chen, J., and Li, Q. (2011) Epigenetics 6, 202–211
26. Kennedy, K. A., Porter, T., Mehta, V., Ryan, S. D., Price, F., Peshdary, V., Karamboulas, C., Savage, J., Drysdale, T. A., Li, S. C., Bennett, S. A., and Skerjanc, I. S. (2009) BMC Biol. 7, 67
27. Chen, J., Ghazawi, F. M., Bakkar, W., and Li, Q. (2006) Mol. Cancer 5, 1
28. Chen, J., Ghazawi, F. M., and Li, Q. (2010) Epigenetics 5, 509–515
29. Chen, J., Halappanavar, S. S., St-Germain, J. R., Tsang, B. K., and Li, Q. (2004) Cell. Mol. Life Sci. 61, 1675–1683
30. McBurney, M. W. (1993) Int. J. Dev. Biol. 37, 135–140
31. Apel, V., Dollè, P., Hindelang, C., Kastner, P., and Chambon, P. (1997) Dev. Biol. 191, 29–41
32. Suov, H. M., Dyson, E., Gumeringer, C. L., Price, J., Chien, K. R., and Evans, R. M. (1994) Genes Dev. 8, 1007–1018
33. Kastner, P., Grondona, J. M., Mark, M., Gansmuller, A., LeMeur, M., Decimo, D., Vonesch, J. L., Dollè, P., and Chambon, P. (1994) Cell 78, 987–1003
34. Petropoulos, H., Gianakopoulos, P. J., Ridgeway, A. G., and Skerjanc, I. S. (2004) J. Biol. Chem. 279, 23874–23881
35. Rigby, A. G., and Skerjanc, I. S. (2005) J. Biol. Chem. 280, 19033–19039
36. Tanaka, T., and De Luca, L. M. (2009) Cancer Res. 69, 4945–4947
37. Umesono, K., and Evans, R. M. (1989) Cell 57, 1139–1146
38. Chen, J., St-Germain, J. R., and Li, Q. (2005) Mol. Cell. Biol. 25, 525–532
39. Pratt, M. A., Kralova, J., and McBurney, M. W. (1990) Mol.Cell. Biol. 10, 6445–6453
40. Costa, S. L., and McBurney, M. W. (1996) Exp. Cell Res. 225, 35–43
41. Jones-Villeneuve, E. M., Rudnicki, M. A., Harris, J. F., and McBurney, M. W. (1983) Mol. Cell. Biol. 3, 2271–2279
42. Yokota, Y., and Okuburo, H. (1996) Exp. Cell Res. 228, 1–7
43. Wang, X. J., Hayes, J. D., Henderson, C. J., and Wolf, C. R. (2007) Proc. Natl. Acad. Sci. U.S.A. 104, 19589–19594
44. Hirst, C. E., Ng, E. S., Azzola, L., Voss, A. K., Thomas, T., Stanley, E. G., and Elefanty, A. G. (2006) Dev. Biol. 293, 90–103
45. Darabi, R., Gehleb, K., Bachoo, R. M., Kamath, S., Osawa, M., Kamm, K. E., Kyba, M., and Perlingeiro, R. C. (2008) Nat. Med. 14, 134–143