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(54)【発明の名称】ＦＫＢＰ５１／５２及びＣＹＰ４０により媒介される哺乳類の発毛

(57)【要約】
本発明は、発毛を調節する非免疫抑制性作用物質を特定するための薬物スクリーニングアッセイと、発毛の調節のためのこうした作用物質の使用法とに関する。

【特許請求の範囲】
【請求項１】
発毛を調節し得る化合物を特定する方法であって、
（ｖ）ＦＫＢＰ５１／５２又はＣｙＰ４０と、ｈｓｐ９０と、無毛、ＡＦＸ－１、及びＧｌｉ３で構成されるグループから選択されたタンパク質とを発現する細胞、或いは、これらを含む調製物を、試験化合物に接触させるステップと、
（ｖｉ）試験化合物に接触させた細胞又は調製物内における、ＦＫＢＰ５１／５２又はＣｙＰ４０と、ｈｓｐ９０と、無毛、ＡＦＸ－１、及びＧｌｉ３で構成されるグループから選択された少なくとも一種類のタンパク質との間での複合体形成レベルを判定するステップと、
（ｖｉｉ）（ｉｉ）で得られた複合体形成レベルを、試験化合物が存在しない状態でのＦＫＢＰ５１／５２又はＣｙＰ４０と、ｈｓｐ９０と、無毛、ＡＦＸ－１、及びＧｌｉ３で構成されるグループから選択された少なくとも一種類のタンパク質との間での複合体形成レベルと比較するステップと、
を含み、
試験化合物が存在する状態と存在しない状態とでの複合体形成レベルの差が、発毛調節活性との正相関を有する方法。
【請求項２】
複合体形成のレベルが、イムノアッセイを使用して検出されることを特徴とする請求項１記載の方法。
【請求項３】
試験化合物が免疫抑制活性を有するか否かを判定するステップを更に含み、こうした活性の欠如が、複合体形成レベルを改変する試験化合物が存在する状態で、発毛調節作用物質としての有用性との正相関を有することを特徴とする請求項１記載の方法。
【請求項４】
発毛を調節し得る化合物を特定する方法であって、
（ｉ）無毛、ＡＦＸ－１、又はＧｌｉ３遺伝子反応要素の転写制御下で核ホルモン受容体及びレポータ遺伝子を発現する細胞を試験化合物と接触させ、細胞内のレポータ遺伝子発現レベルを測定するステップと、
（ｉｉ）試験化合物が存在しない状態でのレポータ遺伝子発現レベルを測定するステップと、
（ｉｉｉ）（ｉ）及び（ｉｉ）で測定されたレポータ遺伝子発現レベルを比較するステップと、
を含み、
ステップ（ｉ）及び（ｉｉ）で測定されたレポータ遺伝子発現レベルの差が、試験化合物の発毛調節活性との正相関を有する方法。
【請求項５】
ステップ（ｉ）が、核ホルモン受容体リガンドの存在する状態で実行されることを特徴とする請求項４記載の方法。
【請求項６】
試験化合物が免疫抑制活性を有するか否かを判定するステップを更に含み、こうした活性の欠如が、レポータ遺伝子発現レベルを改変する試験化合物が存在する状態で、発毛調節作用物質としての有用性との正相関を有することを特徴とする請求項４記載の方法。
【請求項７】
核ホルモン受容体が、アンドロゲン核ホルモン受容体と、ビタミンＤ核ホルモン受容体と、レチノイン酸核ホルモン受容体と、アリル炭化水素核ホルモン受容体と、甲状腺刺激核ホルモン受容体とにより構成されるグループから選択されることを特徴とする請求項４記載の方法。
【請求項８】
発毛を促進し得る化合物を特定する方法であって、

（ｉ）無毛遺伝子産物を含む試料を試験化合物に接触させるステップと、
（ｉｉ）試験化合物が、無毛遺伝子産物と結合するか否かを判定するステップと、
（ｉｉｉ）試験化合物が、無毛遺伝子産物と、ＦＫＢＰ５１／５２、ＣｙＰ４０、核ホルモン受容体、ｈｓｐ９０タンパク質、及びその組み合わせで構成されるグループから選択された結合相手との間での複合体形成を阻害するか否かを判定するステップと、
を含み、
試験化合物が、無毛遺伝子産物と結合する能力と、複合体形成を阻害する能力とは、両方とも、発毛促進活性との正相関を有する方法。
【請求項９】
発毛を調節し得る化合物を特定する方法であって、
（ｉ）ＦＫＢＰ５１／５２又はＣｙＰ４０と、ｈｓｐ９０と、核ホルモン受容体と、無毛遺伝子産物とを発現する細胞を、核ホルモン受容体リガンドが存在する状態で、試験化合物に接触させるステップと、
（ｉｉ）無毛遺伝子産物の細胞核への核転座レベルを判定するステップと、
（ｉｉｉ）試験化合物が存在しない状態での各転座レベルを判定するステップと、
（ｉｖ）（ｉｉ）及び（ｉｉｉ）において測定した核転座レベルを比較するステップと、
を含み、
ステップ（ｉｉ）及び（ｉｉｉ）で測定された核転座レベルの差は、試験化合物の発毛調節活性との正相関を有する方法。
【請求項１０】
試験化合物が免疫抑制活性を有するか否かを判定するステップを更に含む、請求項８又は９記載の方法。
【請求項１１】
発毛を促進し得る化合物を特定する方法であって、
（ｉ）ＡＦＸ－１遺伝子産物を含む試料を試験化合物に接触させるステップと、
（ｉｉ）試験化合物が、ＡＦＸ－１遺伝子産物と結合するか否かを判定するステップと、
（ｉｉｉ）試験化合物が、ＡＦＸ－１遺伝子産物と、ＦＫＢＰ５１／５２、ＣｙＰ４０、核ホルモン受容体、ｈｓｐ９０タンパク質、及びその組み合わせで構成されるグループから選択された結合相手との間での複合体形成を阻害するか否かを判定するステップと、
を含み、
試験化合物が、ＡＦＸ－１遺伝子産物と結合する能力と、複合体形成を阻害する能力とは、両方とも、発毛促進活性との正相関を有する方法。
【請求項１２】
発毛を調節し得る化合物を特定する方法であって、
（ｉ）ＦＫＢＰ５１／５２又はＣｙＰ４０と、ｈｓｐ９０と、核ホルモン受容体と、ＡＦＸ－１遺伝子産物とを発現する細胞を、核ホルモン受容体リガンドが存在する状態で、試験化合物に接触させるステップと、
（ｉｉ）ＡＦＸ－１遺伝子産物の細胞核への核転座レベルを判定するステップと、
（ｉｉｉ）試験化合物が存在しない状態での各転座レベルを判定するステップと、
（ｉｖ）（ｉｉ）及び（ｉｉｉ）において測定した核転座レベルを比較するステップと、
を含み、
ステップ（ｉｉ）及び（ｉｉｉ）で測定された核転座レベルの差は、試験化合物の発毛調節活性との正相関を有する方法。
【請求項１３】
試験化合物が免疫抑制活性を有するか否かを判定するステップを更に含む、請求項１１又は１２記載の方法。
【請求項１４】
発毛を促進し得る化合物を特定する方法であって、
（ｉ）Ｇｌｉ３遺伝子産物を含む試料を試験化合物に接触させるステップと、
（ｉｉ）試験化合物が、Ｇｌｉ３遺伝子産物と結合するか否かを判定するステップと、

（ｉｉｉ）試験化合物が、Ｇｌｉ３遺伝子産物と、ＦＫＢＰ５１／５２、ＣｙＰ４０、核ホルモン受容体、ｈｓｐ９０タンパク質、及びその組み合わせで構成されるグループから選択された結合相手との間での複合体形成を阻害するか否かを判定するステップと、
を含み、
試験化合物が、Ｇｌｉ３遺伝子産物と結合する能力と、複合体形成を阻害する能力とは、両方とも、発毛促進活性との正相関を有する方法。
【請求項１５】
発毛を調節し得る化合物を特定する方法であって、
（ｉ）ＦＫＢＰ５１／５２又はＣｙＰ４０と、ｈｓｐ９０と、核ホルモン受容体と、Ｇｌｉ３遺伝子産物とを発現する細胞を、核ホルモン受容体リガンドが存在する状態で、試験化合物に接触させるステップと、
（ｉｉ）Ｇｌｉ３遺伝子産物の細胞核への核転座レベルを判定するステップと、
（ｉｉｉ）試験化合物が存在しない状態での各転座レベルを判定するステップと、
（ｉｖ）（ｉｉ）及び（ｉｉｉ）において測定した核転座レベルを比較するステップと、
を含み、
ステップ（ｉｉ）及び（ｉｉｉ）で測定された核転座レベルの差は、試験化合物の発毛調節活性との正相関を有する方法。
【請求項１６】
試験化合物が免疫抑制活性を有するか否かを判定するステップを更に含むことを特徴とする請求項１４又は１５の方法。
【請求項１７】
ＦＫＢＰ５１／５２、ＣｙＰ４０、又はｈｓｐ９０と、無毛、Ｇｌｉ３、及びＡＦＸ－１で構成されるグループから選択された少なくとも一つのタンパク質との間での複合体形成を調節する化合物を哺乳類に投与するステップを含む、哺乳類の発毛を調節する方法。
【請求項１８】
無毛、ＡＦＸ－１、及びＧｌｉ３タンパク質で構成されるグループから選択されたタンパク質の核転座を調節する化合物を哺乳類に投与するステップを含む、哺乳類の発毛を調節する方法。
【請求項１９】
ＡＦＸ－１又はＧｌｉ３により媒介される遺伝子発現を調節する化合物を哺乳類に投与するステップを含む、哺乳類の発毛を調節する方法。
【請求項２０】
請求項１のアッセイにより特定された化合物を哺乳類に投与するステップを含む、哺乳類の発毛を調節する方法。
【請求項２１】
請求項４のアッセイにより特定された化合物を哺乳類に投与するステップを含む、哺乳類の発毛を調節する方法。
【請求項２２】
請求項８のアッセイにより特定された化合物を哺乳類に投与するステップを含む、哺乳類の発毛を調節する方法。
【請求項２３】
請求項９のアッセイにより特定された化合物を哺乳類に投与するステップを含む、哺乳類の発毛を調節する方法。
【請求項２４】
請求項１１のアッセイにより特定された化合物を哺乳類に投与するステップを含む、哺乳類の発毛を調節する方法。
【請求項２５】
請求項１２のアッセイにより特定された化合物を哺乳類に投与するステップを含む、哺乳類の発毛を調節する方法。
【請求項２６】

請求項１４のアッセイにより特定された化合物を哺乳類に投与するステップを含む、哺乳類の発毛を調節する方法。
【請求項２７】
請求項１５のアッセイにより特定された化合物を哺乳類に投与するステップを含む、哺乳類の発毛を調節する方法。
【発明の詳細な説明】
【技術分野】
【０００１】
本発明は、皮膚科学、細胞生物学、及び分子生物学の分野に関する。特に、本発明は、発毛を調節する非免疫抑制性作用物質を特定するための薬物スクリーニングアッセイと、発毛の調節のためのこうした作用物質の使用法とに関する。
【背景技術】
【０００２】
免疫抑制物質ＦＫ５０６、ラパマイシン、及びサイクロスポリンＡは、臓器移植患者における拒絶反応を予防するために普通に使用されている公知のＴ細胞特異的免疫抑制物質である。Ｔ細胞において、ＦＫ５０６及びサイクロスポリンＡは、カルシニューリンが転写因子ＮＦ／ＡＴ（活性化Ｔ細胞の核因子）を脱リン酸化するのを防止し、これにより、核への転座を阻害し、受容体の媒介によるインターロイキン－２等のサイトカインの合成及び分泌の増加を防止することで、Ｔ細胞の増殖を防止する（ハイトマン，Ｊ．ら，１９９２，The New Biologist 4: 448-460）。
【０００３】
ＦＫ５０６及びサイクロスポリンＡは、イムノフィリンと呼ばれる内因性の受容体タンパク質と結合することで作用する。その構造、及び特定の薬物との結合親和性に基づき、イムノフィリンは、二クラスのタンパク質に区分されており、ＦＫ５０６との親和性を有するタンパク質は、ＦＫ５０６結合タンパク質（ＦＫＢＰ）と呼ばれ、一方、サイクロスポリンとの親和性を有するものは、サイクロフィリンと呼ばれる。ＦＫＢＰとサイクロフィリンとは、両方とも、同様のペプチジルプロリルイソメラーゼを保有しており、結果として、タンパク質のフォールディングと輸送とにとって重要であると考えられているタンパク質のシストランス異性化を発生させる。加えて、ＦＫＢＰとサイクロフィリンとは、両方とも、情報伝達に関与する様々なタンパク質と相互作用する能力により特徴付けられる。
【０００４】
ＦＫＢＰ群のいくつかの構成要素は、計算された分子量に従って特定及び命名されている（レイン，ＷＳら，１９９１，J. Protein Chem. 10: 151-160、米国特許番号第５，７６３，５９０号）。サイクロフィリンＡ及びＦＫＢＰ１２は、当初、それぞれサイクロスポリンＡ及びＦＫ５０６結合タンパク質として分離され、カルシニューリンの阻害を通じて、免疫抑制活性を発揮することが明らかとなった。ＦＫＢＰ５１は、Ｔ細胞において発現し、遙かに弱い有効性でカルシニューリンを阻害することが発見されており、これはＦＫ５０６の免疫抑制活性の媒介に多数のイムノフィリンが関与する可能性を示唆している。ＦＫＢＰ５１は、プロゲステロン受容体複合体の構成要素であることも明らかになっている（ネア，Ｓ．Ｃ．ら，１９９７，Mol. Cell Biol. 17: 594-603）。ＦＫＢＰ５２は、不活性ステロイド受容体複合体の構成要素として最初に発見された（スミス，Ｄ．Ｆ．ら，１９９３，J. Biol. Chem. 268: 18365-71）。ＦＫＢＰ５２の１乃至１４９番目の残基であるＮ末端領域は、ＦＫＢＰ１２との５５％の相同性を有するが、しかしながら、ＦＫ５０６と複合体形成する時、免疫抑制活性を有しない。ＦＫＢＰ５２は、カゼインキナーゼＩＩによりリン酸化され、イソメラーゼ活性から独立したシャペロン活性を有することが発見されている（ミヤタ，Ｙ．ら，１９９７，Proc. Natl. Accad. Sci. USA 94: 14500-14505）。ＣｙＰ４０は、サイクロスポリンＡとの低い親和性のみを有し、そのため、サイクロスポリンＡの免疫抑制効果をほんの僅かに減少させる能力しかない。
【０００５】

Ｈｓｐ９０は、最も豊富な熱ショックタンパク質である。信号伝達に関与する多数の転写因子及びタンパク質キナーゼは、ｈｓｐ９０との複合体を形成することが発見されている（プラット，Ｗ．Ｂ．ら，１９９９，Cell Signal 11: 938-851、プラット及びトフト，１９９７，Endocrine Rev. 18: 306-360）。転写因子と複合体形成する時、ｈｓｐ９０複合体は、テトラトリコペプチド反復（ＴＰＲ）モチーフを伴う高分子量イムノフィリンを含むことが分かっている（デュイナ，Ａ．Ａ．ら，１９９６，Science 274: 1713-1715、ボーズ，Ｓ．ら，１９９６，Science 274: 1715-1717）。こうしたイムノフィリンは、ＦＫＢＰ５２及びＣｙＰ４０を含む（オーウェンス＝グリロ，Ｊ．Ｋ．，１９９５，J. Biol. Chem. 270: 20479-20484、ミヤタ，Ｙ．ら，１９９７，Proc. Natl. Acad. Sci. USA 94: 14500-14505、シルバースタイン，Ａ．Ｍ．ら，１９９９，J. Biol. Chem. 274: 36980-36986）。

【０００６】

脱毛の原因には、老化と、男性ホルモンの作用と、毛嚢への血液供給の欠失と、頭皮の異常とが含まれる。加えて、遺伝的性質が、脱毛の原因となる場合がある。例えば、男性ホルモン性脱毛症は、遺伝的に決定されると考えられている。最近では、丘疹状の病変を伴う無毛症と言われる、遺伝性脱毛症の希な常染色体性劣性形態が、ヒトの「無毛」遺伝子における突然変異により生じることが分かっている（アフマド，Ｗ．ら，１９９８，Science 279: 720-724）。この形態の脱毛症になる個人では、毛髪は、通常、頭皮からなくなり、患者は、非常に薄い眉毛及び睫毛を有する。マウス無毛遺伝子のヒト相同体における突然変異は、先天性全身脱毛症と丘疹状の病変を伴う無毛症とにつながる。無毛遺伝子における突然変異を有するマウスにおいて、毛母細胞では、早期の大量なアポトーシスが、Ｂｃｌ－２発現の付随的な減少と共に生じると思われ、これは、無毛遺伝子産物が毛嚢における細胞増殖と、分化と、アポトーシスとを規制する役割を果たす可能性を示している。ヒト無毛遺伝子は、最近分離され、ＷＯ９９／３８３９６５において説明されている。

【０００７】

ＦＫ５０６及びサイクロスポリンＡの局所適用は、投与量に依存する形で発毛を刺激することが報告されている（セインズブリ，Ｔ．Ｓ．Ｌ．ら，１９９１，Transplant. Proc. 23: 3332-3334）。例えば、ＦＫ５０６及びサイクロスポリンＡは、マウス及びラットといった実験動物において発毛を刺激することが明らかになっている（ＷＯ９８／５５０９０、マウラ，Ｍ．，１９９７，Am. J. Path. 150: 1433、ヤマモト，Ｓ．ら，１９９３，J. Invest. Dermatol. 102:160）。ＦＫ５０６及びサイクロスポリンＡと関連する作用物質との効果については、説明されている（ツジ，Ｙ．ら，１９９９，Exp. Dermatol. 8: 366-7、マッケルウィ，Ｋ．Ｉ．ら，１９９７，Br. J. Dermatol. 137:491-7、イワブチ，Ｔ．ら，１９９５，J. Dermatol. Sci. 9:64-9、ヤマモト，Ｓ．及びカトウ，Ｒ．，１９９４，J. Dermatol. Sci. 7 Supp. 1: 547-54、ヤマモト，Ｓ．ら，１９９４，J. Invest. Dermatol. 102: 160-4）。

【０００８】

加えて、日本特許出願第１１－１７４０４１号では、ステロイド受容体との複合体を形成できる免疫抑制物質結合タンパク質、つまりＦＫＢＰ５２又はサイクロフィリン４０との結合は可能だが、ＦＫＢＰ１２等、ステロイド受容体との複合体を形成できないＦＫＢＰとは結合しない発毛刺激剤を特定する方法が説明されている。

【０００９】

ＦＫ５０６及びサイクロスポリンＡにより刺激される哺乳類の発毛のメカニズムは、依然として未知である。発毛刺激物質として使用できる可能性にもかかわらず、ＦＫ５０６及びサイクロスポリンＡといった免疫抑制物質は、免疫抑制のような有毒の副作用をも示す。そのため、発毛の調節物質として有効な非免疫抑制物質を特定及び開発する必要がある。本発明は、免疫抑制物質ＦＫ５０６及びサイクロスポリンＡが発毛を調節する際の信号経路の発見に基づくものである。この発見は、発毛を調節し得る非免疫抑制物質のための薬物スクリーニングアッセイを提供する。

【発明の開示】

【発明が解決しようとする課題】
【００１０】
本発明は、発毛を調節する非免疫抑制物質を特定するための薬物スクリーニングアッセイと、発毛の調整のためのこうした物質の使用法方とに関連する。本発明は、特定のイムノフィリン、すなわちＦＫ５０６及びサイクロスポリンＡと、ＣｙＰ４０と、が発毛を調節する際の信号伝達経路の発見に基づくものである。ここで開示するように、ＦＫＢＰ５１／５２タンパク質は、毛嚢の真皮乳頭において発現することが分かっている。加えて、ＦＫＢＰ５１／５２は、ｈｓｐ９０と、Ｇｌｉ３と、ＡＦＸ－１と、細胞内の無毛タンパク質と、との複合体を形成することが分かっている。真皮乳頭細胞のＦＫ５０６又はサイクロスポリンＡとの接触も、Ｇｌｉ３標的遺伝子と、ＢＭＰ４と、ＨＮＦ３βとの発現を刺激することが分かっている。本発明の経路は、発毛を刺激するために使用できる非免疫抑制物質を特定する方法の基盤としての役目を果たす。
【００１１】
本発明は、ＦＫＢＰ５１／５２、ＣｙＰ４０、ｈｓｐ９０、Ｇｌｉ３、ＡＦＸ－１、及び又は無毛タンパク質(hairless protein) を含め、ＦＫＢＰ５１／５２及びＣｙＰ４０信号伝達経路の構成要素を調節する物質、つまり、こうした構成要素のアゴニスト又はアンタゴニストとして作用する物質を、スクリーニングするためのアッセイに関する。本発明の実施形態では、ＦＫＢＰ５１／５２又はＣｙＰ４０の活性及び又はｈｓｐ９０、無毛、ＡＦＸ－１、及び又はＧｌｉ３タンパク質との関連性を特異的に調節する作用物質の合理的ドラッグデザインに関する方法が提供される。本発明は、更に、本発明の経路の構成要素、つまりＦＫＢＰ５１／５２と、ＣｙＰ４０と、ｈｓｐ９０と、Ｇｌｉ３と、ＡＦＸ－１と、無毛タンパク質との相互作用及び又は活性を調節する作用物質を特定するための細胞系アッセイと無細胞系アッセイとを提供する。
【課題を解決するための手段】
【００１２】
具体的には、本発明は、発毛を調節し得る化合物を特定する方法であって、
（ｉ）ＦＫＢＰ５１／５２又はＣｙＰ４０と、ｈｓｐ９０と、無毛、ＡＦＸ－１、及びＧｌｉ３で構成されるグループから選択されたタンパク質とを発現する細胞、或いは、これらを含む調製物を、試験化合物に接触させるステップと、
（ｉｉ）試験化合物に接触させた細胞又は調製物内における、ＦＫＢＰ５１／５２又はＣｙＰ４０と、ｈｓｐ９０と、無毛、ＡＦＸ－１、及びＧｌｉ３で構成されるグループから選択された少なくとも一種類のタンパク質との間での複合体形成レベルを判定するステップと、
（ｉｉｉ）（ｉｉ）で得られた複合体形成レベルを、試験化合物が存在しない状態でのＦＫＢＰ５１／５２又はＣｙＰ４０と、ｈｓｐ９０と、無毛、ＡＦＸ－１、及びＧｌｉ３で構成されるグループから選択された少なくとも一種類のタンパク質との間での複合体形成レベルと比較するステップ、
を含み、試験化合物が存在する状態と存在しない状態とでの複合体形成レベルの差は、発毛調節活性との正相関を有する方法を提供する。
【００１３】
本明細書での使用において、「調製物」という用語は、細胞構成要素を自然な状態で発現する細胞から、或いはその構成要素を発現するように遺伝子操作された細胞から、分離、抽出、又は部分的に精製された少なくとも一つの細胞構成要素を含む、或いは合成的に作成された構成要素を含む、組成物を指し、この組成物は、詳述する方法を遂行するために使用することができる。こうした調製物には、一部として、標準的な手法により作成された細胞の断片と、合成済み又は精製済みの構成要素を組み合わせることで作成された細胞構成要素の水性緩衝液とが含まれる。
【００１４】
本明細書での使用において、「発毛調節活性との正相関」という語句は、試験作用物質により発毛を刺激又は阻害できることを生物活性が示唆する試験化合物の生物活性の観察結

果を指す。
【００１５】
本発明の別の実施形態では、発毛を調節し得る化合物を特定する方法であって、
（ｉ）無毛、ＡＦＸ－１、又はＧｌｉ３遺伝子反応要素の転写制御下で核ホルモン受容体及びレポータ遺伝子を発現する細胞を試験化合物と接触させ、細胞内のレポータ遺伝子発現レベルを測定するステップと、
（ｉｉ）試験化合物が存在しない状態でのレポータ遺伝子発現レベルを測定するステップと、
（ｉｉｉ）（ｉ）及び（ｉｉ）で測定されたレポータ遺伝子発現レベルを比較するステップと、
を含み、ステップ（ｉ）及び（ｉｉ）で測定されたレポータ遺伝子発現レベルの差は、試験化合物の発毛調節活性との正相関を有する方法が提供される。
【００１６】
本発明は、更に、発毛を促進し得る化合物を特定する方法であって、
（ｉ）無毛、ＡＦＸ－１、又はＧｌｉ３遺伝子産物を含む試料を試験化合物に接触させるステップと、
（ｉｉ）試験化合物が、無毛、ＡＦＸ－１、又はＧｌｉ３遺伝子産物と結合するか否かを判定するステップと、
（ｉｉｉ）試験化合物が、無毛、ＡＦＸ－１、又はＧｌｉ３遺伝子産物と、ＦＫＢＰ５１／５２、ＣｙＰ４０、核ホルモン受容体、ｈｓｐ９０タンパク質、及びその組み合わせで構成されるグループから選択された結合相手との間での複合体形成を阻害するか否かを判定するステップと、
を含み、試験化合物が、無毛、ＡＦＸ－１、又はＧｌｉ３遺伝子産物と結合する能力と、複合体形成を阻害する能力とは、両方とも、発毛促進活性との正相関を有する方法を提供する。
【００１７】
本発明は、更に、発毛を調節し得る化合物を特定する方法であって、
（ｉ）ＦＫＢＰ５１／５２又はＣｙＰ４０と、ｈｓｐ９０と、核ホルモン受容体と、無毛、ＡＦＸ－１、又はＧｌｉ３遺伝子産物とを発現する細胞を、核ホルモン受容体リガンドが存在する状態で、試験化合物に接触させるステップと、
（ｉｉ）無毛、ＡＦＸ－１、又はＧｌｉ３遺伝子産物の細胞核への核転座レベルを判定するステップと、
（ｉｉｉ）試験化合物が存在しない状態での各転座レベルを判定するステップと、
（ｉｖ）（ｉｉ）及び（ｉｉｉ）において測定した核転座レベルを比較するステップと、
を含み、ステップ（ｉｉ）及び（ｉｉｉ）で測定された核転座レベルの差は、試験化合物の発毛調節活性との正相関を有する方法に関する。
【００１８】
特定された作用物質は、発毛を調節するために使用できる。こうした作用物質は、遺伝因子と、老化と、局部的な皮膚の状態と、身体全体に影響する病気すなわち全身性疾患と、により生じる禿の治療に特に有用である。こうした疾患には、一部として、男性型禿頭症と、女性型禿頭症と、中毒性禿頭症と、円形脱毛症と、瘢痕性脱毛症とが含まれる。加えて、この作用物質は、放射線又は化学療法に関連する脱毛の対象者を治療するのに使用することができる。
【００１９】
したがって、本発明は、ＦＫＢＰ５１／５２、ＣｙＰ４０、又はｈｓｐ９０と、無毛、Ｇｌｉ３、ＡＦＸ－１、及びｈｓｐ９０で構成されるグループから選択された少なくとも一つのタンパク質との間での複合体形成を調節する化合物を哺乳類に投与するステップを含む、哺乳類の発毛を調節する方法を包含する。
【００２０】
本発明は、無毛、ＡＦＸ－１、及びＧｌｉ３タンパク質で構成されるグループから選択さ

れたタンパク質の核転座を調節する化合物を哺乳類に投与するステップを含む、哺乳類の発毛を調節する方法を提供する。

【００２１】

本発明の更に別の実施形態では、無毛、ＡＦＸ－１、又はＧｌｉ３により媒介される遺伝子発現を調節する化合物を哺乳類に投与するステップを含む、哺乳類の発毛を調節する方法が提供される。

詳細な説明

本発明は、特定のイムノフィリン、すなわちＦＫＢＰ５１と、ＦＫＢＰ５２と、ＣｙＰ４０とが発毛を調節する際の信号伝達経路の発見に基づくものである。ＦＫ５０６及びサイクロスポリンＡといった作用物質が発毛を調節する際の経路の発見は、免疫系への影響を低減しながら発毛を促進するために使用可能な作用物質のスクリーニング標的を提供する。

【００２２】

本発明は、信号伝達経路の構成要素の相互作用及び又は活性を調節する作用物質を特定するためのアッセイを包含する。こうした構成要素は、例えば、ＦＫＢＰ５１／５２と、ＣｙＰ４０と、ｈｓｐ９０と、Ｇｌｉ３と、ＡＦＸ－１と、無毛タンパク質とを含む。細胞系及び無細胞系アッセイの両方を使用して、ＦＫＢＰ５１／５２及びＣｙＰ４０信号伝達経路の活性を増加又は減少させる作用物質を特定することができる。本発明は、更に、ＦＫＢＰ５１／５２又はＣｙＰ４０のｈｓｐ９０、無毛、ＡＦＸ－１、及び又はＧｌｉ３タンパク質との結びつきを特異的に促進又は阻害する作用物質の合理的ドラッグデザインを提供する。

【００２３】

別の実施形態において、本発明は、前記の方法により設計又は特定された作用物質を提供する。更に、本発明は、発毛増強のための前記作用物質の使用法を提供する。特に、こうした作用物質は、遺伝因子、老化、及び又は局部的な皮膚状態から生じる場合がある禿の治療に使用することができる。

【００２４】

ＦＫＢＰ５１／５２／ＣｙＰ４０の活性の調節において有用な作用物質のスクリーニングアッセイ

本発明は、ＦＫＢＰ５１／５２又はＣｙＰ４０活性、或いはＦＫＢＰ５１／５２又はＣｙＰ４０遺伝子発現を調節し、したがって発毛の調節に有用である可能性がある作用物質又は組成物を特定するためのスクリーニングアッセイシステムに関する。

【００２５】

発毛に関与するタンパク質の組み換え発現

発毛を調節する作用物質又は組成物を特定するためのスクリーニングアッセイを開発する目的から、ＦＫＢＰ５１／５２又はＣｙＰ４０タンパク質、及び又はＦＫＢＰ５１／５２及びＣｙＰ４０と相互作用するタンパク質、すなわちｈｓｐ９０、Ｇｌｉ３、ＡＦＸ－１、及び無毛タンパク質を、組み換え発現させる必要性が生じる可能性がある。ＦＫＢＰ５１及びＦＫＢＰ５２のｃＤＮＡ配列及び推定アミノ酸配列は、特徴付けが成されており（ＰｕｂＭｅｄアクセッション番号Ｕ７１３２１及びＭ８８２７９）、これは参照により本明細書に組み込むものとする。ＦＫＢＰ５１／５２という用語は、本明細書での使用において、ＦＫＢＰ５１及びＦＫＢ５２タンパク質の一方又は両方を指す。ＣｙＰ４０のｃＤＮＡ配列及び推定アミノ酸配列は、特徴付けが成されており（アクセッション番号Ｄ６３８６１）、これは参照により本明細書に組み込むものとする。ｈｓｐ９０、Ｇｌｉ３、ＡＦＸ－１、及び無毛のｃＤＮＡ配列及び推定アミノ酸配列についても、特徴付けが成されており（ＰｕｂＭｅｄアクセッション番号ＮＭＯ１８４１１（無毛）、ＮＭ０００１６８（Ｇｌｉ３）、ＮＭ００５３４８（ｈｓｐ９０）、アクセッション番号Ｕ１００７２）、これらは参照により本明細書に組み込むものとする。簡略化のため、以下では、ＦＫＢＰ５１／５２について、組み換え発現の説明が行われるが、しかしながら、この方法は、ＣｙＰ４０、ｈｓｐ９０、Ｇｌｉ３、ＡＦＸ－１、及び無毛タンパク質の組み換え発現に関

して利用することも可能である。

【００２６】

ＦＫＢＰ５１／５２ヌクレオチド配列は、当業者に知られている様々な種類の方法を使用して分離することができる。例えば、ＦＫＢＰ５１／５２を発現することが知られている組織からのＲＮＡを使用して構築したｃＤＮＡライブラリを、標識したＦＫＢＰ５１／５２特異的プローブを使用してスクリーニングすることができる。代わりに、ゲノムライブラリをスクリーニングし、ＦＫＢＰ５１又はＦＫＢ５２タンパク質をコード化する核酸分子を導くことができる。更に、ＦＫＢＰ５１／５２核酸配列は、既知のＦＫＢＰ５１／５２ヌクレオチド配列に基づいて設計された二つのオリゴヌクレオチドプライマを使用したポリメラーゼ鎖反応（ＰＣＲ）を実行して導くことができる。反応のテンプレートは、ＦＫＢＰ５１／５２を発現することが知られている細胞株又は組織から作成されたｍＲＮＡの逆転写により得られるｃＤＮＡにすることができる。

【００２７】

ＦＫＢＰ５１／５２タンパク質、ポリペプチド、及びペプチド断片、ＦＫＢＰ５１／５２の突然変異、切断、又は欠失形態、及び又はＦＫＢＰ５１／５２融合タンパク質は、様々な用途で作成することが可能であり、その一部には、ＦＫＢＰ５１／５２媒介発毛の規制に関与する他の細胞遺伝子産物の特定と、発毛を調節するのに使用可能な作用物質のスクリーニングとが含まれる。ＦＫＢＰ５１／５２融合タンパク質は、酵素、蛍光タンパク質、及びポリペプチドタグ又は発行タンパク質との融合を含み、これらはすべて標識機能を提供する。

【００２８】

ＦＫＢＰ５１／５２ポリペプチド及びペプチドは化学的に合成可能だが（例えば、クレイトン，１９８３，Proteins: Structures and Molecular Principles, W. H. Freeman & Co., N. Y. を参照）、ＦＫＢＰ５１／５２から導かれる大きなポリペプチド及び全長ＦＫＢＰ５１／５２タンパク質は、ＦＫＢＰ５１／５２遺伝子配列及び又はその他のコード配列を含む核酸を発現させるための当業者に広く知られる手法を使用した組み換えＤＮＡ技術により、有利に生産することができる。こうした方法は、ＦＫＢＰ５１／５２ヌクレオチド配列と、適切な転写及び翻訳調節信号とを含む発現ベクタを構築するために使用できる。こうした方法は、例えば、ｉｎ　ｖｉｔｒｏ組み換えＤＮＡ手法と、合成手法と、ｉｎ　ｖｉｖｏ遺伝子組み換えと、を含む（例えば、サンブルックら，１９８９，Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Press, N. Y. 及びオースベルら，１９８９，Current Protocols in Molecular Biology, Green Publishing Associates and Wiley Interscience, N. Y. において説明されている手法を参照）。

【００２９】

様々な宿主－発現ベクタ系を利用して、ＦＫＢＰ５１／５２ヌクレオチド配列を発現させることができる（例えば、ＦＫＢＰ５２の発現に関する米国特許第５，７６３，５９０号を参照）。ＦＫＢＰ５１／５２ペプチド又はポリペプチドが溶性の誘導体として発現し、分泌されない場合には、このペプチド又はポリペプチドは、宿主細胞から回収することができる。代わりに、ＦＫＢＰ５１／５２ペプチド又はポリペプチド分泌される場合には、このペプチド又はポリペプチドは、培地から回収することができる。

【００３０】

本発明の目的において使用できる発現系には、一部として、ＦＫＢＰ５１／５２をコード化するヌクレオチド配列を含む組み換えバクテリオファージ、プラスミド、又はコスミドＤＮＡ発現ベクタにより形質転換させたバクテリア等の微生物、ＦＫＢＰ５１／５２をコード化するヌクレオチド配列を含む組み換え酵母発現ベクタにより形質転換させた酵母、或いは、哺乳類又は昆虫細胞若しくは哺乳類又は昆虫ウイルスから導いたプロモータを含んだＦＫＢＰ５１／５２組み換え発現構成体を含む哺乳類細胞系又は昆虫細胞系が含まれる。

【００３１】

適切な発現系は、ＦＫＢＰ５１／５２の正確な修飾、処理、及び細胞内局在の発生を確保

するために選択できる。この目的のためには、ＦＫＢＰ５１／５２タンパク質を適切に修飾及び処理する能力を備えた真核宿主細胞が好ましい。スクリーニングを目的とする細胞株の作成に望まれるような組み換えＦＫＢＰ５１／５２タンパク質の長期的な高産出量の生産には、安定した発現が好ましい。複製の起源を含む発現ベクタを使用せずに、宿主細胞は、適切な発現調節要素と、例えば、ｔｉｅ、ｈｇｐｒｔ、ｄｈｆｒ、ｎｅｏ、及びｈｙｇｒｏ遺伝子等の選択可能な標識遺伝子とにより制御されたＤＮＡにより形質転換することができる。異種ＤＮＡの導入に続いて、改変細胞は、濃縮培地において一乃至二日間培養し、その後、選択培地に切り替えることができる。こうした改変細胞株は、ＦＫＢＰ５１／５２遺伝子産物の内因性活性を調節する作用物質をスクリーニング及び評価するのに特に有用である可能性がある。

【００３２】

加えて、一部の事例においては、本発明のスクリーニングアッセイにおいて使用するために、ＣｙＰ４０、ｈｓｐ９０、Ｇｌｉ３、ＡＦＸ－１、及び無毛タンパク質といった不活性結合タンパク質を共発現させる必要がある。ＦＫＢＰ５１／５２の発現に関する上で説明した方法は、こうした結合タンパク質を共発現させるために、同様に使用することができる。

【００３３】

無細胞系アッセイ

本発明に従って、ＦＫＢＰ５１／５２又はＣｙＰ４０と相互作用すなわち結合し、こうしたタンパク質の活性を調節する作用物質を特定するために、無細胞系アッセイシステムを使用することができる。こうした作用物質は、ＦＫＢＰ５１／５２又はＣｙＰ４０活性のアンタゴニスト又はアゴニストとして作用する場合があり、発毛の調節に使用することができる。特に、こうした作用物質は、ＦＫＢＰ５１／５２又はＣｙＰ４０と、その結合相手、すなわちｈｓｐ９０、Ｇｌｉ３、ＡＦＸ－１、及び又は無毛タンパク質との間での複合体の形成を妨害又は防止する機能を果たすことができる。簡略化のため、以下では、ＦＫＢＰ５１／５２について、無細胞系アッセイの説明が行われるが、しかしながら、これはＣｙＰ４０についても同様に利用することが可能である。

【００３４】

様々な機能領域に対応するペプチドを含む組み換えＦＫＢＰ５１／５２、又はＦＫＢＰ５１／５２融合タンパク質は、ＦＫＢＰ５１／５２と相互作用する作用物質を特定するために、発現させ、アッセイにおいて使用することができる。

【００３５】

この目的のために、溶性ＦＫＢＰ５１／５２を組み換え発現させ、ＦＫＢＰ５１／５２と結合する作用物質を特定するために、無細胞系アッセイにおいて利用することができる。組み換え発現ＦＫＢＰ５１／５２ポリペプチド、又は一つ以上のＦＫＢＰ５１／５２機能領域を含む融合タンパク質は、上で説明したように作成可能であり、無細胞系スクリーニングアッセイにおいて使用することができる。こうした機能領域の一つは、タンパク質／タンパク質相互作用にとって重要なテトラトリコペプチド反復（ＴＰＲ）である。例えば、全長ＦＫＢＰ５１／５２、又は一つ以上の領域が欠失しているがＴＲＰは保持されているような溶性切断ＦＫＢＰ５１／５２、ＴＰＲモチーフに対応するペプチド、或いはアッセイシステムにおける利点（結果として生じる複合体を標識又は分離する等）を提供するタンパク質又はポリペプチドと融合したＦＫＢＰ５１／５２ＴＰＲモチーフを含む融合タンパク質を利用することが可能である。ＴＰＲモチーフと相互作用する作用物質を特定したい場合には、ＦＫＢＰ５１／５２ＴＰＲモチーフに対応するペプチドと、ＦＫＢＰ５１／５２ＴＰＲモチーフを含む融合タンパク質とを使用することができる。ＦＫＢＰ５１／５２タンパク質は、未処理又は半精製抽出物の一部として存在することもある。

【００３６】

ＦＫＢＰ５１／５２と結合する作用物質を特定するために使用するアッセイの原理には、ＦＫＢＰ５１／５２と試験作用物質との反応混合物を、二種類の構成要素が相互作用及び結合可能であり、したがって反応混合物内で除去及び又は検出可能な複合体が形成される

条件下において、これらに十分な時間に渡って、調製することが含まれる。その後、結合した試験作用物質の独自性が判断される。

【００３７】

スクリーニングアッセイは、一般に知られる様々な方法のいずれかにより達成される。例えば、こうしたアッセイを実施する方法の一つには、ＦＫＢＰ５１／５２タンパク質、ポリペプチド、ペプチド、融合タンパク質、又は試験物質を固相に固定し、反応の終わりに、固相に付着したＦＫＢＰ５１／５２／試験作用物質複合体を検出することが含まれる。こうした方法の一実施形態では、ＦＫＢＰ５１／５２反応物が固体面に固定され、試験作用物質は、固定されず、直接又は間接的に標識することができる。

【００３８】

実際には、マイクロタイタプレートを固体面として都合良く利用することができる。固定構成要素は、非共有又は共有結合により固体面で不動化する。固体面は、事前に準備し、保管することができる。アッセイを実施するためには、非不動化構成要素を、固定構成要素で覆われた固体面に追加する。反応完了後には、形成された任意の複合体が固体面で不動化された状態を維持するような条件下で、未反応構成要素を（例えば洗浄により）除去する。固体面で不動化された複合体の検出は、多数の方法で達成することができる。事前に不動化されない構成要素が前もって標識されている場合、表面で不動化された標識の検出は、複合体の形成を示す。前もって不動化されない構成要素が事前に標識されない場合は、例えば前もって不動化されない構成要素に特異的な標識済みの抗体を使用する等、間接的な標識を使用して、固体面上の複合体を検出することができる。

【００３９】

代わりに、反応は液相において実施され、反応生成物は、ＦＫＢＰ５１／５２タンパク質、融合タンパク質、又は試験作用物質に特異的な不動化抗体を使用して、未反応構成要素から分離され、複合体は、複合体のその他の構成要素に特異的な標識済みの抗体を使用して検出される。

【００４０】

本発明に従って、無細胞系アッセイシステムは、細胞内でのＦＫＢＰ５１／５２と一つ以上の他のタンパク質との間での相互作用に直接的に干渉する作用物質を特定するために使用することができる。ＦＫＢＰ５１／５２と相互作用するタンパク質は、この解説の目的から、「結合相手」と呼ばれる。こうした結合相手は、ＦＫＢＰ５１／５２信号伝達経路に関与する可能性が高い。こうした結合相手には、一部として、ｈｓｐ９０と、Ｇｌｉ３と、ＡＦＸ－１と、無毛タンパク質とが含まれる。そのため、一つ以上のこうした結合相手のＦＫＢＰ５１／５２との相互作用を調節する作用物質を特定することが望ましい。こうした作用物質は、一つ以上のこうした結合相手のＦＫＢＰ５１／５２との相互作用に干渉すること、或いはこれを妨害することが可能であり、発毛の調節において有用である可能性がある。代わりに、作用物質は、一つ以上の結合相手とＦＫＢＰ５１／５２との間の親和性を増加させるもの、或いは相互作用を向上させるものとして特定される可能性がある。

【００４１】

ＦＫＢＰ５１／５２部分と一つ以上の結合相手との間での相互作用に干渉する作用物質を特定するために使用されるアッセイシステムの基本原理には、ＦＫＢＰ５１／５２タンパク質、ポリペプチド、ペプチド、又は融合タンパク質と、一つ以上の結合相手とを含む反応混合物を調製し、構成要素が相互作用及び結合可能であり、したがって複合体が形成される条件下において、これらに十分な時間に渡って、反応混合物を培養することが含まれる。阻害活性に関して作用物質を試験するためには、試験作用物質が存在する状態と存在しない状態との両方において、反応混合物を調製する。試験作用物質は、最初から反応混合物に含めることが可能であり、或いはＦＫＢＰ５１／５２部分を（複数の）結合相手に追加した後の時点で追加することも可能である。対照反応混合物は、試験作用物質なしで、或いはプラシーボと共に、培養される。その後、ＦＫＢＰ５１／５２部分と（複数の）結合相手との間での任意の複合体の形成が検出される。試験作用物質を含む反応混合物に

存在せず、対照反応には存在する複合体の形成は、ＦＫＢＰ５１／５２と（複数の）相互作用での結合相手との相互作用に試験作用物質が干渉したことを示す。

【００４２】

ＦＫＢＰ５１／５２と結合相手との相互作用に干渉する作用物質のアッセイは、不均質又は均質なフォーマットにおいて実施することができる。不均質アッセイには、ＦＫＢＰ５１／５２部分の生成物、又は結合相手を固体面に固定し、反応の終わりに、固体面に付着した複合体を検出することが含まれる。均質アッセイでは、全ての反応が液相で実施される。いずれのアプローチにおいても、反応物の追加順序は、試験する作用物質に関する様々な情報を取得するために変化させることができる。例えば、競合により相互作用に干渉する試験作用物質は、試験物質が存在する状態で反応を実施することで、すなわち、ＦＫＢＰ５１／５２部分及び相互作用での結合相手よりも先に、或いはこれらと同時に、試験物質を反応混合物に追加することで、特定できる。代わりに、予備形成される複合体を妨害する試験作用物質、例えば、複合体から構成要素の一つを駆逐する高い結合定数を備えた作用物質は、複合体が形成された後で反応混合物に試験作用物質を追加することで試験できる。様々なフォーマットについては、下で簡単に説明する。

【００４３】

特定の実施形態では、不動化のためにＦＫＢＰ５１／５２の融合を準備することができる。例えば、ＦＫＢＰ５１／５２又は例えばＴＰＲモチーフに対応するペプチド断片は、ｐＧＥＸ－５Ｘ－１等の融合ベクタを使用して、結果として生じる融合タンパク質において結合活性が維持されるような形で、グルタチオンＳ－トランスフェラーゼ（ＧＳＴ）と融合させることができる。相互作用での結合相手、すなわちｈｓｐ９０、Ｇｌｉ３、ＡＦＸ－１、又は無毛タンパク質は、この技術で通常実施される方法を使用して、モノクローナル抗体を発生させるために精製及び使用することができる。モノクローナル抗体の生成は、こうした抗体が存在しており、広く利用可能である場合、省略することができる。この抗体は、この技術で通常実施される方法により、例えば、放射性同位体^{125}Ｉにより標識することができる。例えば、不均質アッセイにおいては、ＧＳＴ－ＦＫＢＰ５１／５２融合タンパク質を、グルタチオンアガロースビーズに固定することができる。相互作用での結合相手は、その後、試験作用物質が存在する状態又は存在しない状態において、相互作用及び結合が発生可能な形で、追加することができる。反応期間の終わりには、未結合物質を洗い流し、このシステムに標識済みモノクローナル抗体を追加し、複合化した構成要素に結合させることができる。ＦＫＢＰ５１／５２遺伝子産物と相互作用での結合相手との間の相互作用は、グルタチオンアガロースビーズに関連して残存する放射能の量を測定することで検出できる。試験作用物質による相互作用の阻害が成功すれば、測定される放射能が減少することになる。

【００４４】

代わりに、ＧＳＴ－ＦＫＢＰ５１／５２融合タンパク質と相互作用での結合相手とは、固体のグルタチオンアガロースビーズが存在しない状態で、水中において混合することができる。試験作用物質は、化学種の相互作用が可能となっている間に、又はその後で、追加することができる。この混合物は、その後、グルタチオンアガロースビーズに追加することが可能であり、未結合物質は洗い流す。同様に、ＦＫＢＰ５１／５２の結合相手との相互作用の阻害の程度は、標識済み抗体を追加し、ビーズに関連する放射能を測定することで検出できる。

【００４５】

本発明の代替実施形態では、均質アッセイを使用することができる。このアプローチにおいて、ＦＫＢＰ５１／５２部分の予備形成複合体と相互作用での結合相手とは、ＦＫＢＰ５１／５２又はその結合相手のいずれかが標識されるが、複合体の形成により、標識の生成する信号が消えるように調製される。予備形成複合体の化学種の一つと競合し駆逐する試験物質を追加すると、バックグラウンドを上回る信号が生成されることになる。これにより、ＦＫＢＰ５１／５２の結合相手との相互作用を妨害する試験物質は、特定可能となる。

【００４６】
本発明に従って、無細胞系アッセイは、更に、ＦＫＢＰ５１／５２に関連する酵素活性を直接的に阻害する作用物質をスクリーニングするために使用することができる。こうした活性には、その一部として、プロリンイソメラーゼ活性が含まれる。例えば、ハリソン及びスタイン（１９８０，Biochem. 29: 3813-3816）に従って、パークら（１９９２，Biol. Chem. 267: 3316-3324）により説明された修正と共に実行されたペプチジルプロリル・シストランスイソメラーゼアッセイは、ＦＫＢＰ５１／５２の活性レベルを測定するために使用できる。この目的のために、ＦＫＢＰ５１／５２及び試験作用物質の反応混合物質は、基質が存在する状態で調製され、ＦＫＢＰ５１／５２の酵素活性は、試験作用物質が存在しない状態で観察された活性と比較される。

【００４７】
本発明の限定されない実施形態では、ＦＫＢＰ５１／５２と、試験作用物質と、基質との反応混合物が調製され、ＦＫＢＰ５１／５２の酵素活性は、試験作用物質が存在しない状態で観察された活性と比較され、これにおいて、試験作用物質が存在する状態でのＦＫＢＰ５１／５２の酵素活性レベルの減少は、ＦＫＢＰ５１／５２の阻害物質が特定されたことを示す。代わりに、ＦＫＢＰ５１／５２と、試験作用物質と、基質との反応混合物が調製され、ＦＫＢＰ５１／５２の酵素活性は、試験作用物質が存在しない状態で観察された活性と比較され、これにおいて、試験作用物質が存在する状態でのＦＫＢＰ５１／５２の酵素活性レベルの増加は、ＦＫＢＰ５１／５２のアゴニストが特定されたことを示す。

【００４８】
細胞系アッセイ
本発明に従って、ＦＫＢＰ５１／５２又はＣｙＰ４０の活性を規制する作用物質を特定するために、細胞系アッセイシステムを使用することができる。加えて、ＦＫＢＰ５１／５２／ｈｓｐ９０／無毛／ＡＦＸ－１／Ｇｌｉ３複合体又はＣｙＰ４０／ｈｓｐ９０／無毛／ＡＦＸ－１／Ｇｌｉ３は、細胞内で核ホルモン受容体と結合すると考えられている。したがって、細胞系アッセイは、核ホルモン受容体を発現する細胞を使用して実行することができる。こうした核ホルモン受容体には、一部として、アンドロゲン、ビタミンＤ、レチノイン酸、アリル炭化水素、及び甲状腺ホルモン受容体が含まれる。こうした細胞を使用する時、試験作用物質の活性は、核ホルモン受容体リガンドが存在する状態、或いは存在しない状態で、テストすることができる。以下では、ＦＫＢＰ５１／５２の活性を規制する作用物質の特定について、細胞系アッセイの説明が行われるが、しかしながら、こうした細胞系アッセイは、ＣｙＰ４０の活性を規制する作用物質を同様に特定するために使用することが可能である。

【００４９】
本発明は、ＦＫＢＰ５１／５２の酵素活性を活性化する作用物質を特定する方法を提供し、これは、（ｉ）ＦＫＢＰ５１／５２を発現する細胞を試験作用物質に接触させ、ＦＫＢＰ５１／５２の酵素活性レベルを測定することと、（ｉｉ）別個の実験において、ＦＫＢＰ５１／５２タンパク質を発現する細胞をビヒクル対照と接触させ、パート（ｉ）と本質的に同じ条件で、ＦＫＢＰ５１／５２の酵素活性レベルを測定することと、その後、（ｉｉ）パート（ｉ）において測定されたＦＫＢＰ５１／５２の活性レベルを、パート（ｉｉ）におけるＦＫＢＰ５１／５２の活性レベルと比較することと、を含み、これにおいて、ビヒクル対照が存在する状態でのＦＫＢＰ５１／５２の酵素活性レベルと比較して、試験作用物質が存在する状態でのＦＫＢＰ５１／５２の活性レベルが増加している場合、これは試験作用物質がＦＫＢＰ５１／５２の酵素活性化物質であることを示す。

【００５０】
本発明は、更に、ＦＫＢＰ５１／５２の酵素活性を阻害する作用物質を特定する方法を提供し、これは、（ｉ）ＦＫＢＰ５１／５２を発現する細胞を、ＦＫ５０６が存在する状態で、試験作用物質に接触させ、ＦＫＢＰ５１／５２の酵素活性レベルを測定することと、（ｉｉ）別個の実験において、ＦＫＢＰ５１／５２を発現する細胞を、ＦＫ５０６が存在する状態で接触させ、パート（ｉ）と本質的に同じ条件で、ＦＫＢＰ５１／５２の酵素活

性レベルを測定することと、その後、（ｉｉｉ）パート（ｉ）において測定されたＦＫＢＰ５１／５２の活性レベルを、パート（ｉｉ）におけるＦＫＢＰ５１／５２の酵素活性レベルと比較することと、を含み、これにおいて、ビヒクル対照が存在する状態でのＦＫＢＰ５１／５２の酵素活性レベルと比較して、試験作用物質が存在する状態でのＦＫＢＰ５１／５２の活性レベルが減少している場合、これは試験作用物質がＦＫＢＰ５１／５２の酵素阻害物質であることを示す。

【００５１】

こうした細胞系を利用する際には、ＦＫＢＰ５１／５２タンパク質を発現する細胞を、試験作用物質又はビヒクル対照（例えば、プラシーボ）に曝露する。曝露後又は曝露中には、細胞を検査し、ＦＫＢＰ５１／５２の酵素活性、或いはＦＫＢＰ５１／５２に依存する信号伝達経路自体の活性を測定することができる。

【００５２】

試験分子がＦＫＢＰ５１／５２の酵素活性を調節する能力は、標準的な生化学的又は生理学的手法を使用して測定可能であり、例えば、化学的、生理学的、生物学的、又は表現型変化と、宿主細胞遺伝子又はレポータ遺伝子の誘導と、宿主細胞のキナーゼ活性の変化と、その他とにより測定される。例えば、ＦＫＢＰ５１／５２に関連するペプチジルプロピルイソメラーゼ活性を測定することができる。こうした活性のアッセイは、ハリソン及びスタイン（１９８０，Biochem. 29: 3813-3816）、パーク，Ｓ．Ｔ．ら（１９９２，J. Biol. Chem. 267: 3316-3324）、及び米国特許第５，７６３，５９０号において説明されるものを含む。代わりに、ＢＭＰ４又はＨＮＦ３βといった、ＦＫＢＰ５１／５２信号伝達経路の活性により調節されることが知られている遺伝子の発現を検査し、ＦＫＢＰ５１／５２又は活性の調節物質を特定することができる。

【００５３】

加えて、動物モデルを利用して、脱毛を改善することが可能な作用物質を特定することができる。こうした動物モデルは、このような疾患を治療するのに有効である可能性がある薬物と、製薬と、治療法と、介入法とを特定するための試験基質として使用することができる。例えば、動物モデルは、発毛を調節する能力を示すと思われる作用物質に曝露することが可能であり、曝露する動物でこうした発毛を引き出すのに十分な濃度で、十分な時間時間に渡って、これを行うことができる。この曝露に対する動物の反応は、発毛の調節を検査することでモニタできる。本発明の特定の実施形態では、Ｃ３Ｈマウスモデルを使用して、試験化合物が発毛を開始させる能力を測定することができる。通常は、生後七週間のメスのＣ３Ｈマウスを実験に使用する。マウスの腰部の毛を電気バリカンで剪毛し、その後、試験作用物質を投与する。試験動物発毛の目視観測により、試験作用物質が発毛を調節する能力に関する判定が行われることになる。加えて、Ｄｕｎｄｅｅ　Ｂａｌｄラットモデル動物又は化学療法により処置したマウスを使用することもできる。介入法に関して、疾患に似た症状の何らかの態様を逆転させる任意の処置は、ヒトにおける治療的介入法の候補と考えるべきである。試験作用物質の投与量は、下で説明するように、用量反応曲線を導くことで判定できる。

【００５４】

合理的ドラッグデザイン

本発明の実施形態では、コンピュータモデリング及び検索技術を、ＦＫＢＰ５１／５２、ＣｙＰ４０、ｈｓｐ９０、Ｇｌｉ３、ＡＦＸ－１、及び又は無毛タンパク質の間でのタンパク質相互作用を調節できる作用物質の特定に使用することができる。例えば、ＦＫＢＰ５１／５２又はＣｙＰ４０の結合部位の知識と、ＦＫＢＰ５１／５２又はＣｙＰ４０とｈｓｐ９０、Ｇｌｉ３、ＡＦＸ－１、及び又は無毛といったタンパク質との間での複合体の研究と、に基づいて、ＦＫＢＰ５１／５２又はＣｙＰ４０信号伝達経路の潜在的な調節物質を特定することが可能である。

【００５５】

結合部位の三次元幾何学構造は、既知の方法を使用して決定することが可能であり、これには、完全な分子構造を決定可能なＸ線結晶法が含まれる。一方、固相又は液相ＮＭＲを

使用して、特定の分子内距離を決定することもできる。他の任意の実験的構造決定方法を使用して、部分的又は完全な幾何学構造を取得することができる。この幾何学構造は、複合タンパク質又は作用物質により測定することが可能であり、これは決定された活性部位の精度を高める可能性がある。

【００５６】

不完全又は不十分な精度の構造が決定された場合、コンピュータに基づく数値モデリング方法を使用して、構造を完成させること、或いは精度を向上させることができる。認められた任意のモデリング方法を使用可能であり、これには、タンパク質等の特定のバイオポリマに限定されたパラメータ化モデル、分子運動の計算に基づく分子力学モデル、熱のアンサンブルに基づく統計力学モデル、又は複合モデルが含まれる。殆どのタイプのモデルに関して、構成する原子及び基の間の力を表す標準分子力場が必要であり、これは、物理化学において知られている力場から選択することができる。不完全な又は精度の低い実験構造は、こうしたモデリング方法により計算された完全な精度の高い構造に対する制約として働く可能性がある。

【００５７】

実験、モデリング、或いはこうした方法の組み合わせのいずれかにより、結合部位の構造が決定された後、調節作用物質の候補は、作用物質を、その分子構造に関する情報と共に収容するデータベースを検索することで特定できる。こうした検索では、決定された結合部位構造と一致し、活性部位を定める基と相互作用する構造を有する作用物質が求められる。こうした検索は、手動で行うことが可能だが、好ましくは、コンピュータにより支援される。この検索で発見された作用物質は、潜在的な発毛調節作用物質である。

【００５８】

代わりに、こうした方法は、既知の発毛調節作用物質を修飾し、その活性を改善するために使用することができる。既知の作用物質は修飾可能であり、修飾の構造的影響は、上で説明した実験的及びコンピュータモデリング方法を使用して判定することができる。改変構造は、その後、作用物質の活性部位構造と比較し、適合性又は相互作用の改善が生じたかを判断することができる。これにより、側鎖基の変化等による組成における体系的変化を迅速に評価し、例えば、ＣｙＰ４０との親和性を強め、同時にサイクロスポリンＡ又はＢとの親和性を弱めるためのサイクロスポリンＡの修飾等、特異性又は活性が改善された修飾調節作用物質又はリガンドを得ることができる。

【００５９】

ＦＫＢＰ５１／５２又はＦＫＢＰ５１／５２結合タンパク質の結合部位の特定に基づいて、調節作用物質を特定するのに有用な、その他の実験的及びコンピュータモデリング方法は、当業者に明らかである。加えて、ＣｙＰ４０又はＣｙＰ４０結合タンパク質の結合部位の特定に基づいて、調節作用物質を特定するのに有用な実験的及びコンピュータモデリング方法は、当業者に明らかである。

【００６０】

分子モデリングシステムの例には、ＣＨＡＲＭｍ及びＱＵＡＮＴＡプログラム（マサチューセッツ州ウォルサム、Ｐｏｌｙｇｅｎ　Ｃｏｒｐｏｒａｔｉｏｎ）がある。ＣＨＡＲＭｍは、エネルギ最小化及び分子力学関数を実行する。ＱＵＡＮＴＡは、分子構造の構築、グラフィックモデリング、及び解析を実行する。ＱＵＡＮＴＡでは、分子相互の行動を対話式に構築、修飾、視覚化、及び解析することができる。

【００６１】

特定のタンパク質と相互作用する薬物のコンピュータモデリングは、多数の論文において検討されており、これには、ロティビネンら，１９８８，Acta Pharmaceut. Fennica 97: 159-166、リプカ，１９８８，New Scientist 54-57、マッキナリ及びロスマン，１９８９，Ann. Rev. Pharmacol. Toxiciol. 29: 111-122、ペリー及びデイビス，１９８９，OSAR: Quantitative Structure-Activity Relationships in Drug Design, pp. 189-193 (Alan R. Liss, Inc.)、ルイス及びディーン，１９８９，Proc. R. Soc. Lond. 236: 125-140 and 141-162等があり、核酸構成要素のモデル受容体に関しては、アスキュら，１９８

９，Ｊ．Ａｍ．Ｃｈｅｍ．Ｓｏｃ．１１１：１０８２-１０９０がある。化学物質を選別し画像で表現するその他のコンピュータプログラムは、ＢｉｏＤｅｓｉｇｎ（カリフォルニア州パサディナ）、Ａｌｌｅｌｉｘ，Ｉｎｃ．（カナダ、オンタリオ州ミシサーガ）、及びＨｙｐｅｒｃｕｂｅ，Ｉｎｃ．（オンタリオ州ケンブリッジ）といった企業から入手できる。ここで説明するように、ＦＫＢＰ５１／５２は、多数の既知の転写因子と結合し、その一部には、ＡＦＸ－１と、Ｇｌｉ３と、無毛タンパク質が含まれる。したがって、上で説明したモデリングが、最初、特定のタンパク質に特異的な薬物への応用のために設計されたとしても、領域が特定された後、ＤＮＡ又はＲＮＡの領域に特異的な薬物の設計に適合させることが可能である。

【００６２】

ＦＫＢＰ５１／５２又はＣｙＰ４０の発現を規制する作用物質のアッセイ

本発明に従って、細胞系アッセイシステムを使用して、細胞内でＦＫＢＰ５１／５２又はＣｙＰ４０の発現を調節する作用物質をスクリーニングすることができる。アッセイは、転写又は翻訳レベルのいずれかで、ＦＫＢＰ５１／５２又はＣｙＰ４０の発現を規制する作用物質をスクリーニングするために設計することができる。以下で説明するアッセイは、ＦＫＢＰ５１／５２遺伝子発現を規制することが可能な作用物質を特定するために設計されているが、しかしながら、こうしたアッセイは、ＣｙＰ４０遺伝子発現を規制する作用物質を特定するために、同様に使用することができる。

【００６３】

一実施形態では、レポータ分子をコード化するＤＮＡを、ＦＫＢＰ５１／５２遺伝子の規制因子に結び付け、適宜の完全な細胞、細胞抽出物、又はライセートにおいて使用し、ＦＫＢＰ５１／５２遺伝子発現を調節する作用物質を特定することができる。こうしたレポータ分子には、一部として、クロラムフェニコールアセチルトランスフェラーゼ（ＣＡＴ）、ルシフェラーゼ、β－グルクロニダーゼ（ＧＵＳ）、成長ホルモン、又は胎盤性アルカリフォスファターゼが含まれる。こうした構成物を細胞に導入することで、ＦＫＢＰ５１／５２遺伝子発現の調節物質を特定するためのスクリーニングアッセイに有用な組み換え細胞が提供される。

【００６４】

細胞を試験作用物質に曝露させた後、レポータ遺伝子の発現レベルは、ＦＫＢＰ５１／５２発現を規制する試験作用物質の能力を判定するために、数量化することができる。酵素が細胞から分泌される場合、アルカリフォスファターゼアッセイは、本発明の実施において特に有用であり、その後、分泌されたアルカリフォスファターゼに関して、組織培養の上澄みを検査することが可能である。加えて、アルカリフォスファターゼ活性は、ブロンスタイン，Ｉ．ら，１９９４，Ｂｉｏｔｅｃｈｎｉｑｕｅｓ １７：１７２-１７７において説明されているような熱量測定、生物発光、又は化学発光アッセイにより測定することができる。こうしたアッセイは、薬品スクリーニングのための単純で、感度が高く、容易に自動化可能な検出システムを提供する。

【００６５】

ＦＫＢＰ５１／５２の翻訳を調節する作用物質を特定するためには、ＦＫＢＰ５１／５２転写物を含む細胞又はｉｎ　ｖｉｔｒｏ細胞ライセートを、ＦＫＢＰ５１／５２のｍＲＮＡの翻訳の調節に関して試験することができる。ＦＫＢＰ５１／５２の翻訳の阻害物質を検査するためには、ｉｎ　ｖｉｔｒｏ翻訳抽出物において、ＦＫＢＰ５１／５２のｍＲＮＡの翻訳を調節する能力に関して、試験作用物質を試験する。

【００６６】

本発明の実施形態において、ＦＫＢＰ５１／５２の発現レベルは、ＦＫＢＰ５１／５２のｍＲＮＡ転写の翻訳を阻害又は防止するアンチセンス又はリボザイムアプローチ、或いはＦＫＢＰ５１／５２遺伝子の転写を阻害する三重螺旋アプローチを使用して調節することができる。こうしたアプローチは、発毛を調節するために利用することができる。

【００６７】

アンチセンスアプローチには、ＦＫＢＰ５１／５２のｍＲＮＡの少なくとも一部に相補的

なオリゴヌクレオチド（ＤＮＡ又はＲＮＡ）の設計が含まれる。アンチセンスオリゴヌクレオチドは、相補ｍＲＮＡ転写物と結合し、翻訳を妨げる。完全な相補性は、好ましいものの、必要ではない。当業者は、ハイブリッド複合体の融点を判定する標準的な手順を使用して、許容できる不一致の度合いを確認することができる。

【００６８】

本発明の更に別の実施形態では、ＦＫＢＰ５１／５２ｍＲＮＡ転写物を触媒的に切断するように設計されたリボザイム分子を使用して、ＦＫＢＰ５１／５２のｍＲＮＡの翻訳とＦＫＢＰ５１／５２の発現を妨げることができる（例えば、１９９０年１０月４日発行のＰＣＴ公報ＷＯ９０／１１３６４、サーバら，１９９０，Science 247: 1222-1225を参照）。

【００６９】

代わりに、内因性ＦＫＢＰ５１／５２遺伝子発現は、ＦＫＢＰ５１／５２遺伝子の規制領域に相補的なデオキシリボヌクレオチド配列（つまり、ＦＫＢＰ５１／５２のプロモータ又はエンハンサ）を標的にすることで、体内の標的細胞においてＦＫＢＰ５１／５２遺伝子の転写を妨げる三重螺旋構造を形成し、低減することができる（ヘレン，Ｃ．ら，１９９１，Anticancer Drug Des. 6: 569-584、及びマーヘル，ＬＪ，１９９２，Biassays 14: 807-815を参照）。

【００７０】

本発明のオリゴヌクレオチド、すなわちアンチセンス、リボザイム、及び三重螺旋形成オリゴヌクレオチドは、この技術で知られている標準的な方法により、例えば自動化されたＤＮＡシンセサイザ（Ｂｉｏｓｅａｒｃｈ、Ａｐｐｌｉｅｄ　Ｂｉｏｓｙｓｔｅｍｓ、その他で市販されるもの等）を使用して、合成することができる。代わりに、組み換え発現ベクタを、本発明のオリゴヌクレオチドの発現を導くように構築することができる。こうしたベクタは、この技術において標準的な組み換えＤＮＡ技法により構築できる。特定の実施形態では、ウイルスベクタ等のベクタは、標的細胞における抑制性オリゴヌクレオチドのｉｎ　ｖｉｖｏ発現を目標とした遺伝子治療用途で設計することができる。

【００７１】

Ｇｌｉ３、ＡＦＸ－１、及び又は無毛転写因子の転写活性を規制する作用物質のアッセイ

本発明に従って、ＦＫＢＰ５１／５２、ＣｙＰ４０、Ｇｌｉ３、ＡＦＸ－１、及び又は無毛タンパク質により媒介される転写活性化を調節する作用物質を特定するためにアッセイを策定することができる。任意の特定の理論に拘束されるものではないが、ＦＫ５０６をＦＫＢＰ５１／５２／ｈｓｐ９０複合体に結合させること、或いはサイクロスポリンＡをＣｙＰ４０／ｈｓｐ９０複合体に結合させることは、複合体からのジンクフィンガ転写因子の無毛又はＧｌｉ３の活性化及び又は放出を促進する。無毛及び又はＧｌｉ３タンパク質の核転座は、結果として標的遺伝子の転写促進と毛の生産の刺激とを発生させる。

【００７２】

本発明に従って、調節する作用物質を特定するためにアッセイを使用することができる。Ｇｌｉ３、ＡＦＸ－１、及び又は無毛タンパク質の核への転座を調節する作用物質を特定するためにアッセイを使用することができる。このアッセイの目的から、無毛、ＡＦＸ－１、及び又はＧｌｉ３タンパク質は、ＧＦＰ等の容易に検出可能なペプチドタグによりタグを付けることができる。こうしたアッセイは、タグ付きの無毛、ＡＦＸ－１、又はＧｌｉ３タンパク質を発現する細胞を、ＦＫ５０６又はサイクロスポリンＡが存在する状態で、試験作用物質に接触させることを含む。代わりに、このアッセイでは、核ホルモン受容体リガンドが存在する状態で、核ホルモン受容体を発現する細胞を使用することができる。試験作用物質を曝露させるのに続いて、例えば、核の中に存在するタグ付きタンパク質の量を測定することで、核の中に位置するタグ付きの無毛、ＡＦＸ－１、又はＧｌｉ３タンパク質の量を測定する。ビヒクル対照が存在する状態で実施した同じアッセイと比較して、試験作用物質が存在する状態で、核の中に検出されるタグ付きタンパク質の量が減少した場合には、無毛及び又はＧｌｉ３核転座の調節物質が特定される。

【００７３】

加えて、Ｇｌｉ３、ＡＦＸ－１、又は無毛タグ付きタンパク質を発現する細胞は、ＦＫＢＰ５１／５２／ｈｓｐ９０又はＣｙＰ４０／ｈｓｐ９０複合体からの無毛、ＡＦＸ－１、及び又はＧｌｉ３の解離を調節する作用物質を検査するのに使用できる。こうしたアッセイは、ＦＫ５０６又はサイクロスポリンＡが存在する状態で、ＦＫ５０６－－又はサイクロスポリンＡ－－により媒介される前記複合体からの無毛、ＡＦＸ－１、及び又はＧｌｉ３の解離を阻害する作用物質を特定するために実行することができる。例えば、タグ付きの無毛、ＡＦＸ－１、又はＧｌｉ３タンパク質を発現する細胞は、ＦＫ５０６が存在する状態で、試験作用物質に接触させる。試験作用物質との接触に続いて、細胞ライセートを作成することが可能であり、その後、ＦＫＢＰ５１／５２又はＣｙＰ４０タンパク質複合体の免疫沈降が生じる。この免疫沈降複合体は、その後、タグ付きのＧｌｉ３、ＡＦＸ－１、又は無毛タンパク質の存在又は欠如を判定するために分析される。

【００７４】

Ｇｌｉ３転写因子の下流標的遺伝子は、ＦＫ５０６及びサイクロスポリンＡ処理により規制される。例えば、ＢＭＰ４は、Ｇｌｉ３経路の下流標的遺伝子であり、ＢＭＰ４の発現は、ＦＫ５０６及びサイクロスポリンＡが存在する状態で刺激される。したがって、本発明の特定の実施形態において、Ｇｌｉ３反応因子、例えば５’ＴＧＧＧＴＧＧＴＣ－３’を含む構成物は、様々な種類のレポータ遺伝子のいずれかと結び付けることが可能であり、ＦＫＢＰ５１／５２を発現する細胞に導入することができる。こうしたレポータ遺伝子には、上で述べたように、その一部として、クロラムフェニコールアセチルトランスフェラーゼ（ＣＡＴ）、ルシフェラーゼ、ＧＵＳ、成長ホルモン、又は胎盤性アルカリフォスファターゼが含まれる。試験作用物質に対する細胞の曝露に続いて、レポータ遺伝子の発現レベルを数量化し、試験作用物質がレポータ遺伝子の転写を規制する能力を判定することができる。ＦＫ５０６により誘導される転写のアンタゴニストの特定が望ましい事例において、この細胞は、ＦＫ５０６と試験作用物質との両方に接触させる。この細胞から分泌される酵素であるため、アルカリフォスファターゼアッセイは、本発明の実施において特に有用である。したがって、分泌されたアルカリフォスファターゼに関して、組織培養の上澄みを検査することが可能である。加えて、アルカリフォスファターゼ活性は、上で説明したような熱量測定、生物発光、又は化学発光アッセイにより測定することができる。

【００７５】

試験作用物質の免疫抑制活性

本発明は、免疫抑制の副作用なしで、発毛を調節し得る作用物質の特定に関する。したがって、本発明に従って、発毛の潜在的な調節物質として特定された任意の作用物質は、免疫反応を抑制する能力に関しても試験される。

【００７６】

試験作用物質の免疫抑制効果を測定するためのアッセイは、例えば、リンパ球刺激アッセイを含み、サイトカイン産出量、すなわちＩＬ－２産出量を測定するためのアッセイを実行することが可能である。こうしたアッセイの一つは、次のように実行される。

【００７７】

安楽死（CO_2により窒息）させた生後七週乃至１６週の範囲の成体のオスＣ３Ｈマウス（インディアナ州インディアナポリスのＨａｒｌａｎ　Ｓｐｒａｇｕｅ　Ｄａｗｌｅｙ，Ｉｎｃが市販する生きたマウス）から脾臓を摘出する。この脾臓を、直ちにハンクス平衡塩類溶液（ＨＢＳＳ、メリーランド州ゲイサーズバーグのＧｉｂｃｏ－ＢＲＬが市販）に入れる。次に、この脾臓を磨りガラスのスライドグラスの間ですりつぶし、殺菌したスクリーンで濾過し、組織の破片を取り除く。結果として生じた細胞懸濁物を、等しい体積のＦｉｃｏｌｌ－Ｐａｑｕｅ　Ｐｌｕｓ（ニュージャージ州ピスカタウェイが市販）により沈降させ、脾細胞を収集するために、２０℃で、約４０分間、４００Ｇで遠心分離する。この脾細胞は、使い捨てのピペットを使用して界面から収集し、ＨＢＳＳにより二回洗浄し、その後、２０℃で、１０分間、１００Ｇで遠心分離する。脾細胞は、１０％熱不活化ウシ胎仔血清（Ｇｉｂｃｏ－ＢＲＬ）と、ペニシリン（５０Ｕ／ｍｌ）と、ストレプトマ

イシン（１００μｇ／ｍｌ）と、Ｌ－グルタミン（２ｍＭ）と、２－メルカプトエタノール（10^{-5}Ｍ）と、Ｎ－２ヒドロキシエチルピペラジン－Ｎ’－２－エタンスルホン酸（ＨＥＰＥＳ）（１０ｍＭ）とを含むフェノールレッドフリーのＲＰＭＩ１６４０（Ｇｉｂｃｏ－ＢＲＬが市販する培地）で構成される５乃至１０ｍｌの細胞培地に再懸濁させる。この細胞について、例えばトリパンブルーを使用して、計数し、生存率をチェックする。脾細胞を10^{6}細胞／ｍｌで溶媒に再懸濁し、ピペットにより10^{5}細胞／ウェルで９６ウェル丸底プレートに入れる。脾細胞は、試験化合物が存在する状態又は存在しない状態で、５０μｌ／ウェルのコンカナバリンＡ（最終アッセイ濃度＝５μｇ／ｍｌ）を追加して活性化させる。試験化合物は、ジメチルスルホキシド（ＤＭＳＯ）による原液として作成され、その後、溶媒で稀釈して、５０μｌ／ウェルで追加し、アッセイにおけるＤＭＳＯの最終濃度が０．０５％未満になるようにする。このプレートを、３７℃、５％CO_2で、４８時間培養する。細胞は、１μＣｉ／ウェルのメチ－^{3}Ｈ－チミジン（イングランド、バッキンガムシャのＡｍｅｒｓｈａｍが市販）によりパルス標識し、更に２４時間培養する。その後、この細胞をＧＦ／Ｃフィルタプレート（イリノイ州ダウナーズグローブのＰａｃｋａｒｄが市販）上に取り出し、Ｍｉｃｒｏｓｃｉｎｔ２０（Ｐａｃｋａｒｄ）において可溶化し、ＴｏｐＣｏｕｎｔマイクロプレートシンチレーション及びルミネッセンスプレートカウンタ（Ｐａｃｋａｒｄ）において計数する。活性は、試験化合物が存在しない状態での対照活性のパーセンテージとして測定され、試験化合物濃度に対してプロットされる。このデータを、四つのパラメータによる曲線の当てはめ（Ｓｉｇｍａｐｌｏｔ）に適合させ、IC_{50}値を計算する。ここでの使用において、試験化合物が非免疫抑制性であるとみなされるのは、この方法を使用することで、（サイクロスポリンＡのIC_{50}／試験化合物のIC_{50}）×１００の比率が０．０２以下となる場合、すなわち、ここで定義されるように、非免疫抑制性試験化合物がサイクロスポリンＡの＃２％の免疫抑制活性を有する場合である。

【００７８】

細胞生存率は、ＭＴＴ（３－［４，５－ジメチル－チアゾール－２－イル］２，５－ジフェニル－テトラゾリウムブロミド）色素アッセイにより、ネルソンら，Ｊ．Immunol.，1993，150(6):2139-2147において説明されるように評価されるが、無血清、フェノールレッドフリーのＲＰＭＩ１６４０においてアッセイが実施される場合は例外となり、この色素は１００μＬ／ウェルのＤＭＳＯにおいて可溶化し、ＳｐｅｃｔｒａＭａｘ　Ｐｌｕｓマイクロプレートリーダ（カリフォルニア州メンロパークのＭｏｌｅｃｕｌａｒ　Ｄｅｖｉｃｅ）により、５４０ｎｍのＯＤにおいて、６５０ｎｍのバックグラウンド補正で読み取りが行われる。

【００７９】

代わりに、試験作用物質が免疫抑制効果を有するか否かを判定するために、動物実験を実施することができる。

【００８０】

本発明に従ってスクリーニングできる作用物質

上で説明したアッセイでは、ＦＫＢＰ５１／５２の活性を調節する作用物質を特定することができる。例えば、ＦＫＢＰ５１／５２の活性に影響を与える作用物質には、一部として、ＦＫＢＰ５１／５２と結合して、ＦＫＢＰ５１／５２の活性を調節する作用物質が含まれる。代わりに、作用物質は、ＦＫＢＰ５１／５２とは直接的に結合しないが、ＦＫＢＰ５１／５２の信号伝達に関与するタンパク質の活性を改変することでＦＫＢＰ５１／５２の活性を改変できるものとして特定できる。更に、（ＦＫＢＰ５１／５２の全長又は切断形態を調節できるように、転写に影響を与える、或いはスプライシング事象に干渉する分子、例えばタンパク質又は小有機分子等、を含め、ＦＫＢＰ５１／５２遺伝子発現に影響を与えることで）ＦＫＢＰ５１／５２遺伝子活性に影響を与える作用物質を、本発明のスクリーンを使用して特定することができる。

【００８１】

本発明に従ってスクリーニングできる作用物質には、一部として、小有機又は無機作用物

質と、ペプチドと、抗体及びその断片と、ＦＫＢＰ５１／５２に結合し、ＦＫＢＰ５１／５２の任意の既知又は未知の基質により誘発される活性を模倣するか（つまりアゴニスト）、或いはＦＫＢＰ５１／５２の任意の既知又は未知の基質により誘発される活性を阻害する（つまりアンタゴニスト）その他の有機作用物質（例えばペプチドミメティック）とが含まれる。ＦＫＢＰ５１／５２と結合して、ＦＫＢＰ５１／５２の活性を高める（アゴニスト）或いはＦＫＢＰ５１／５２の活性を阻害する（アンタゴニスト）作用物質が、特定されることになる。ＦＫＢＰ５１／５２の活性を改変／調節するタンパク質と結合する作用物質が、特定されることになる。

【００８２】

作用物質には、一部として、例えばランダムペプチドライブラリの構成要素のような可溶性ペプチド等のペプチド（ラム，Ｋ．Ｓ．ら，１９９１，Nature 354: 82-84、ホートン，Ｒ．ら，１９９１，Nature 354: 84-86等を参照）と、Ｄ及び又はＬ型アミノ酸により作成された、組み合わせ化学に由来する分子ライブラリと、ホスホペプチド（ランダム、又は部分縮重した方向性のあるホスホペプチドライブラリの構成要素等）（ソンヤン，Ｚ．ら，１９９３，Cell 72: 767-778等を参照）と、抗体（ポリクローナル、モノクローナル、ヒト化、抗イディオタイプ、キメラ、又は一本鎖抗体、及びＦａｂ、Ｆ（ａｂ’）$_2$ＦＶ、及びＦＡｂ発現ライブラリ断片、及びそのエピトープ結合断片）と、小有機又は無機分子とが含まれる。

【００８３】

本発明に従ってスクリーニング可能なその他の作用物質には、一部として、（遺伝子発現に関与する規制領域又は転写因子と相互作用すること等により）ＦＫＢＰ５１／５２遺伝子又はＦＫＢＰ５１／５２信号伝達経路に関与するその他の遺伝子の発現に影響を与える小有機分子、或いは、ＦＫＢＰ５１／５２の活性又はＦＫＢＰ５１／５２の活性を調節することに関与する他の何らかの因子の活性に影響を与えるような作用物質、例えばＦＫＢＰ５１／５２を修飾し、これによりＦＫＢＰ５１／５２の酵素活性を不活性化するタンパク質等が含まれる。

【００８４】

発毛の調節物質を含む組成物及びその使用法

本発明は、上で述べたアッセイを使用して特定したＦＫＢＰ５１／５２又はＣｙＰ４０アゴニスト又はアンタゴニスト等、有効な量のＦＫＢＰ５１／５２又はＣｙＰ４０調節作用物質に細胞を接触させることを含む、発毛を調節する方法を提供する。ＦＫＢＰ５１／５２又はＣｙＰ４０阻害物質すなわちアンタゴニストの「有効な量」とは、発毛を検出可能なほどに減少させる量である。ＦＫＢＰ５１／５２又はＣｙＰ４０活性化物質すなわちアゴニストの「有効な量」とは、発毛を検出可能なほどに増加させる量である。

【００８５】

本発明は、更に、こうした処置が必要な対象において発毛を調節する方法を提供し、これは、上で述べたように特定されたＦＫＢＰ５１／５２又はＣｙＰ４０の活性を調節する有効な量の作用物質を対象に投与することを含む。

【００８６】

本発明は、更に、ＦＫＢＰ５１／５２又はＣｙＰ４０の活性の一つ以上の活性化物質又は阻害物質を含む組成物を提供する。この組成物は、ＦＫＢＰ５１／５２又はＣｙＰ４０に直接的に作用することが可能であり、或いは代わりに、ＦＫＢＰ５１／５２又はＣｙＰ４０信号伝達経路に関与するタンパク質に作用することができる。

【００８７】

本発明は、更に、ＦＫＢＰ５１／５２、ＣｙＰ４０、或いはＦＫＢＰ５１／５２、ＣｙＰ４０が媒介する信号伝達の活性、及び又はＦＫＢＰ５１／５２又はＣｙＰ４０の発現を調節し、これにより発毛を調節し得る有効な量の作用物質を含む薬品組成と、製薬において許容される担体を提供する。特定の実施形態において、「製薬において許容される」という用語は、動物での使用、特に人間での使用に関して、連邦又は州政府の規制機関により承認されること、或いは、米国薬局方又はその他の一般に認められる薬局方に記載される

ことを意味する。「担体」という用語は、治療物質が投与される際に伴う稀釈剤、アジュバント、賦形剤、又はビヒクルを指す。適切な薬品担体の例は、ゲンナロら（編）のRemington: The Science and Practice of Pharmacy, 20th Edition, Lippincott Williams & Wilkins, Philadelphia, PA (ISBN 0-683-306472)において説明されている。

【００８８】

本発明は、更に、ＦＫＢＰ５１／５２又はＣｙＰ４０の発現又は活性を調節する作用物質の投与による、発毛に関連する様々な疾患の治療を提供する。こうした作用物質には、一部として、ＦＫＢＰ５１／５２又はＣｙＰ４０アゴニスト及びアンタゴニストが含まれる。こうした疾患には、一部として、男性型禿頭症と、女性型禿頭症と、中毒性禿頭症と、円形脱毛症と、瘢痕性脱毛症とが含まれる。加えて、この作用物質は、放射線又は化学療法に関連する脱毛の対象を治療するのに使用することができる。

【００８９】

本発明の作用物質は、好ましくは、ヒトでの試験及び使用の前に、望ましい治療的又は予防的活性に関して、動物系において、ｉｎ　ｖｉｔｒｏで、及びその後、ｉｎ　ｖｉｖｏで、試験される。例えば、特定の治療物質の投与が示唆されるか否かを決定するのに使用されるｉｎ　ｖｉｔｒｏアッセイには、ＦＫＢＰ５１／５２又はＣｙＰ４０を発現する細胞を治療物質に曝露させるか、或いはこの細胞にその他の方法で治療物質を投与する、ｉｎ　ｖｉｔｒｏ細胞株アッセイが含まれ、これにおいて、こうした治療物質のＦＫＢＰ５１／５２又はＣｙＰ４０に対する効果は、その後、ＦＫＢＰ５１／５２又はＣｙＰ４０の活性が観察された時に観察される。本発明の特定の実施形態においては、作用物質がＣｙＰ４０又はＦＫＢＰ５１／５２により媒介される信号伝達経路を規制する能力が検査される。

【００９０】

本発明は、必要な対象に有効な量の本発明の発毛調節作用物質を投与することを含む治療及び又は予防の方法を提供する。好適な態様において、この作用物質は、十分に精製されている。対象は、好ましくは動物であり、更に好ましくは哺乳類であり、最も好ましくはヒトである。

【００９１】

発毛を調節し得る作用物質を投与するためには、リポソームによるカプセル化、微粒子、マイクロカプセル等、様々な伝達システムが知られており、これらを使用することが可能である。導入の方法には、一部として、皮内と、局所と、筋肉内と、腹腔内と、静脈と、皮下と、鼻腔内と、硬膜外と、経口との経路が含まれる。作用物質は、例えば、注入又は静脈内ボーラス、及び上皮又は皮膚粘膜内層（例えば、口腔粘膜、直腸及び腸粘膜）からの吸収により、任意の都合の良い経路で投与可能であり、その他の生物活性作用物質と一緒に投与することができる。投与は、全身又は局所にすることが可能であり、好ましくは、局所適用のものが採用される。

【００９２】

特定の実施形態では、本発明の組成物を身体の特定のエリアに局部的に投与することが望ましい場合がある。これは、制限的ではない例として、局所適用により達成できる。本発明の方法に従って特定された活性化合物は、一般に、少なくとも一つのこうした化合物を、薬品において許容されるビヒクル又は稀釈剤と共に含む、薬品組成物の形態で投与される。こうした組成物は、一般に、局所適用のために固体又は液体ビヒクル又は稀釈剤を必要に応じて利用する従来の方法で、溶液と、オイルと、ゲルと、クリームと、ゼリーと、ペーストと、ローションと、軟膏と、塗剤と、リーブオン及びリンスオフヘアコンディショナと、シャンプと、エアゾールと、その他との形態に調製される。

【００９３】

本発明の方法に従って特定された活性化合物の適用のためのビヒクルの例には、水溶液又は水アルコール溶液、水中油型又は油中水型の乳液、乳化ゲル、又は二相系が含まれる。好ましくは、本発明による組成物は、ローション、クリーム、ミルク、ゲル、マスク、ミクロスフィア又はナノスフィア、或いはベシクルの分散の形態をとる。ベシクルの分散の

場合、ベシクルを作成する脂質は、イオン型又は非イオン型、或いはこれらの混合にすることが可能である。

【００９４】

活性化合物を含む局所組成物は、例えば、水、アルコール、アロエゲル、アラントイン、グリセリン、ビタミンＡ及びＥオイル、鉱油、プロピレングリコール、プロピオン酸ＰＰＧ－２ミリスチル、及びその他といった、この技術で公知の様々な担体物質と、任意の様々なタイプの透過促進剤、増粘剤、ｐＨ安定剤、酸化防止剤、防腐剤、香料、着色剤、及びその他と、に混合することができる。

【００９５】

局所的担体において使用するのに適したその他の物質には、この技術において知られているように、例えば、エモリエントと、溶剤と、湿潤剤と、濃化剤と、パウダとが含まれる。

【００９６】

本発明の組成物は、更に、カリウムチャネル開放剤と、抗アンドロゲン物質と、甲状腺ホルモン及びその誘導体及び類縁物質と、プロスタグランジンアゴニスト又はアンタゴニストと、レチノイドと、トリテルペンと、この技術で知られている、或いは今後特定されるその他の物質と、といった、その他の発毛調節作用物質を、随意的に含むことができる。

【００９７】

特定の疾患の治療において効果的となる本発明の作用物質の量は、疾患の性質に依存し、標準的な臨床手法により決定することができる。最適な投与量の範囲の特定を支援するために、ｉｎ　ｖｉｔｒｏアッセイを随意的に採用することができる。処方において採用する正確な投与量は、投与経路と疾患の性質とに依存し、医師の判断に応じて、各患者の状況を考慮して、決定するべきである。有効な投与量は、ｉｎ　ｖｉｔｒｏ又は動物モデル試験系から導かれた用量反応曲線から推定することができる。

【００９８】

本発明は、更に、本発明の薬品組成物の一つ以上の材料を含んだ一つ以上のコンテナを備える薬品パック又はキットを提供する。こうした（複数の）コンテナには、薬品又は生物学商品の製造、使用、又は販売を規制する政府機関により規定された形式の通告を随意的に関連付けることが可能であり、この通告は、ヒトへの投与に関する、製造、使用、又は販売規制機関による承認を反映したものとなる。このキットは、更に、発毛を調節する、すなわち刺激又は阻害する、組成物の使用法を指示する印刷済みの取扱説明書又は印刷ラベルを備えてもよい。

【発明を実施するための最良の形態】

【００９９】

以下の例は、本発明の作成及び実施の好適な形態を例示しているが、代替の方法を利用して同じものを得ることができるため、これは本発明の範囲を制限することを意図するものではない。

【０１００】

例：ＦＫＢＰ５１／５２により媒介される発毛

以下に提示する例は、（ｉ）ＦＫＢＰ５１及びＦＫＢＰ５２が、真皮乳頭において選択的に発現することと、（ｉｉ）ＦＫＢＰ５１／５２が、ｈｓｐ９０、ＡＦＸ－１、Ｇｌｉ３、及び無毛タンパク質と共沈することと、（ｉｉｉ）ＢＭＰ４及びＨＮＦ３β遺伝子、及びＧｌｉ３反応性遺伝子の発現が、ＦＫ５０６により活性化されることを実証する。

【０１０１】

材料及び方法

逆転写

様々なヒト細胞におけるＦＫＢＰ５１、ＦＫＢＰ５２、及びＣｙＰ４０のｍＲＮＡの検出には、市販のＲＴ－ＰＣＲキット（Ｐｒｏｍｅｇａ　Ａｃｃｅｓｓ　ＲＴ－ＰＣＲキット）を使用した。ヒトＦＫＢＰ５１に関して使用されたプライマの配列は、ＴＧＡＡＧＡＡＡＧＣＣＣＣＡＣＡＧＣ（ＳＥＱ　ＩＤ　ＮＯ：１）（フォワードプライマ）と、ＣＴＣ

ＣＡＡＡＡＣＣＡＴＡＴＣＴＴＧＧＴＣＣ（ＳＥＱ　ＩＤ　ＮＯ：２）（リバースプライマ）とである。ヒトＦＫＢＰ５２に関するプライマ配列は、ＡＣＡＴＴＧＣＣＡＴＡＧＣＣＡＣＣＡ（ＳＥＱ　ＩＤ　ＮＯ：３）（フォワードプライマ）と、ＡＧＣＣＡＡＧＡＣＡＣＧＡＴＣＴＴＣ（ＳＥＱ　ＩＤ　ＮＯ：４）（リバースプライマ）とである。ヒトＣｙＰ４０に関するプライマ配列は、ＴＧＡＡＧＧＡＡＧＧＡＧＡＴＧＡＣＧＧＧ（ＳＥＱ　ＩＤ　ＮＯ：５）（フォワードプライマ）と、ＴＣＣＴＣＡＧＧＧＡＡＡＴＣＴＧＧＡＴＧＡ（ＳＥＱ　ＩＤ　ＮＯ：６）（リバースプライマ）とである。

【０１０２】

トータルＲＮＡは、ＴＲＩＺＯＬ試薬（Ｌｉｆｅ　Ｔｅｃｈｎｏｌｏｇｉｅｓ）を使用し、製造会社の指示に従って、細胞株から抽出した。このＲＮＡを、ＤＮａｓｅにより処理し、ゲノムＤＮＡによる潜在的な汚染を取り除き、その後、ＲＴ－ＰＣＲ反応に使用した。ＰＣＲ反応産物は、２％アガロースゲル上で動かし、増幅された産物を視覚化し、適切な制限酵素により消化し、産物を確認した。

【０１０３】

組織の抽出

ヒト皮膚組織は、氷上でＴＰＥＲ試薬（イリノイ州ロックフォードのＰｉｅｒｃｅ）を１０：１（抽出緩衝液：組織、体積：重量）の比率で使用し、プロテアーゼ阻害物質（Ｐｒｏｔｅａｓｅ　Ｉｎｈｉｂｉｔｏｒ　Ｃｏｃｋｔａｉｌ、１：５０の稀釈、ミズーリ州セントルイスのＳｉｇｍａ）が存在する状態で、Ｐｏｌｙｔｒｏｎホモジナイザを使用して抽出し、ライセートを形成した。

【０１０４】

抗体のマグネチックレジン・プロテインＧセファロースとの結合

使用される抗体には、抗ｈｓｐ９０（１ｍｇ／ｍｌ　ＢＳＡを伴うモノクローナルＩｇＧ１、ＴＬ）及び抗ＦＫＢＰ５２（抗ペプチドポリクローナルＮ１７及びＣ１９　Ａｂｓ、Ｓａｎｔａ　Ｃｒｕｚ　Ｂｉｏｔｅｃｈｎｏｌｏｇｉｅｓ）が含まれる。抗体は、Ａｍｉｃｏｎ　Ｍｉｃｒｏｃｏｎ－３０を使用して、濃縮し、ＰＢＳにより三回洗浄した。抗体は、中性ｐＨのＰＢＳ緩衝液において、３７℃で一晩、トシル活性化Ｄｙｎａ　Ｍ－４５０ビーズと結合させた。レジンは、トリスＨＣｌ、ｐＨ８により、４時間、３７℃でブロックした。

【０１０５】

免疫沈降

ＦＫ５０６は、最終濃度１μＭで、ライセートに追加した。抗体複合体は、ＴＰＥＲ緩衝液において、６℃で一晩、免疫沈降させた。複合体は、トリス／生理食塩水（４℃）により十回洗浄し、その後、１Ｍ　NH_4CO_3又は２０ｍＭトリス、ｐＨ７．４、０．３ＭＮａＣｌ（５×１００μｌ）、及び０．１％ＴＦＡ、５％ＭｅＯＨ（５×１００μｌ）により溶出させた。複合体を濃縮し、緩衝液は、食塩溶出のために交換するか、或いは、ＴＦＡ／ＭｅＯＨ溶出のために真空下で体積を低減した。試料は、－２０℃で保存した。

【０１０６】

プロテアーゼ消化

タンパク質は、１Ｍ　ＧｎＨＣｌ、１００ｍＭ　NH_4CO_3、０．５ｍＭ　ＤＴＴに懸濁し、トリプシン（Ｐｒｏｍｅｇａ）又はｇｌｕＣ（Ｒｏｃｈｅ）により、約１８時間、３７℃で、消化を施した。消化物は、６０％アセトニトリル、０．１％ＴＦＡにより溶出したＣ１８　ＺｉｐＴｉｐｓ（Ｍｉｌｌｉｐｏｒｅ）を使用して脱塩した。

【０１０７】

質量分析による解析

ペプチド消化物は、マトリックス支援レーザ脱離イオン化（ＭＡＬＤＩ）飛行時間型（ＴＯＦ）質量分析計により解析した。基本的に、ペプチドは、ドライドロップレット法が使用されたマトリックス（１：１の試料：α－シアノ－４－ヒドロキシ桂皮酸、２０ｍｇ／ｍＩ－ＨＣＣＡ、３０％アセトニトリル、０．１％ＴＦＡ）と混合した。ペプチドは、リフレクタモードのＶｏｙａｇｅｒ　ＤＥ－Ｐｒｏスペクトロメータ（ＰＥ　ＢｉｏＳｙｓ

ｔｅｍｓ）を使用して、陽イオン加速電圧２０ｋＶ、グリッド電圧１２．８ｋＶ、誘導線電圧１４００Ｖで、１００ｎｓ遅延抽出を使用して解析した。６４スキャンより大きいものをスペクトル毎に平均化した。ブラジキニン及びＡＣＴＨ（１８乃至３９番目のアミノ酸残基）の３０乃至５０ｆｍｏｌ毎に、内部質量標準として使用した。

【０１０８】

データベース検索

ペプチドスペクトルは、ＰｒｏＦｏｕｎｄ［ｈｔｔｐ：／／ｎｔ２／ｐｒｏｗｌ／ｐｒｏｗｌ．ｈｔｍｌ］又はＲＡＤＲＡＳ（ＯＳＩ　ｉｎｔｅｒｎａｌ）を使用して比較した。質量誤差許容量は、通常３０ｐｐｍとした。不完全な開裂部を一つ許容した。修飾は、最初に考慮しなかった。ＳＷＩＳＳＰＲＯＴとＧＥＮＢＡＮＫ　ＮＲデータベースとの両方を検索した。

【０１０９】

サイクロスポリンＡ及びＦＫ５０６処理によるＢＭＰ４及びＨＮＦ３βのｍＲＮＡの誘導一次的ヒト皮膚繊維芽細胞（ＢＭＰ４検出のため）及び皮膚ケラチノサイト（ＨＮＦ３β検出のため）を培養し、１ｕＭのサイクロスポリンＡ又はＦＫ５０６により処理した。トータルＲＮＡは、処理二日目及び四日目に取り出した。リアルタイムＰＣＲを使用して、ＢＭＰ４及びＨＮＦ３βのｍＲＮＡレベルを数量化した。ＡＢＩ　ＰＲＩＳＭ　７７００配列検出装置と、ＴａｑＭａｎ　ＰＣＲキットと、蛍光染料で標識され、市販ソフトで設計されたＰＣＲプライマとを使用して、ＲＮＡ数量化を行った。図３に提示した結果は、サイクロスポリンＡとＦＫ５０６との両方がＢＭＰ４及びＨＮＦ３βのｍＲＮＡを上昇規制することを示している。

【０１１０】

加えて、Ｇｌｉ３の下流標的遺伝子は、皮膚繊維芽細胞及び皮膚ケラチノサイトといった皮膚細胞におけるサイクロスポリンＡ及びＦＫ５０６処理により上昇規制されることが分かった。図３に示すように、ＢＭＰ４及びＨＮＦ３βのｍＲＮＡレベルは、サイクロスポリンＡ又はＦＫ５０６処理により誘導された。

【０１１１】

ＦＫ５０６及びサイクロスポリンＡによる甲状腺ホルモン受容体（ＴＲ）媒介転写の誘導四つのタンデム甲状腺ホルモン応答要素（ＴＲＥ）（ＡＧＧＴＣＡ　ＣＡＧＧ　ＡＧＧＴＣＡ）（下線の配列は反復される）（ＳＥＱ　ＩＤ　ＮＯ：７）を、単一のオリゴヌクレオチドとチミジンキナーゼ（ＴＫ）プロモータの結紮された５’において、標準の手順を使用して合成した。プラスミド（ＴＲＥ－ＴＫ／ｐＵＶ１２０ｐｕｒｏ）は、結果として生じた、デウェットＪｒ．ら、１９８６，Methods Enzymol 133: 3-14のＨ．ｐｙｒａｌｉｓからのルシフェラーゼレポータ遺伝子のＴＲＥ／ＴＫプロモータ５’を、ＳＶ４０プロモータの制御下で発現する抗生物質ピューロマイシンに対する耐性を与えるタンパク質をコード化する遺伝子と共に結び付けることで構築した。このプラスミドにおいて、ルシフェラーゼ遺伝子の発現は、ＴＲＥ－ＴＫプロモータの直接的な制御下にあり、甲状腺ホルモン核受容体（ＴＲ）のアゴニストにより誘導できる。ＨｅＬａ細胞（ＡＴＣＣ、Ｍａｎａｓｓａｓ、ＶＡ２０１０８、＃ＣＣＬ－２）には、標準的な手順を使用したエレクトロポレーションで、プラスミドＤＮＡによるトランスフェクションを行い、薬物耐性細胞株は、ミシガン州セントルイスのＳｉｇｍａ－Ａｌｄｒｉｃｈ　Ｃｏｒｐ．のピューロマイシンを使用して選択した。薬物耐性細胞株（ＨｅＬａ／ＴＲＥ）は、甲状腺ホルモンに対する感応性により選択し、その後の実験では、トランスフェクションが安定した単一のクローン株を使用した。

【０１１２】

質量分析実験は、ｈｓｐ９０と、サイクロフィリン４０、ＦＫＢＰ５１、又は５２のいずれかと、無毛のジンクフィンガ転写因子と、核ホルモン受容体（甲状腺ホルモン受容体、アンドロゲン受容体、ビタミンＤ受容体、又はグルココルチコイド受容体）とをある程度含むタンパク質複合体により、発毛が媒介される可能性が高いことを示唆した。

【０１１３】

イムノフィリン信号の送信における甲状腺ホルモン核受容体の役割を確立するために、甲状腺ホルモン核受容体信号の送信を増加させる能力について、サイクロスポリンＡ、ＦＫ５０６、及び甲状腺ホルモンＴ３を評価した。ＨｅＬａ／ＴＲＥ細胞を、１ウェル当たり１０，０００細胞の濃度で、９６ウェルマイクロタイタプレートにおいて、１％チャコール処理ＦＣＳと、２ｍＭグルタミンと、抗体（ペニシリン及びストレプトマイシン）とを含むＤＭＥＭ培地に接種した。この細胞は、サイクロスポリンＡ又はＦＫ５０６（ミシガン州セントルイスのＳｉｇｍａ－Ａｌｄｒｉｃｈ Ｃｏｒｐ．、カリフォルニア州サンディエゴのＣａｌｂｉｏｃｈｅｍ－Ｎｏｖａ ｂｉｏｃｈｅｒｙｌ Ｃｏｒｐ．）の濃度を上昇させ（２０ｎＭ、２ｎｍ、２００ｐｍ、及び２０ｐＭ）、１６時間に渡って処理し、ルシフェラーゼレポータ遺伝子の活性を測定した（デウェットＪｒ．ら、１９８６，Methods Enzymol 133: 3-14）。

【０１１４】

結果

真皮乳頭（ＤＰ）細胞は、発毛にとって決定的である。培養したヒト真皮乳頭細胞において、ＣｙＰ４０及びＦＫＢＰ５１／５２を、ＲＴ－ＰＣＲを使用して検出した（図２）。ＦＫＢＰ５１／５２のレベルは、ＦＫＢＰ１２／１３のレベルよりも遙かに大きい。ＦＫ５０６の免疫抑制効果を媒介するＦＫＢＰ１２／１３のレベルは、検出限界に近く、その発現レベルが非常に小さいことを示している。ヒト真皮乳頭繊維芽細胞及びケラチノサイトにおける発現パターンについても研究し、同一であることを発見した。

【０１１５】

ヒト頭皮皮膚に由来するｈｓｐ９０及びＦＫＢＰ５２免疫沈降複合体の質量分析による解析から、三種類の転写因子の存在が明らかになっている。この因子には、無毛のジンクフィンガタンパク質、ソニックヘッジホッグ誘導因子Ｇｌｉ－３、及びＡＦＸ－１が含まれる。

【０１１６】

データは、ヒトの皮膚のＦＫ５０６がｈｓｐ９０タンパク質複合体との無毛の相互作用を改変できることも示唆している。無毛は、その後、核に転座し、発毛を規制する遺伝子の転写を刺激することが可能である。

【０１１７】

加えて、ＢＭＰ４及びＨＮＦ３といったＧｌｉ３の下流標的遺伝子は、ＦＫ５０６処理により上昇規制されることが発見された（図３）。

【０１１８】

イムノフィリン作用に関連するｈｓｐ９０及びＦＫＢＰ５２複合体は、モノクローナル抗体と、プロテアーゼ消化と、ペプチド質量の質量分析測定と、データベース検索により、ヒト頭皮皮膚抽出物から特定された。以下のタンパク質は、質量分析による解析を行ったトリプシン及び又はＧｌｕＣ消化物から特定された。

【０１１９】

表１

抗ｈｓｐ９０及び抗ＦＫＢＰ５２

ヒト皮膚抽出物＋ＦＫ５０６

３０ｐｐｍ（ＳＷＩＳＳＰＲＯＴ）

タンパク質の折り畳み、イムノフィリン、ストレス応答

	トリプシン	GluC
hsp90-β	×	×
hsp90-α	×	×
hsp70s	×	×
FKBP-51	×	×
hsp27	×	×
FKBP-52	×	×
FRAP	×	×
hsp110	×	×
hsp40タンパク質-3（DNAJホモログ1）	×	×
プロリル4-ヒドロキシラーゼ（αおよびβサブユニット）	×	
タンパク質ジスルフィドイソメラーゼ	×	×
浸透圧ストレスタンパク質94（hsp70関連）	×	×

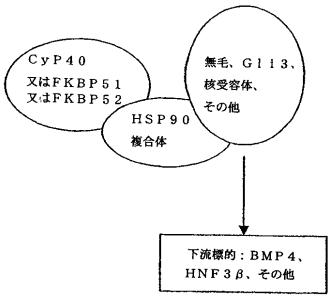
サイクロフィリン４０	×	
ステロイド状受容体		
エストロゲン受容体	×	×
グルココルチコイド受容体	×	×
アンドロゲン受容体	×	
ＲＸＲ－β		×
ＴＲＩＰ－１２		×
甲状腺ホルモン受容体	×	×
タンパク質分解		
ｃｕｌｌｉｎ－２	×	×
ｃｕｌｌｉｎ－３	×	×
ユビキチンカルボキシ末端加水分解酵素	×	×
２６Ｓプロテオソームサブユニット	×	×

ユビキチン活性化酵素Ｅ１		×
転写及びクロマチンリモデリング		
ヒト無毛（ｚｆｐ）	×	×
熱ショック因子タンパク質（ＨＳＦ－２）	×	
ＧＬＩ－３	×	×
ＡＦＸ１推定ｗｈｎ因子（フォークヘッドドメイン）	×	

【０１２０】
このデータは、サイクロスポリンＡ又はＦＫ５０６が、投与量に依存する形で、甲状腺ホルモン受容体媒介転写を誘導することを示している。ｈｓｐ９０複合体におけるサイクロスポリン４０（サイクロスポリンＡ）又はＦＫＢＰ５１／５２（ＦＫ５０６）タンパク質とのリガンドの結合は、甲状腺ホルモン受容体の転写活性を明らかに活性化する。甲状腺ホルモン受容体の修飾タンパク質である無毛タンパク質も、サイクロスポリンＡ又はＦＫ５０６の作用を調節し、これにより発毛を規制することができる。
【０１２１】
等価物
本発明は、本発明の個別の態様の単一の例示を意図した本明細書記載の特定の実施形態により範囲を限定されることはなく、機能的に同等の方法及び構成要素は、本発明の範囲に含まれる。実際に、本発明の様々な変形は、本明細書で図示及び説明したものとは別に、前記の説明と添付図面とから当業者には明らかとなろう。本明細書では様々な印刷物が引用されており、その内容は、参照により全体を本明細書に組み込むものとする。
【図面の簡単な説明】
【０１２２】
本発明の前記その他の目的、その様々な特徴、及び本発明自体は、以下の添付図面と共に以下の説明を読むことで、より完全に理解されよう。
【図１】ＦＫＢＰ５１／５２と、ＣｙＰ４０と、ステロイド受容体と、ｈｓｐ９０と、その他の結合相手との間での相互作用の提案モデルを表す説明図である。
【図２】培養したヒト真皮乳頭細胞において、ＦＫＢＰ５１と、ＦＫＢＰ５２と、ＣｙＰ４０との発現がＲＴ－ＰＣＲを使用して検出されることを実証する、ヒト真皮乳頭ＲＮＡのＲＴ－ＰＣＲ解析を示す図である。
【図３Ａ】ＦＫ５０６及びサイクロスポリンＡ治療の四日後におけるＢＭＰ４転写の刺激を実証するグラフである。
【図３Ｂ】ＦＫ５０６及びサイクロスポリンＡ治療の四日後におけるＨＮＦ３β転写の刺激を実証するグラフである。
【図４】サイクロスポリンＡ又はＦＫ５０６によるＴＲ媒介転写の誘導を示すグラフであ

る。

【図１】



【図２】

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	真皮繊維芽細胞	真皮乳頭	ケラチノサイト
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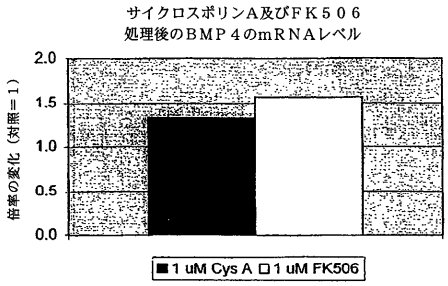

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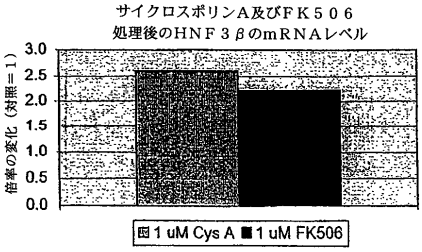

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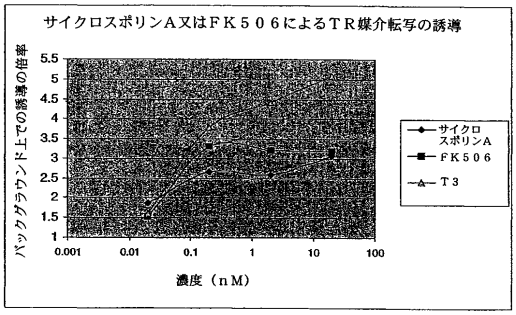
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(54) Title: FKBP51/52 AND CYP40-MEDIATED MAMMALIAN HAIR GROWTH

(57) Abstract: The present invention related to drug screening assays designed to identify non-immunosuppressive agents that modulate hair growth and the use of such agents for modulation of hair growth.

FKBP51/52 AND CYP40-MEDIATED MAMMALIAN HAIR GROWTH

BACKGROUND OF THE INVENTION

Field of the Invention

The invention relates to the fields of dermatology, cell biology, and molecular biology. More specifically, the present invention relates to drug screening assays designed to identify non-immunosuppressive agents that modulate hair growth and the use of such agents for modulation of hair growth.

Summary of the Related Art

The immunosuppressant drugs FK506, rapamycin and cyclosporin A are well known T-cell specific immunosuppressants that are routinely used to prevent graft rejection in organ transplant patients. In T cells, FK506 and cyclosporin A prevent calcineurin from dephosphorylating the transcription factor NF/AT (nuclear factor of activated T-cells), thereby blocking its translocation into the nucleus and preventing the receptor-mediated increase in synthesis and secretion of cytokines, such as interleukin-2 and, hence, T-cell proliferation (Heitman, J. et al., 1992, *The New Biologist* 4:448-460).

FK506 and cyclosporin A act by binding to endogenous intracellular receptor proteins termed immunophilins. Based on their structure and binding affinity for specific drugs, immunophilins have been divided into two classes of proteins; those proteins having an affinity for FK506 are referred to as FK506-binding proteins (FKBPs), while those having an affinity for cyclosporin are referred to as cyclophilins. Both FKBPs and cyclophilins possess a similar peptidyl-prolyl isomerase activity resulting in cis-trans-isomerization of proteins, which is believed to be important for protein folding and trafficking. In addition, both FKBPs and cyclophilins are characterized by their ability to interact with a variety of different proteins involved in signal transduction.

Several members of the FKBP family have been identified and named according to their calculated molecular mass (Lane, W.S. et al, 1991, *J. Protein*

Chem. 10:151-160; U.S. Patent No. 5,763,590). Cyclophilin A and FKBP12 were originally isolated as cyclosporin A and FK506 binding proteins, respectively, and were shown to exert immunosuppressive activity through inhibition of calcineurin. FKBP-51 was found to be expressed in T-cells where it inhibits calcineurin with much weaker potency, suggesting that multiple immunophilins may participate in mediating FK506 immunosuppressant activity. FKBP-51 has also been shown to be a component of the progesterone receptor complex (Nair, S.C. et al., 1997, *Mol. Cell Biol.* 17:594-603). FKBP52 was initially discovered as a component of the inactive steroid receptor complex (Smith, D.F. et al., 1993, *J. Biol. Chem.* 268:18365-71). The N-terminal domain, residues 1-149 of FKBP52, shares 55% homology with FKBP12, however, it does not have immunosuppressant activity when complexed with FK506. FKBP52 is phosphorylated by casein kinase II and has been found to have chaperone activity independent of isomerase activity (Miyata, Y. et al., 1997, *Proc. Natl. Acad. Sci. USA* 94:14500-14505). CyP40 has only a low affinity for cyclosporin A and is therefore capable of only slight reduction of the immunosuppressive effect of cyclosporin A.

Hsp90 is the most abundant of the heat shock proteins. A number of transcription factors and protein kinases involved in signal transduction are found complexed with hsp90 (Pratt, W.B. et al., 1999, *Cell Signal* 11:839-851; Pratt and Toft, 1997, *Endocrine Rev.* 18:306-360). When complexed with transcription factors, the hsp90 complexes are found to contain high molecular weight immunophilins with tetratricopeptide repeat (TPR) motifs (Duina, A.A. et al., 1996, *Science* 274:1713-1715; Bose, S. et al., 1996, *Science* 274:1715-1717). Such immunophilins include FKBP52 and CyP40 (Owens-Grillo, J.K., 1995, *J. Biol. Chem.* 270:20479-20484; Miyata, Y. et al., 1997, *Proc. Natl. Acad. Sci. USA* 94:14500-14505; Silverstein, A.M. et al., 1999, *J. Biol. Chem.* 274:36980-36986).

Causes of hair loss include aging, the action of male hormones, the loss of blood supply to hair follicles, and scalp abnormalities. In addition, genetic disposition may account for hair loss. For example, androgenic alopecia is considered to be genetically determined. Recently, a rare autosomal recessive form of hereditary alopecia, referred to as atrichia with papular lesions, was found to result from mutations in the human "*hairless*" gene (Ahmad, W. et al., 1998, *Science* 279:720-724). In individuals affected with this form of hair loss, hairs are typically absent from the scalp, and patients have very sparse eyebrows and eyelashes. Mutations in

the human homolog of the mouse *hairless* gene lead to congenital alopecia universalis and atrichia with papular lesions. In mice bearing a mutation in the *hairless* gene, the hair matrix cells appear to undergo premature and massive apoptosis together with a concomitant decline in Bcl-2 expression indicating that the *hairless* gene product may play a role in regulating cell proliferation, differentiation and apoptosis in the hair follicle. The human *hairless* gene has recently been isolated and is described in WO 99/38965.

It has been reported that topical application of FK506 and cyclosporin A stimulate hair growth in a dose dependent manner (Sainsbury, T.S.L. et al., 1991, *Transplant. Proc.* 23:3332-3334). For example, FK506 and cyclosporin A have been shown to stimulate hair growth in experimental animals, such as mice and rats (WO 98/55090; Maurer, M., 1997, *Am. J. Path.* 150:1433; Yamamoto, S. et al., 1993, *J. Invest. Dermatol.* 102:160). The effects of FK506 and cyclosporin A and related agents have been described (Tsuji Y. et al., 1999, *Exp. Dermatol.* 8:366-7; McElwee, K.J. et al., 1997, *Br. J. Dermatol.* 137:491-7; Iwabuchi T. et al., 1995, *J. Dermatol. Sci.* 9:64-9; Yamamoto S. and Kato R., 1994, *J. Dermatol. Sci. 7 Supp.* 1:547-54; and Yamamoto S. et al., 1994, *J. Invest. Dermatol*102:160-4).

In addition, Japanese patent application No. 11-174041 describes methods for identifying hair stimulating agents that can bind to immunosuppressive agent-bound proteins that can form a complex with steroid receptors, *i.e.*, FKBP 52 or cyclophilin 40, but do not bind to FKBPs that cannot form complexes with steroid receptors, *e.g.*, FKBP12.

The mechanism of mammalian hair growth stimulated by FK506 and cyclosporin A remains unknown. Despite their potential use as hair stimulating agents, immunosuppressive agents such as FK506 and cyclosporin A also exhibit toxic side effects such as immunosuppression. Thus, there is the need to identify and develop non-immunosuppressive agents that are useful as modulators of hair growth. The present invention is based on the discovery of the signaling pathway by which the immunosuppressive agents FK506 and cyclosporin A modulate hair growth. This discovery provides drug screening assays for identification of non-immunosuppressive agents capable of modulating hair growth.

Summary of the Invention

The present invention relates to drug screening assays designed to identify non-immunosuppressive agents that modulate hair growth and the use of such agents for modulation of hair growth. The invention is based on the discovery of the signal transduction pathway by which specific immunophilins, namely FKBP51 and FKBP52, and CyP40, modulate hair growth. As disclosed herein, the FKBP51/52 proteins are found to be expressed in hair follicle dermal papillae. In addition, FKBP51/52 are found complexed with the hsp90, Gli3, AFX-1 and hairless protein within the cell. Contact of dermal papillae cells with FK506 or cyclosporin A was also found to stimulate expression of the Gli3 target gene, BMP4 and HNF3β. The pathway of the invention serves as a basis for methods designed to identify non-immunosuppressive agents which can be used to modulate hair growth.

The invention relates to assays designed to screen for agents that modulate the components of the FKBP51/52 and CyP40 signal transduction pathway, *i.e.*, agents that act as agonists or antagonists of such components, including FKBP51/52, CyP40, hsp90, Gli3, AFX-1 and/or the hairless protein. In an embodiment of the invention, a method is provided for rational drug design of agents which specifically modulate activity and/or the association of FKBP51/52 or CyP40 with the hsp90, hairless, AFX-1 and/or Gli3 protein. The present invention further provides cell based and non-cell based assays for identifying agents which modulate the interaction and/or activity of the components of the inventive pathway, *i.e.*, the FKBP51/52, CyP40, hsp90, Gli3 AFX-1 and hairless protein.

Specifically, the invention provides a method for identifying a compound capable of modulating hair growth comprising:

(i) contacting a cell that expresses, or a preparation containing, FKBP51/52 or CyP40, hsp90 and a protein selected from the group consisting of hairless, AFX-1, and gli3, with a test compound;

(ii) determining the level of complex formation between FKBP51/52 or CyP40, hsp90 and at least one protein selected from the group consisting of the hairless, AFX-1, and gli3, in the cell, or preparation, contacted with the test compound; and

(iii) comparing the level of complex formation obtained in (ii) to the level of complex formation between FKBP51/52 or CyP40, hsp90, and at

least one protein selected from the group consisting of the hairless, AFX-1, and gli3, in the absence of test compound;

wherein a difference in the level of complex formation in the presence versus in the absence of test compound has a positive correlation with hair growth modulating activity.

As used herein, the term "preparation" refers to a composition comprising at least one cellular component that has been isolated, extracted or partially purified either from a cell in which it is naturally expressed or from a cell which has been genetically engineered to express the component, or a component that has been synthetically prepared, which composition can be used to carry out the recited method. Such preparations include, but are not limited to, cell fractions prepared by standard techniques, as well as aqueous, buffered solutions of cellular components prepared by combining together previously synthesized or purified components.

As used herein, the phrase "positive correlation with hair growth modulating activity" refers to an observation of the biological activity of a test compound wherein the activity indicates that the test agent is capable of either stimulating or inhibiting hair growth.

In another embodiment of the invention, a method is provided for identifying a compound capable of modulating hair growth comprising:

(i) contacting a cell that expresses a nuclear hormone receptor and a reporter gene under the transcriptional control of a *hairless*, AFX-1, or gli3 gene responsive element with a test compound and measuring the level of reporter gene expression in the cell;

(ii) measuring the level of reporter gene expression in the absence of the test compound; and

(iii) comparing the levels of reporter gene expression measured in (i) and (ii);

wherein a difference in the levels of reporter gene expression measured in steps (i) and (ii) has a positive correlation with hair growth modulating activity of the test compound.

The invention further provides a method for identifying a compound capable of promoting hair growth comprising:

(i) contacting a sample containing a *hairless,* AFX-1 or gli3 gene product with a test compound;

(ii) determining whether the test compound binds to the *hairless*, AFX-1 or gli3 gene product; and

(iii) determining whether the test compound inhibits complex formation between the *hairless*, AFX-1 or gli3 gene product and a binding partner selected from the group consisting of FKBP51/52, CyP40, a nuclear hormone receptor, a hsp90 protein, and a combination thereof;

wherein the ability of a test compound to both bind to the *hairless,* AFX-1 or gli3 gene product and inhibit complex formation has a positive correlation with hair growth promoting activity.

The invention further relates to a method for identifying a compound capable of modulating hair growth comprising:

(i) contacting a cell that expresses FKBP51/52 or CyP40, and hsp90, a nuclear hormone receptor, and the *hairless,* AFX-1 or gli3 gene product with a test compound in the presence of a nuclear hormone receptor ligand;

(ii) determining the level of nuclear translocation of the *hairless*, AFX-1 or gli3 gene product into the nucleus of the cell;

(iii) determining the level of nuclear translocation in the absence of the test compound; and

(iv) comparing the level of nuclear translocation measured in (ii) and (iii);

wherein a difference in the level of nuclear translocation measured in steps (ii) and (iii) has a positive correlation with hair growth modulating activity of the test compound.

Identified agents can be used to modulate hair growth. Such agents are particularly useful for treating baldness resulting from genetic factors, aging, local skin conditions and diseases that affect the body generally, *i.e.*, systemic diseases. Such disorders include, but are not limited to, male pattern baldness, female pattern

baldness, toxic baldness, alopecia areata and scarring alopecia. In addition, the agents can be used to treat subjects with hair loss associated with radiation or chemotherapy.

Thus, the invention encompasses a method for modulating hair growth in a mammal comprising administering to the mammal a compound that modulates complex formation between FKBP51/52, CyP40 or hsp90 and at least one protein selected from the group consisting of hairless, gli3, AFX-1, and hsp90.

The invention provides a method for modulating hair growth in a mammal comprising administering to the mammal a compound that modulates the nuclear translocation of a protein selected from the group consisting of the hairless, AFX-1 and gli3 protein.

In yet another embodiment of the invention, a method is provided for modulating hair growth in a mammal comprising administering to the mammal a compound that modulates *hairless*, AFX-1-or gli-3-mediated gene expression.

Description of the Figures

The foregoing and other objects of the present invention, the various features thereof, as well as the invention itself may be more fully understood from the following description, when read together with the accompanying drawings in which:

Figure 1 is a schematic representation depicting the proposed model of interactions between FKBP51/52, CyP40, steroid receptors, hsp 90 and other binding partners.

Figure 2 is a representation of RT-PCR analysis of human dermal papilla RNA, demonstrating that in cultured human dermal papilla cells, expression of FKBP51, FKBP52 and CyP40 is detected using RT-PCR.

Figure 3A is a graphic representation demonstrating stimulation of BMP4 transcription four days after FK506 and cyclosporin A treatment.

Figure 3B is a graphic representation demonstrating stimulation of HNF3β transcription four days after FK506 and cyclosporin A treatment.

Figure 4 is a graphic presentation of the induction of TR mediated transcription by cyclosporin A or FK506.

Detailed Description

The present invention is based on the discovery of the signal transduction pathway by which specific immunophilins, namely FKBP51, FKBP52 and CyP40, modulate hair growth. The discovery of the pathway by which agents such as FK506 and cyclosporin A modulate hair growth provides screening targets for agents that can be used to promote hair growth while having a reduced effect on the immune system.

The present invention encompasses assays designed to identify agents that modulate the interaction and/or activity of the components of the signal transduction pathway. Such components include, for example, the FKBP51/52, CyP40, hsp90, Gli3, AFX-1 and hairless proteins. Both cell based and non-cell based assays can be used to identify agents that either increase or decrease the activity of the FKBP51/52 and CyP40 signal transduction pathway. The present invention further provides for rational drug design of agents that specifically promote or inhibit association of FKBP51/52 or CyP40 with the hsp90, hairless, AFX-1 and/or Gli3 proteins.

In other embodiments, the present invention provides for agents designed or identified by the foregoing methods. Further, the present invention provides for the use of said agents for enhancement of hair growth. In particular, such agents can be used to treat baldness which can result from genetic factors, aging, and/or local skin conditions.

SCREENING ASSAYS FOR AGENTS USEFUL IN MODULATING THE ACTIVITY OF FKBP51/52/CYP40

The present invention relates to screening assay systems designed to identify agents or compositions that modulate FKBP51/52 or CyP40 activity or FKBP51/52 or CyP40 gene expression, and thus, may be useful for modulation of hair growth.

RECOMBINANT EXPRESSION OF PROTEINS INVOLVED IN HAIR GROWTH

For purposes of developing screening assays designed to identify agents or compositions that modulate hair growth, it may be necessary to recombinantly express the FKBP51/52 or CyP40 proteins and/or the proteins that

interact with FKBP51/52, and CyP40, *i.e.*, hsp90, Gli3, AFX-1 and hairless proteins. The cDNA sequences and deduced amino acid sequences of FKBP51 and FKBP52 have been characterized (PubMed Accession Nos.U71321 and M88279) and are incorporated herein by reference. The term FKB51/52 as used herein refers to either or both the FKBP51 and FKBP52 proteins. The cDNA sequence and deduced amino acid sequences of CyP40 have been characterized (Accession No. D63861) and is incorporated herein by reference. The cDNA sequences and deduced amino acid sequences of hsp90, Gli3, AFX-1 and *hairless* have also been characterized (PubMed Accession Nos. NMO18411 (hairless); NM000168 (Gli3) and NM005348 (hsp90); Accession No. U10072 and are incorporated herein by reference. For simplicity, recombinant expression is described below for FKBP51/52; however, the methods can also be utilized for recombinant expression of CyP40, hsp90, Gli3, AFX-1 and/or the hairless protein.

FKBP51/52 nucleotide sequences can be isolated using a variety of different methods known to those skilled in the art. For example, a cDNA library constructed using RNA from a tissue known to express FKBP51/52 can be screened using a labeled FKBP51/52 specific probe. Alternatively, a genomic library can be screened to derive nucleic acid molecules encoding the FKBP51 or FKBP52 protein. Further, FKBP51/52 nucleic acid sequences can be derived by performing a polymerase chain reaction (PCR) using two oligonucleotide primers designed on the basis of known FKBP51/52 nucleotide sequences. The template for the reaction can be cDNA obtained by reverse transcription of mRNA prepared from cell lines or tissue known to express FKBP51/52.

FKBP51/52 protein, polypeptides and peptide fragments, mutated, truncated or deleted forms of FKBP51/52 and/or FKBP51/52 fusion proteins can be prepared for a variety of uses, including, but not limited to, the identification of other cellular gene products involved in the regulation of FKBP51/52-mediated hair growth, and the screening for agents that can be used to modulate hair growth. FKBP51/52 fusion proteins include fusions to an enzyme, fluorescent protein, and a polypeptide tag or luminescent protein, all of which provide a marker function.

While the FKBP51/52 polypeptides and peptides can be chemically synthesized (*e.g.*, see Creighton, 1983, Proteins: Structures and Molecular Principles, W.H. Freeman & Co., N.Y.), large polypeptides derived from FKBP51/52 and the full

length FKBP51/52 proteins can be advantageously produced by recombinant DNA technology using techniques well known in the art for expressing nucleic acids containing FKBP51/52 gene sequences and/or other coding sequences. Such methods can be used to construct expression vectors containing the FKBP51/52 nucleotide sequences and appropriate transcriptional and translational control signals. These methods include, for example, *in vitro* recombinant DNA techniques, synthetic techniques, and *in vivo* genetic recombination. (See, for example, the techniques described in Sambrook et al., 1989, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Press, N.Y.; and Ausubel et al., 1989, Current Protocols in Molecular Biology, Green Publishing Associates and Wiley Interscience, N.Y.).

A variety of host-expression vector systems can be utilized to express the FKBP51/52 nucleotide sequences (See, *e.g.*, U.S. Patent No. 5,763,590 for expression of FKBP 52). Where the FKBP51/52 peptide or polypeptide is expressed as a soluble derivative and is not secreted, the peptide or polypeptide can be recovered from the host cell. Alternatively, where the FKBP51/52 peptide or polypeptide is secreted, the peptide or polypeptides can be recovered from the culture media.

The expression systems that can be used for purposes of the invention include, but are not limited to, microorganisms such as bacteria transformed with recombinant bacteriophage, plasmid or cosmid DNA expression vectors containing FKBP51/52 encoding nucleotide sequences, yeast transformed with recombinant yeast expression vectors containing FKBP51/52 encoding nucleotide sequences or mammalian cell systems, or insect cell systems containing FKBP51/52 recombinant expression constructs containing promoters derived from the genome of mammalian or insect cells or from mammalian or insect viruses.

Appropriate expression systems can be chosen to ensure that the correct modification, processing, and sub-cellular localization of the FKBP51/52 protein occurs. To this end, eukaryotic host cells that possess the ability to properly modify and process the FKBP51/52 protein are preferred. For long-term, high yield production of recombinant FKBP51/52 protein, such as that desired for development of cell lines for screening purposes, stable expression is preferred. Rather than using expression vectors which contain origins of replication, host cells can be transformed with DNA controlled by appropriate expression control elements and a selectable marker gene, *e.g.*, *tk*, *hgprt*, *dhfr*, *neo*, and *hygro* genes, to name a few. Following the

introduction of the foreign DNA, engineered cells may be allowed to grow for 1-2 days in enriched media, and then switched to a selective media. Such engineered cell lines can be particularly useful in screening and evaluating agents that modulate the endogenous activity of the FKBP51/52 gene product.

In addition, in some instances it may be necessary to co-express interactive binding proteins such as the CyP40, hsp90, Gli3, AFX-1 and hairless proteins for use in the screening assays of the invention. Methods described above for expression of FKBP51/52 can be similarly used to co-express such binding proteins.

NON-CELL BASED ASSAYS

In accordance with the invention, non-cell based assay systems can be used to identify agents that interact with, *i.e.*, bind to, FKBP51/52 or CyP40, and regulate the activity of such proteins. Such agents may act as antagonists or agonists of FKBP51/52 or CyP40 activity and can be used to regulate hair growth. In particular, such agents may function to disrupt or prevent the formation of a complex between FKBP51/52 or CyP40 and their binding partners, *i.e.*, the hsp90, Gli3, AFX-1 and/or hairless protein. For simplicity, the non-cell based assays are described below for FKBP51/52; however, they can be similarly utilized for CyP40 as well.

Recombinant FKBP51/52, including peptides corresponding to different functional domains, or FKBP51/52 fusion proteins, can be expressed and used in assays to identify agents that interact with FKBP51/52.

To this end, soluble FKBP51/52 can be recombinantly expressed and utilized in non-cell based assays to identify agents that bind to FKBP51/52. Recombinantly expressed FKBP51/52 polypeptides or fusion proteins containing one or more of the FKBP51/52 functional domains can be prepared as described above, and used in the non-cell based screening assays. One such functional domain is the tetratricopeptide repeat (TPR) which is important for protein/protein interactions. For example, the full length FKBP51/52, or a soluble truncated FKBP51/52, *e.g.*, in which one or more domains is deleted from the molecule but the TPR is retained, a peptide corresponding to the TPR motifs, or a fusion protein containing the FKBP51/52 TPR motif fused to a protein or polypeptide that affords advantages in the assay system (*e.g.*, for labeling or isolating the resulting complex) can be utilized. Where agents that interact with the TPR motif are sought to be identified, peptides corresponding to

the FKBP51/52 TPR motif and fusion proteins containing the FKBP51/52 TPR motif can be used. The FKBP51/52 protein can also be present as part of a crude or semi-purified extract.

The principle of the assays used to identify agents that bind to FKBP51/52 involves preparing a reaction mixture of FKBP51/52 and the test agent under conditions and for time sufficient to allow the two components to interact and bind, thus forming a complex which can be removed and/or detected in the reaction mixture. The identity of the bound test agent is then determined.

The screening assays are accomplished by any of a variety of commonly known methods. For example, one method to conduct such an assay involves anchoring the FKBP51/52 protein, polypeptide, peptide, fusion protein, or the test substance onto a solid phase and detecting FKBP51/52/test agent complexes adhered to the solid phase at the end of the reaction. In one embodiment of such a method, the FKBP51/52 reactant is anchored onto a solid surface, and the test agent, which is not anchored, may be labeled, either directly or indirectly.

In practice, microtitre plates conveniently can be utilized as the solid surface. The anchored component is immobilized to the solid surface by non-covalent or covalent attachment. The solid surfaces may be prepared in advance and stored. In order to conduct the assay, the non-immobilized component is added to the solid surface coated with the anchored component. After the reaction is completed, unreacted components are removed (*e.g.*, by washing) under conditions such that any complexes formed will remain immobilized on the solid surface. The detection of complexes anchored on the solid surface can be accomplished in a number of ways. Where the previously non-immobilized component is pre-labeled, the detection of label immobilized on the surface indicates that complexes have been formed. Where the previously non-immobilized component is not pre-labeled, an indirect label can be used to detect complexes on the solid surface, *e.g.*, using a labeled antibody specific for the previously non-immobilized component.

Alternatively, a reaction is conducted in a liquid phase, the reaction products are separated from unreacted components using an immobilized antibody specific for FKBP51/52 protein, fusion protein or the test agent, and complexes are detected using a labeled antibody specific for the other component of the complex.

In accordance with the invention, non-cell based assay systems can be used to identify agents that directly interfere with the interaction between FKBP 51/52 and one or more other proteins within the cell. The proteins that interact with the FKBP51/52 are referred to, for purposes of this discussion, as "binding partners." These binding partners are likely to be involved in the FKBP51/52 signal transduction pathway. Such binding partners include, but are not limited to, the hsp90, Gli3, AFX-1 and hairless protein. Therefore, it is desirable to identify agents that modulate the interaction of one or more of such binding partners with FKBP51/52. Such agents may interfere with or disrupt the interaction of one or more such binding partners with FKBP51/52 and may be useful in modulating hair growth. Alternatively, agents can be identified that increase the affinity or improve the interaction between the one or more binding partners and FKBP51/52.

The basic principle of the assay systems used to identify agents that interfere with the interaction between a FKBP51/52 moiety and one or more of its binding partners involves preparing a reaction mixture containing FKBP51/52 protein, polypeptide, peptide or fusion protein, and the one or more binding partner, and incubating the reaction mixture under conditions and for a time sufficient to allow the components to interact and bind, thus forming a complex. In order to test an agent for inhibitory activity, the reaction mixture is prepared both in the presence and absence of the test agent. The test agent may be initially included in the reaction mixture, or may be added at a time subsequent to the addition of the FKBP51/52 moiety with its binding partner(s). Control reaction mixtures are incubated without the test agent or with a placebo. The formation of any complexes between the FKBP51/52 moiety and the binding partner(s) is then detected. The formation of a complex in the control reaction, but not in the reaction mixture containing the test agent, indicates that the test agent interferes with the interaction of the FKBP51/52 and the interactive binding partner(s).

The assay for agents that interfere with the interaction of FKBP51/52 and binding partners can be conducted in a heterogeneous or homogeneous format. Heterogeneous assays involve anchoring either the FKBP51/52 moiety product or the binding partner onto a solid surface and detecting complexes attached to the solid surface at the end of the reaction. In homogeneous assays, the entire reaction is carried out in a liquid phase. In either approach, the order of addition of reactants can be varied to obtain different information about the agents being tested. For example,

test agents that interfere with the interaction by competition can be identified by conducting the reaction in the presence of the test substance, *i.e.*, by adding the test substance to the reaction mixture prior to or simultaneously with FKBP51/52 moiety and interactive binding partner. Alternatively, test agents that disrupt preformed complexes, *e.g.* agents with higher binding constants that displace one of the components from the complex, can be tested by adding the test agent to the reaction mixture after complexes have been formed. The various formats are described briefly below.

In a particular embodiment, an FKBP51/52 fusion can be prepared for immobilization. For example, the FKBP51/52 or a peptide fragment, *e.g.*, corresponding to the TPR motif, can be fused to a glutathione-S-transferase (GST) gene using a fusion vector, such as pGEX-5X-1, in such a manner that its binding activity is maintained in the resulting fusion protein. The interactive binding partner, *i.e.*, the hsp90, Gli3, AFX-1 or hairless protein, can be purified and used to raise a monoclonal antibody, using methods routinely practiced in the art. The generation of monoclonal antibodies can be omitted if such antibodies exist and are publicly available. This antibody can be labeled with the radioactive isotope ^{125}I, for example, by methods routinely practiced in the art. In a heterogeneous assay, for example, the GST-FKBP51/52 fusion protein can be anchored to glutathione-agarose beads. The interactive binding partner can then be added in the presence or absence of the test agent in a manner that allows interaction and binding to occur. At the end of the reaction period, unbound material can be washed away, and the labeled monoclonal antibody can be added to the system and allowed to bind to the complexed components. The interaction between the FKBP51/52 gene product and the interactive binding partner can be detected by measuring the amount of radioactivity that remains associated with the glutathione-agarose beads. A successful inhibition of the interaction by the test agent will result in a decrease in measured radioactivity.

Alternatively, the GST-FKBP51/52 fusion protein and the interactive binding partner can be mixed together in liquid in the absence of the solid glutathione-agarose beads. The test agent can be added either during or after the species are allowed to interact. This mixture can then be added to the glutathione-agarose beads and unbound material is washed away. Again, the extent of inhibition of the FKBP51/52 binding partner interaction can be detected by adding the labeled antibody and measuring the radioactivity associated with the beads.

In alternate embodiments of the invention, a homogeneous assay can be used. In this approach, a preformed complex of the FKBP51/52 moiety and the interactive binding partner is prepared in which either the FKBP51/52 or its binding partner is labeled, but the signal generated by the label is quenched due to formation of the complex. The addition of a test substance that competes with and displaces one of the species from the preformed complex will result in the generation of a signal above background. In this way, test substances which disrupt FKBP51/52 binding partner interaction can be identified.

In accordance with the invention, non-cell based assays can also be used to screen for agents that directly inhibit enzymatic activities associated with FKBP51/52. Such activities include, but are not limited to, proline isomerase activity. For example, a peptidyl-prolyl cis-trans isomerase assay performed according to Harrison and Stein (1980, *Biochem.* 29:3813-3816) with modifications described by Park et al. (1992, *Biol. Chem.* 267:3316-3324) can be used to measure the level of FKBP51/52 activity. To this end, a reaction mixture of FKBP51/52 and a test agent is prepared in the presence of substrate, and the enzymatic activity of FKBP51/52 is compared to the activity observed in the absence of test agent.

In non-limiting embodiments of the invention, a reaction mixture of FKBP51/52, a test agent and substrate is prepared and the enzymatic activity of FKBP51/52 is compared to the activity observed in the absence of the test agent, wherein a decrease in the level of FKBP51/52 enzymatic activity in the presence of the test agent indicates that an inhibitor of FKBP51/52 has been identified. Alternatively, a reaction mixture of FKBP51/52, a test agent and substrate is prepared and the enzymatic activity of FKBP51/52 is compared to the activity observed in the absence of the test agent, wherein an increase in the level of FKBP51/52 enzymatic activity in the presence of the test agent indicates that a FKBP51/52 agonist has been identified.

CELL BASED ASSAYS

In accordance with the invention, cell based assay systems can be used to identify agents that regulate the activity of FKBP51/52 or CyP40. In addition, it is believed that the FKBP51/52/hsp90/hairless/AFX-1/gli3 complex or CyP40/hsp90/hairless/AFX-1/gli3 is bound to nuclear hormone receptors within the cell. Thus, the cell based assays may be performed using cells expressing a nuclear

hormone receptor. Such nuclear hormone receptors include, but are not limited to, androgen, vitamin D, retinoic acid, aryl hydrocarbon and thyroid hormone receptors. When using such cells, the activity of a test agent can be tested in the presence or absence of a nuclear hormone receptor ligand. Cell based assays are described below for identification of agents that regulate the activity of FKBP51/52 proteins; however, such cell based assays may be used to similarly identify agents that regulate the activity of CyP40.

The present invention provides methods for identifying an agent that activates FKBP51/52 enzymatic activity comprising (i) contacting a cell expressing FKBP51/52 with a test agent and measuring the level of FKBP51/52 enzymatic activity; (ii) in a separate experiment, contacting a cell expressing FKBP51/52 protein with a vehicle control and measuring the level of FKBP51/52 enzymatic activity where the conditions are essentially the same as in part (i), and then (iii) comparing the level of FKBP51/52 activity measured in part (i) with the level of FKBP51/52 activity in part (ii), wherein an increased level of FKBP51/52 enzymatic activity in the presence of the test agent compared to the level of FKBP51/52 enzymatic activity in the presence of vehicle control indicates that the test agent is a FKBP51/52 enzyme activator.

The present invention also provides methods for identifying an agent that inhibits FKBP51/52 enzymatic activity comprising (i) contacting a cell expressing FKBP51/52 with a test agent in the presence of FK506 and measuring the level of FKBP51/52 enzymatic activity; (ii) in a separate experiment, contacting a cell expressing FKBP51/52 in the presence of FK506 and measuring the level of FKBP51/52 enzymatic activity, where the conditions are essentially the same as in part (i); and then (iii) comparing the level of FKBP51/52 enzymatic activity measured in part (i) with the level of FKBP51/52 enzymatic activity in part (ii), wherein a decrease in the level of FKBP51/52 enzymatic activity in the presence of the test agent compared to the level of FKBP51/52 enzymatic activity in the presence of vehicle control indicates that the test agent is a FKBP51/52 enzyme inhibitor.

In utilizing such cell systems, the cells expressing the FKBP51/52 protein are exposed to a test agent or to a vehicle control (*e.g.*, placebo). After or during exposure, the cells can be assayed to measure the enzymatic activity of

FKBP51/52 or the activity of the FKBP51/52 dependent signal transduction pathway itself.

The ability of a test molecule to modulate the enzymatic activity of FKBP51/52 can be measured using standard biochemical and physiological techniques, *e.g.*, as measured by a chemical, physiological, biological or phenotypic change, induction of a host cell gene or reporter gene, change in host cell kinase activity, etc. For example, FKBP51/52 associated peptidyl-prolyl isomerase activity can be measured. Assays for such activity include those described in Harrison and Stein (1980, *Biochem*, 29:3813-3816); Park, S.T. et al., (1992, *J. Biol. Chem.* 267:3316-3324); and U.S. Patent No. 5,763,590. Alternatively, the expression of genes known to be modulated by activation of the FKBP51/52 signal transduction pathway, such as BMP4 or HNF3β, can be assayed to identify modulators of FKBP51/52 or activity.

In addition, animal models can be utilized to identify agents capable of ameliorating hair loss. Such animal models can be used as test substrates for the identification of drugs, pharmaceuticals, therapies and interventions that can be effective in treating such disorders. For example, animal models can be exposed to an agent suspected of exhibiting an ability to modulate hair growth at a sufficient concentration and for a time sufficient to elicit such hair growth in the exposed animals. The response of the animals to the exposure can be monitored by assessing the modulation of hair growth. In a specific embodiment of the invention, a C3H mouse model can be used to measure the capacity of a test compound to initiate hair growth. Typically, approximately seven-week-old female C3H mice are used for experiments. The lower back hair of mice are sheared with an electrical clipper, followed by administration of the test agent. Visual observation of the test animals' hair growth will result in a determination regarding the ability of a test agent to modulate hair growth. In addition, the Dundee Bald rat model animal or chemotherapy treated mice can be used. With regard to intervention, any treatments which reverse any aspect of disorder-like symptoms should be considered as candidates for human therapeutic intervention. Dosages of test agents may be determined by deriving dose-response curves, as discussed below.

RATIONAL DRUG DESIGN

In an embodiment of the invention, computer modeling and searching technologies can be used for identification of agents that can modulate the protein interactions between FKBP51/52, CyP40, hsp90, Gli3, AFX-1 and/or hairless protein. For example, based on the knowledge of the FKBP51/52 or CyP40 binding sites and the study of complexes between FKBP51/52 or CyP40 and proteins such as hsp90, Gli3, AFX-1 and hairless, potential modulators of the FKBP51/52 or CyP40 signal transduction pathway can be identified.

The three dimensional geometric structure of binding sites can be determined using known methods, including X-ray crystallography, which can determine a complete molecular structure. On the other hand, solid or liquid phase NMR can be used to determine certain intra-molecular distances. Any other experimental method of structure determination can be used to obtain partial or complete geometric structures. The geometric structures may be measured with a complexed protein or agent, which may increase the accuracy of the active site structure determined.

If an incomplete or insufficiently accurate structure is determined, the methods of computer based numerical modeling can be used to complete the structure or improve its accuracy. Any recognized modeling method may be used, including parameterized models specific to particular biopolymers such as proteins, molecular dynamic models based on computing molecular motions, statistical mechanics models based on thermal ensembles, or combined models. For most types of models, standard molecular force fields, representing the forces between constituent atoms and groups, are necessary, and can be selected from force fields known in physical chemistry. The incomplete or less accurate experimental structures can serve as constraints on the complete and more accurate structures computed by these modeling methods.

Having determined the structure of the binding site, either experimentally, by modeling, or by a combination of such methods, candidate modulating agents can be identified by searching databases containing agents along with information on their molecular structure. Such a search seeks agents having structures that match the determined binding site structure and that interact with the groups defining the active site. Such a search can be manual, but is preferably

computer assisted. Agents found from this search are potential hair growth modulating agents.

Alternatively, these methods can be used to modify known hair growth modulating agents to improve their activity. A known agent can be modified and the structural effects of modification can be determined using the experimental and computer modeling methods described above. The altered structure can then be compared to the active site structure of the agent to determine if an improved fit or interaction results. In this manner, systematic variations in composition, such as by varying side groups, can be quickly evaluated to obtain modified modulating agents or ligands of improved specificity or activity, *e.g.*, modifying cyclosporin A to increase its affinity to CyP40 while reducing its affinity to cyclosporin A or B.

Further experimental and computer modeling methods useful to identify modulating agents based upon identification of the binding sites of FKBP51/52 or FKBP51/52 binding proteins will be apparent to those of skill in the art. In addition, experimental and computer modeling methods useful to identify modulating agents based upon identification of the binding sites of CyP40 or CyP40 binding proteins will be apparent to those of skill in the art.

Examples of molecular modeling systems are the CHARMm and QUANTA programs (Polygen Corporation, Waltham, MA). CHARMm performs the energy minimization and molecular dynamics functions. QUANTA performs the construction, graphic modeling and analysis of molecular structure. QUANTA allows interactive construction, modification, visualization, and analysis of the behavior of molecules with each other.

A number of articles review computer modeling of drugs interactive with specific proteins, such as Rotivinen, et al., 1988, *Acta Pharmaceut. Fennica* 97:159-166; Ripka, 1988, *New Scientist* 54-57; McKinaly and Rossmann, 1989, *Ann. Rev. Pharmacol. Toxiciol.* 29:111-122; Perry and Davies, 1989, QSAR: Quantitative Structure-Activity Relationships in Drug Design, pp. 189-193 (Alan R. Liss, Inc.); Lewis and Dean, 1989 *Proc. R. Soc. Lond.* 236:125-140 and 141-162; and, with respect to a model receptor for nucleic acid components, Askew, et al., 1989, *J. Am. Chem. Soc.* 111:1082-1090. Other computer programs that screen and graphically depict chemicals are available from companies such as BioDesign, Inc. (Pasadena, CA.), Allelix, Inc. (Mississauga, Ontario, Canada), and Hypercube, Inc. (Cambridge,

Ontario). As described herein, FKBP51/52 bind to a number of known transcription factors, including, but not limited to, AFX-1, gli3 and hairless protein. Thus, although the modeling described above is primarily designed for application to drugs specific to particular proteins, they can be adapted to design of drugs specific to regions of DNA or RNA, once that region is identified.

ASSAY FOR AGENTS THAT REGULATE THE EXPRESSION OF FKBP51/52 OR CYP40

In accordance with the invention, a cell based assay system can be used to screen for agents that modulate the expression of FKBP51/52 or CyP40 within a cell. Assays can be designed to screen for agents that regulate FKBP51/52 or CyP40 expression at either the transcriptional or translational level. The assays described below are designed for identification of agents capable of regulating FKBP51/52 gene expression; however, such assays can be similarly used to identify agents that regulate CyP40 gene expression.

In one embodiment, DNA encoding a reporter molecule can be linked to a regulatory element of the FKBP51/52 gene and used in appropriate intact cells, cell extracts or lysates to identify agents that modulate FKBP51/52 gene expression. Such reporter molecules include, but are not limited to, chloramphenicol acetyltransferase (CAT), luciferase, β-glucuronidase (GUS), growth hormone, or placental alkaline phosphatase. Such constructs are introduced into cells, thereby providing a recombinant cell useful for screening assays designed to identify modulators of FKBP51/52 gene expression.

Following exposure of the cells to the test agent, the level of reporter gene expression can be quantitated to determine the test agent's ability to regulate FKBP51/52 expression. Alkaline phosphatase assays are particularly useful in the practice of the invention where the enzyme is secreted from the cell, and tissue culture supernatant can then be assayed for secreted alkaline phosphatase. In addition, alkaline phosphatase activity can be measured by calorimetric, bioluminescent or chemiluminescent assays such as those described in Bronstein, I. et al., 1994, *Biotechniques* 17:172-177. Such assays provide a simple, sensitive, easily automatable detection system for pharmaceutical screening.

To identify agents that regulate FKBP51/52 translation, cells or *in vitro* cell lysates containing FKBP51/52 transcripts can be tested for modulation of

FKBP51/52 mRNA translation. To assay for inhibitors of FKBP51/52 translation, test agents are assayed for their ability to modulate the translation of FKBP51/52 mRNA in *in vitro* translation extracts.

In an embodiment of the invention, the level of FKBP51/52 expression can be modulated using antisense or ribozyme approaches to inhibit or prevent translation of FKBP51/52 mRNA transcripts, or triple helix approaches to inhibit transcription of the FKBP51/52 gene. Such approaches can be utilized to modulate hair growth.

Antisense approaches involve the design of oligonucleotides (either DNA or RNA) that are complementary to at least a portion of FKBP51/52 mRNA. The antisense oligonucleotides bind to the complementary mRNA transcripts and prevent translation. Absolute complementarity, although preferred, is not required. One skilled in the art can ascertain a tolerable degree of mismatch by use of standard procedures to determine the melting point of the hybridized complex.

In yet another embodiment of the invention, ribozyme molecules designed to catalytically cleave FKBP51/52 mRNA transcripts can be used to prevent translation of FKBP51/52 mRNA and expression of FKBP51/52. (See, *e.g.*, PCT International Publication WO 90/11364, published October 4, 1990; Sarver et al., 1990, *Science* 247:1222-1225).

Alternatively, endogenous FKBP51/52 gene expression can be reduced by targeting deoxyribonucleotide sequences complementary to the regulatory region of the FKBP51/52 gene (*i.e.*, the FKBP51/52 promoter and/or enhancers) to form triple helical structures that prevent transcription of the FKBP51/52 gene in targeted cells in the body. (See generally, Helene, C. et al., 1991, *Anticancer Drug Des.* 6:569-584; and Maher, LJ, 1992, *Bioassays* 14:807-815).

The oligonucleotides of the invention, *i.e.*, antisense, ribozyme, and triple helix forming oligonucleotides, can be synthesized by standard methods known in the art, *e.g.*, by use of an automated DNA synthesizer (such as are commercially available from Biosearch, Applied Biosystems, etc.). Alternatively, recombinant expression vectors can be constructed to direct the expression of the oligonucleotides of the invention. Such vectors can be constructed by recombinant DNA technology methods standard in the art. In a specific embodiment, vectors such as viral vectors

can be designed for gene therapy applications where the goal is *in vivo* expression of inhibitory oligonucleotides in targeted cells

ASSAY FOR AGENTS THAT REGULATE THE TRANSCRIPTIONAL ACTIVITY OF THE GLI3 AFX-1 AND/OR *HAIRLESS* TRANSCRIPTION FACTORS

In accordance with the invention, assays can be developed to identify agents that modulate transcriptional activation mediated by FKBP51/52, CyP40, Gli3, AFX-1 and the hairless protein. While not being bound to any one particular theory, it is believed that the binding of FK506 to the FKBP51/52/hsp90 complex, or cyclosporin A to the CyP40/hsp90 complex, promotes the activation and/or release of the zinc finger transcription factors *hairless* and/or Gli3 from the complex. Nuclear translocation of the hairless and/or Gli3 proteins results in transactivation of target genes and stimulation of hair production.

In accordance with the invention, an assay can be used to identify agents that modulate translocation of the Gli-3, AFX-1 and/or hairless protein into the nucleus. For purposes of the assay, the hairless, AFX-1 and/or Gli3 protein can be tagged with an easily detectable peptide tag such as GFP. Such an assay involves contacting a cell expressing a tagged hairless, AFX-1 or Gli3 protein with a test agent in the presence of FK506 or cyclosporin A. Alternatively, the assay can be performed using a cell expressing a nuclear hormone receptor in the presence of nuclear hormone receptor ligand. Following exposure to the test agent, the amount of tagged hairless, AFX-1 or Gli3 protein located within the nucleus is measured, *e.g.*, by measuring the amount of tagged protein present in the nucleus. If the amount of tagged protein detected in the nucleus is decreased in the presence of the test agent, as compared to the same assay conducted in the presence of a vehicle control, a modulator of hairless and/or Gli3 nuclear translocation has been identified.

In addition, cells expressing Gli3, AFX-1 or hairless tagged proteins can be used to assay for agents that modulate the dissociation of hairless, AFX-1 and/or Gli3 from the FKBP51/52/hsp90 or CyP40/hsp90 complexes. Such assays can be done in the presence of FK506 or cyclosporin A to identify agents that inhibit the FK506 – or cyclosporin A – mediated dissociation of hairless, AFX-1 and/or Gli3 from said complexes. For example, a cell expressing a tagged hairless, AFX-1 or Gli3 protein is contacted with a test agent in the presence of FK506. Following

contact with the test agent, a cell lysate can be prepared followed by immunoprecipitation of the FKBP51/52 or CyP40 protein complex. The immunoprecipitated complex is then analyzed to determine the presence or absence of the tagged Gli3, AFX-1 or hairless protein.

Downstream target genes of the Gli3 transcription factor are regulated by FK506 and cyclosporin A treatment. For example, BMP4 is a downstream target gene of the Gli3 pathway and expression of BMP4 is stimulated in the presence of FK506 and cyclosporin A. Thus, in a specific embodiment of the invention, constructs containing a Gli3 responsive element, *e.g.*, 5'TGGGTGGTC-3', can be linked to any of a variety of different reporter genes and introduced into cells expressing FKBP51/52. Such reporter genes, as set forth above, can include, but are not limited to, those encoding chloramphenicol acetyltransferase (CAT), luciferase, GUS, growth hormone, or placental alkaline phosphatase. Following exposure of the cells to the test agent, the level of reporter gene expression can be quantitated to determine the test agent's ability to regulate transcription of the reporter gene. In instances where identification of antagonists of FK506 induced transcription is desired, the cells are contacted with both FK506 and the test agent. Alkaline phosphatase assays are particularly useful in the practice of the invention because the enzyme is secreted from the cell. Therefore, tissue culture supernatant can be assayed for secreted alkaline phosphatase. In addition, alkaline phosphatase activity can be measured by calorimetric, bioluminescent or chemilumenscent assays such as those described above.

IMMUNOSUPPRESSIVE ACTIVITY OF TEST AGENTS

The present invention relates to the identification of agents capable of modulating hair growth without the side effect of immunosuppression. Thus, in accordance with the invention, any agents identified as possible modulators of hair growth are also tested for their ability to immunosuppress.

Assays designed to measure the immunosuppressive effect of a test agent include, for example, lymphocyte stimulation assays and assays designed to measure cytokine production, *i.e.*, IL-2 production may be performed. One such assay is conducted as follows.

Spleens are excised from euthanized (CO_2 asphyxiation) adult male C3H mice ranging in age from seven to sixteen weeks old (live mice commercially available from Harlan Sprague Dawley, Inc., Indianapolis, IN). The spleens are placed immediately in cold Hanks Balanced Salt Solution (HBSS, commercially available from Gibco-BRL, Gaithersburg, MD). The spleens are then ground up between frosted glass slides and filtered through a sterile screen to remove tissue debris. The resulting cell suspension is underlayed with an equal volume of Ficoll-Paque Plus (commercially available from Pharmacia Biotech, Piscataway, NJ) and centrifuged at 400 x g for approximately forty minutes at 20°C in order to collect the splenocytes. The splenocytes are collected from the interface using a disposable pipet and are washed twice with HBSS, followed by centrifugation at 100 x g for ten minutes at 20°C. Splenocytes are resuspended in five to ten mL of cell culture media consisting of phenol red-free RPMI 1640 (culture media commercially available from Gibco-BRL) containing 10% heat-inactivated fetal bovine serum (Gibco-BRL), penicillin (50 U/mL), streptomycin (100 µg/mL), L-glutamine (2 mM), 2-mercaptoethanol (10^{-5} M), and N-2 hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) (10 mM). The cells are counted and checked for viability using, for example, trypan blue. Splenocytes are resuspended in medium at 10^6 cells/mL and pipetted into 96 well round bottom plates at 10^5 cells/well. Splenocytes are activated by addition of 50 µL/well of conconavalin A (final assay concentration = 5 µg/ml) in the presence or absence of a test compound. Test compounds are made up as stock solutions in dimethyl sulfoxide (DMSO), then diluted in medium and 50 µL/well added, so that the final concentration of DMSO in the assay is below 0.05%. The plates are incubated at 37°C with 5% CO_2 for 48 hours. The cells are pulsed with 1 µCi/well of methy-^{3}H-thymidine (commercially available from Amersham, Buckinghamshire, England) and incubated an additional 24 hours. The cells are then harvested onto GF/C filter plates (commercially available from Packard, Downers Grove, IL), solubilized in Microscint 20 (Packard), and counted on a TopCount microplate scintillation and luminescence plate counter (Packard). Activity is measured as a percentage of control activity in the absence of test compound and plotted *versus* test compound concentration. The data are fit to a 4-parameter curve fit (Sigmaplot) and IC_{50} values are calculated. As used herein, test compounds are considered non-immunosuppressive if, by using this method, the ratio of (cyclosporin A IC_{50}/test compound IC_{50}) x 100 is less than or equal to 0.02, *i.e.*, as defined herein,

a non-immunosuppressive test compound has # 2% of the immunosuppressive activity of cyclosporin A.

Cell viability is assessed using the MTT (3-[4,5-dimethyl-thiazoyl-2-yl]2,5-diphenyl-tetrazolium bromide) dye assay as described by Nelson et al., *J. Immunol.*, 1993, 150(6):2139 –2147, with the exception that the assay is carried out in serum-free, phenol red-free RPMI 1640 and the dye is solubilized in 100 µL/well DMSO and read at an OD of 540 nm with a background correction at 650 nm on a SpectraMax Plus microplate reader (Molecular Devices, Menlo Park, CA).

Alternatively, animal studies can be performed to determine whether a test agent has an immunosuppressive effect.

AGENTS THAT CAN BE SCREENED IN ACCORDANCE WITH THE INVENTION

The assays described above can identify agents that modulate FKBP51/52 activity. For example, agents that affect FKBP51/52 activity include, but are not limited to, agents that bind to FKBP51/52 and modulate the activity of FKBP51/52. Alternatively, agents can be identified that do not bind directly to FKBP51/52, but are capable of altering FKBP51/52 activity by altering the activity of a protein involved in FKBP51/52 signal transduction. Further, agents that affect FKBP51/52 gene activity (by affecting FKBP51/52 gene expression, including molecules, *e.g.*, proteins or small organic molecules, that affect transcription or interfere with splicing events so that expression of the full length or the truncated form of the FKBP51/52 can be modulated) can be identified using the screens of the invention.

The agents which may be screened in accordance with the invention can include, but are not limited to, small organic or inorganic agents, peptides, antibodies and fragments thereof, and other organic agents (*e.g.*, peptidomimetics) that bind to FKBP51/52 and either mimic the activity triggered by any of the known or unknown substrates of FKBP51/52 (*i.e.*, agonists) or inhibit the activity triggered by any of the known or unknown substrates of FKBP51/52 (*i.e.*, antagonists). Agents that bind to FKBP51/52 and either enhance FKBP51/52 activities (*i.e.*, agonists) or inhibit FKBP51/52 activities (*i.e.*, antagonists), will be identified. Agents that bind to proteins that alter/modulate the activity of FKBP51/52 will be identified.

Agents can include, but are not limited to, peptides such as, for example, soluble peptides, such as members of random peptide libraries (see, *e.g.*, Lam, K.S. et al., 1991, *Nature* 354:82-84; Houghten, R. et al., 1991, *Nature* 354:84-86); and combinatorial chemistry-derived molecular libraries made of D- and/or L-configuration amino acids, phosphopeptides (such as members of random or partially degenerate, directed phosphopeptide libraries); (see, *e.g.*, Songyang, Z. et al., 1993, *Cell* 72:767-778), antibodies (such as polyclonal, monoclonal, humanized, anti-idiotypic, chimeric or single chain antibodies, and FAb, $F(ab')_2$ FV, and FAb expression library fragments, and epitope binding fragments thereof), and small organic or inorganic molecules.

Other agents that can be screened in accordance with the invention include, but are not limited to, small organic molecules that affect the expression of the FKBP51/52 gene or some other gene involved in the FKBP51/52 signal transduction pathway (*e.g.*, by interacting with the regulatory region or transcription factors involved in gene expression); or such agents that affect the activities of the FKBP51/52 or the activity of some other factor involved in modulating FKBP51/52 activity, such as for example, a protein that modifies FKBP51/52 and thereby inactivates FKBP51/52 enzyme activities.

COMPOSITIONS CONTAINING MODULATORS
OF HAIR GROWTH AND THEIR USES

The present invention provides methods of modulating hair growth comprising contacting a cell with an effective amount of a FKBP51/52 or CyP40 modulating agent, such as an FKBP51/52 or CyP40 agonist or antagonist identified using the assays as set forth above. An "effective amount" of the FKBP51/52 or CyP40 inhibitor, *i.e.*, antagonist, is an amount that detectably decreases hair growth. An "effective amount" of the FKBP51/52 or CyP40 activator, *i.e.*, agonist, is an amount that detectably increases hair growth.

The present invention further provides methods of modulating hair growth in a subject in need of such treatment, comprising administering to the subject an effective amount of an agent that modulates FKBP51/52 or CyP40 activity identified as set forth above.

The present invention further provides compositions comprising one or more activators or inhibitors of FKBP51/52 and CyP40 activity. The composition may act directly on FKBP51/52 or CyP40, or alternatively may act on proteins involved in the FKBP51/52 and CyP40 signal transduction pathway.

The present invention further provides pharmaceutical compositions comprising an effective amount of an agent capable of modulating the activity of FKBP51/52, CyP40-, or FKBP51/52, CyP40-mediated signal transduction and/or the expression of FKBP51/52 or CyP40, thereby regulating hair growth, and a pharmaceutically acceptable carrier. In a specific embodiment, the term "pharmaceutically acceptable" means approved by a regulatory agency of the federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopoeia for use in animals, and more particularly in humans. The term

"carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the therapeutic is administered. Examples of suitable pharmaceutical carriers are described in Remington: The Science and Practice of Pharmacy, Gennaro et al. (eds), 20th Edition, Lippincott Williams & Wilkins, Philadelphia, PA (ISBN 0-683-306472).

The invention further provides for the treatment of various disorders associated with hair growth by administration of an agent that regulates the expression or activity of FKBP51/52 or CyP40. Such agents include, but are not limited to, FKBP51/52 or CyP40 agonists and antagonists. Such disorders include, but are not limited to, male pattern baldness, female pattern baldness, toxic baldness, alopecia areata and scarring alopecia. In addition, the agent can be used to treat subjects with hair loss associated with exposure to radiation or chemotherapy.

The agents of the invention are preferably tested *in vitro*, and then *in vivo* in an animal system for a desired therapeutic or prophylactic activity, prior to testing and use in humans. For example, *in vitro* assays that can be used to determine whether administration of a specific therapeutic is indicated include *in vitro* cell culture assays in which cells expressing FKBP51/52 or CyP40 are exposed to or otherwise administered a therapeutic agent, where the effect of such a therapeutic agent on FKBP51/52 or CyP40 is then observed upon FKBP51/52 or CyP40 activity is then observed. In a specific embodiment of the invention the ability of an agent to regulate the signal transduction pathway mediated by CyP40 or FKBP51/52 is assayed.

The invention provides methods of treatment and/or prophylaxis comprising administering to a subject in need thereof an effective amount of a hair growth modulating agent of the invention. In a preferred aspect, the agent is substantially purified. The subject is preferably an animal, more preferably a mammal, and most preferably a human.

Various delivery systems are known and can be used to administer an agent capable of regulating hair growth, *e.g.*, encapsulation in liposomes, microparticles, microcapsules. Methods of introduction include, but are not limited to, intradermal, topical, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal, epidural, and oral routes. The agents may be administered by any convenient route, for example, by infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (*e.g.*, oral mucosa, rectal and intestinal mucosa,

etc.), and may be administered together with other biologically active agents. Administration can be systemic or local, and is preferably adopted for topical applications.

In a specific embodiment, it may be desirable to administer the compositions of the invention locally to a specific area of the body. This may be achieved, for example, and not by way of limitation, by topical application. The active compounds identified according to the methods of the present invention are generally administered in the form of pharmaceutical compositions comprising at least one of such compounds together with a pharmaceutically acceptable vehicle or diluent. Such compositions are generally formulated in a conventional manner utilizing solid or liquid vehicles or diluents as appropriate for topical administration, in the form of solutions, oils, gels, creams, jellies, pastes, lotions, ointments, salves, leave-on and rinse-out hair conditioners, shampoos, aerosols and the like.

Examples of vehicles for application of an active compound identified according to a method of the present invention include an aqueous or water-alcohol solution, an emulsion of the oil-in-water or water-in-oil type, an emulsified gel, or a two-phase system. Preferably, the compositions according to the invention are in the form of lotions, creams, milks, gels, masks, microspheres or nanospheres, or vesicular dispersions. In the case of vesicular dispersions, the lipids of which the vesicles are made can be of the ionic or nonionic type, or a mixture thereof.

Topical compositions containing the active compound can be admixed with a variety of carrier materials well known in the art, such as, for example, water alcohols, aloe vera gel, allantoin, glycerin, vitamin A and E oils, mineral oil, propylene glycol, PPG-2 myristyle propionate, and the like, as well as any of various types of penetration enhancers, viscosity enhancing agents, pH stabilizers, anti-oxidants, preservatives, perfumes, coloring agents, etc.

Other materials suitable for use in topical carriers include, for example, emollients, solvents, humectants, thickeners and powers, as known in the art.

The compositions of the present invention may also optionally comprise other hair growth modulating agents such as potassium channel openers, anti-androgens, thyroid hormones and derivatives and analogs thereof, prostaglandin agonists or antagonists, retinoids, triterpenes, and others known in the art or to be identified.

The amount of the agent of the invention which will be effective in the treatment of a particular disorder will depend on the nature of the disorder and can be determined by standard clinical techniques. *In vitro* assays may optionally be employed to help identify optimal dosage ranges. The precise dose to be employed in the formulation will depend on the route of administration and the nature of the disorder, and should be decided according to the judgment of the medical practitioner and in view of each patient's circumstances. Effective doses can be extrapolated from dose response curves derived from *in vitro* or animal model test systems.

The invention also provides a pharmaceutical pack or kit comprising one or more containers comprising one or more of the ingredients of the pharmaceutical compositions of the invention. Optionally associated with such container(s) can be a notice in a form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration. The kit may further comprise printed instructions or a printed label directing the use of the composition to modulate, *i.e.*, to stimulate or inhibit, hair growth.

The following examples illustrate the preferred modes of making and practicing the present invention but are not meant to limit the scope of the invention since alternative methods may be utilized to obtain similar

Example: FKBP51/52 Mediated Hair Growth

The examples presented below demonstrate that (i) FKBP51 and FKBP52 are selectively expressed in dermal papillae; (ii) FKBP51/52 co-precipitate with hsp90, AFX-1 Gli3 and the hairless protein; and (iii) expression of the BMP4 and HNF3β gene, a Gli3 responsive gene is activated by FK506.

MATERIALS AND METHODS

REVERSE TRANSCRIPTION

A commercial RT-PCR kit (Promega Access RT-PCR kit) was used for detection of FKBP51, FKBP52, and CyP40 mRNA in different human cells. The sequence of primers used for human FKBP51 is: TGAAGAAAGCCCCACAGC (SEQ ID NO:1) (forward primer) and CTCCAAAACCATATCTTGGTCC (SEQ ID NO:2) (reverse primers). Primer sequence for human FKBP52 is: ACATTGCCATAGCCACCA (SEQ ID NO:3) (forward primer) and AGCCAAGACACGATCTTC (SEQ ID NO:4) (reverse primer). Primer sequence for human CyP40 is: TGAAGGAAGGAGATGACGGG (SEQ ID NO:5) (forward primer) and TCCTCAGGGAAATCTGGATGA (SEQ ID NO:6) (reverse primer).

Total RNA was extracted from cultured cells using TRIZOL reagent (Life Technologies) according to manufacturer's instruction. The RNA was treated with DNase to remove potential contamination by genomic DNA and then used in RT-PCR reaction. The PCR reaction product was run on a 2% agarose gel to visualize the amplified product and digested with appropriate restriction enzymes to confirm the products.

TISSUE EXTRACTION

Human skin tissue was extracted using TPER reagent (Pierce, Rockford, IL) at a ratio of 10:1 (extraction buffer:tissue; vol:wt) on ice using a Polytron homogenizer in the presence of protease inhibitors (Protease Inhibitor Cocktail, 1:50 dilution; Sigma, St. Louis, MO) to form a lysate.

COUPLING OF ANTIBODIES TO MAGNETIC RESIN PROTEIN G SEPHAROSE

Antibodies used included anti-hsp90 (monoclonal IgG1 with 1 mg/ml BSA; TL) and anti-FKBP52 (anti-peptide polyclonal N17 and C19 Abs; Santa Cruz Biotechnologies). Antibodies were concentrated and washed 3x with PBS using Amicon Microcon-30. Antibodies were coupled to tosyl-activated Dyna M-450 beads in neutral pH PBS buffer overnight at 37°C. Resins were blocked with Tris-HCl, pH 8 for 4 hours, 37°C.

IMMUNOPRECIPITATION

FK506 was added to the lysate to a final concentration of 1 μM. Antibody complexes were immunoprecipitated in TPER buffer at 6°C overnight. Complexes were washed 10x with Tris/saline (4°C) and sequentially eluted with 1M NH_4CO_3 or 20 mM Tris, pH 7.4, 0.3 M NaCl: (5 x 100 μl) and 0.1% TFA, 5% MeOH (5 x 100 μl). Complexes were concentrated and buffer was exchanged (mw 3500) for salt elutions or reduced in volume under vacuum for TFA/MeOH elutions. Samples were stored at -20°C.

PROTEASE DIGESTION

Proteins were suspended in 1 M GnHCl, 100 mM NH_4CO_3, 0.5 mM DTT and subject to digestion with trypsin (Promega) or gluC (Roche) for about 18 hours at 37°C. Digests were desalted using C18 ZipTips (Millipore) eluted with 60% acetonitrile, 0.1% TFA.

MASS SPECTROMETRY ANALYSIS

The peptide digests were analyzed by matrix-assisted laser disorption ionization (MALDI) time of flight (TOF) spectrometry. Essentially, peptides were mixed with matrix (1:1 sample: α-cyano-4-hydroxycinnamic acid; 20 mg/ml-HCCA, 30% acetonitrile, 0.1% TFA) in which the dried droplet method was used. Peptides were analyzed using a Voyager DE-Pro spectrometer (PE BioSystems) in reflector mode (2 m flight length) with a positive ion accelerating voltage of 20 kV, a grid voltage of 12.8 kV, guide wire voltage of 1400 V, using 100 ns delayed extraction. Greater than 64 scans were averaged per spectra. 30 - 50 fmols each of bradykinin and ACTH (amino acid residues 18-39) were used as internal mass standards.

DATABASE SEARCHING

Peptide spectra were compared using ProFound [http://nt2/prowl/prowl.html] or RADARS (OSI internal). Mass error tolerance was typically 30 ppm. One missed cleavage was allowed. Modifications were not initially considered. Both SWISSPROT and GENBANK NR databases were searched.

INDUCTION OF BMP4 AND HNF3β
mRNA BY CYCLOSPORIN A AND FK506 TREATMENT

Primary human dermal fibroblasts (for detectin of BMP4) and skin keratinocytes (for detection of HNF3β) were cultured and treated with 1 uM of cyclosporin A or FK506. Total RNA was harvested at days 2 and 4 of treatment. Real time PCR was used to quantify the BMP4 and HNF3β mRNA level. ABI PRISM 7700 sequence detector, TaqMan PCR kit, and PCR primers designed with commercial software labeled with fluorescence dyes were used for RNA quantification. The results as presented in Figure 3 show that cyclosporin A and FK506 both up-regulate the level of BMP4 and HNF3β mRNA.

In addition, downstream target genes of Gli3 were found to be up regulated by cyclosporin A and FK506 treatment in skin cells such as dermal fibroblasts and skin keratinocytes. As indicated in Figure 3, BMP4 and HNF3β mRNA level were induced by cyclosporin A or FK506 treatment.

INDUCTION OF THYROID HORMONE RECEPTOR (TR)
MEDIATED TRANSCRIPTION BY Fk506 AND CYCLOSPORIN A

Four tandem thyroid hormone response elements (TRE) (AGGTCA CAGG AGGTCA) (underlined sequence is repeated) (SEQ ID NO:7) were synthesized in a single oligonucleotide and ligated 5' of thymidine kinase (TK) promoter using standard procedures. A plasmid (TRE-TK/pUV120puro) was constructed by linking the resulting TRE/TK promoter 5' of the luciferase reporter gene from *H. pyralis,* de Wet Jr. et al, 1986, *Methods Enzymol* 133:3-14, together with a gene encoding a protein conferring resistance to the antibiotic puromycin expressed under the control of the SV40 promoter. In this plasmid the expression of the luciferase gene is under the direct control of the TRE-TK promoter and is inducible by agonists of the thyroid hormone nuclear receptor (TR). HeLa cells (ATCC, Manassas, VA 20108, #CCL-2)

were transfected with plasmid DNA by electroporation using standard procedures, and drug resistant cell lines were selected using puromycin Signa-Aldrich Corp., St. Louis, MI. Drug resistant cell lines (HeLa/TRE) were selected for responsiveness to thyroid hormone, and a single, stably transfected, clonal line was used in subsequent experiments.

Mass spectrometry experiments indicated that hair growth was likely mediated by a protein complex comprising in part hsp90, either cyclophillin 40, FKBP 51 or 52, the zinc finger transcription factor hairless, and a nuclear hormone receptor (thyroid hormone receptor, androgen receptor, vitamin D receptor or glucocorticoid receptor).

In order to establish the role of the thyroid hormone nuclear receptor in immunophillin signaling, cyclosporine A, FK506 and thyroid hormone T3 were evaluated for their ability to increase thyroid hormone nuclear receptor signaling. HeLa/TRE cells were seeded at a density of 10,000 cells per well in 96 well microtiter plates in DMEM culture media containing 1% charcoal stripped FCS, 2mM glutamine and antibiotics (penicillin and streptomycin). The cells were treated with increasing concentrations (20 nM, 2 nM, 200 pM, and 20 pM) of cyclosporin A or FK506) Sigma-Aldrich Corp., St. Louis, MI; (Calbiochem-Nova biocheryl Corp., San Diego, CA) for 16 hours and the activity of the luciferase reporter gene was measured) de Wet Jr. et al, 1986, *Methods Enzymol* 133:3-14.

RESULTS

Dermal Papillar (DP) cells are critical for hair growth. In cultured human dermal papillar cells, CyP40 and FKBP51/52 were detected using RT-PCR (Figure 2). The levels of FKBP51/52 are much greater than those of FKBP 12/13. The levels of FKBP12/13, which mediate the immunosuppressive effect of FK506, was close to the detection limit, indicating that their levels of expression were very low. The expression patterns in human dermal fibroblasts and keratinocytes have also been studied and were found to be identical.

Mass spectrometry analysis of hsp90 and FKBP52 immunoprecipitated complexes derived from human scalp skin reveal the presence of three transcription factors. The factors include the zinc finger protein hairless, the sonic hedgehog stimulated factor Gli-3, and AFX-1.

Data also suggest that in human skin FK506 might alter hairless interaction with hsp90 protein complexes. Hairless may then translocate to the nucleus and stimulate transcription of genes regulating hair growth.

In addition, downstream target genes of Gli3 such as BMP4 and HNF3 were found to be upregulated by FK506 treatment (Figure 3).

Hsp90 and FKBP52 complexes relevant to immunophillin action were identified from human scalp skin extracts using monoclonal antibodies, protease digestion, mass spectrometric measurement of peptide masses and protein database searching. The following proteins were identified from trypsin and/or GluC digests subject to mass spectrometric analysis:

Table 1

Anti-hsp90 and anti-FKBP52 *human skin extract + FK506* 30ppm (SWISSPROT)	Trypsin	GluC
Protein folding, immunophilins, stress-response		
hsp90-beta	x	x
hsp90-alpha	x	x
hsp70s	x	x
FKBP-51	x	x
hsp27	x	x
FKBP-52	x	x
FRAP	x	x
hsp110	x	x
hsp40 protein-3 (DNAJ homolog 1)	x	x
prolyl 4-hydroxylase (alpha and beta subunits)	x	
protein disulfide isomerases	x	x
osmotic stress protein 94 (hsp70 related)	x	x
cyclophilin 40	x	
Steroid-like receptors		
estrogen receptors	x	x
glucocorticoid receptor	x	x
androgen receptor	x	
RXR-beta		x
TRIP-12		x
thyroid hormone receptor	x	x
Protein degradation		
cullin-2	x	x
cullin-3	x	x
ubiquitin carboxy terminal hydrolases	x	x
26S proteosome subunits	x	x
ubiquitin activating enzyme E1		x
Transcription and chromatin remodeling		
human hairless (zfp)	x	x
heat shock factor protein-2 (HSF-2)	x	
GLI-3	x	x
AFX1 putative whn factor (forkhead domain)	x	

The data show that cyclosporin A or FK506 induces thyroid hormone receptor mediated transcription in a dose dependent manner (Figure 4). Binding of ligand to cyclosphilin 40 (cyclosporin A) or FKBP51/52 (FK506) proteins in the hsp90

complex clearly activates the transcriptional activity of thyroid hormone receptor. The hairless protein, an accessory protein of thyroid hormone receptor, also can modulate cyclosporin A or FK506 action and thereby regulate hair growth.

Equivalents

The present invention is not to be limited in scope by the specific embodiments described herein which are intended as single illustrations of individual aspects of the invention, and functionally equivalent methods and components are within the scope of the invention. Indeed, various modifications of the invention, in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Various publications are cited herein, the contents of which are hereby incorporated, by reference, in their entireties.

WE CLAIM:

1. A method for identifying a compound capable of modulating hair growth comprising:

(v) contacting a cell that expresses, or a preparation containing, FKBP51/52 or CyP40, hsp90 and a protein selected from the group consisting of hairless, AFX-1, and gli3, with a test compound;

(vi) determining the level of complex formation between FKBP51/52 or CyP40, hsp90 and at least one protein selected from the group consisting of the hairless, AFX-1, and gli3 in the cell or preparation, contacted with the test compound; and

(vii) comparing the level of complex formation obtained in (ii) to the level of complex formation between FKBP51/52 or CyP40, hsp90, and at least one protein selected from the group consisting of the hairless, AFX-1, and gli3, in the absence of test compound;

wherein a difference in the level of complex formation in the presence versus in the absence of test compound has a positive correlation with hair growth modulating activity.

2. The method of claim 1, wherein the level of complex formation is detected using an immunoassay.

3. The method of claim 1, further comprising the step of determining whether the test compound has immunosuppressive activity, where the absence of such activity, in the presence of a test compound that alters the level of complex formation has a positive correlation with usefulness as a hair growth modulating agent.

4. A method for identifying a compound capable of modulating hair growth comprising:

(i) contacting a cell that expresses a nuclear hormone receptor and a reporter gene under the transcriptional control of a *hairless*, AFX-1, or gli3 gene responsive element with a test compound and measuring the level of reporter gene expression in the cell;

(ii) measuring the level of reporter gene expression in the absence of the test compound; and

(iii) comparing the levels of reporter gene expression measured in (i) and (ii);

wherein a difference in the levels of reporter gene expression measured in steps (i) and (ii) has a positive correlation with hair growth modulating activity of the test compound.

5. The method of claim 4, wherein step (i) is performed in the presence of a nuclear hormone receptor ligand.

6. The method of claim 4, further comprising the step of determining whether the test compound has immunosuppressive activity where the absence of such activity, in the presence of a test compound that alters the level of reporter gene expression, has a positive correlation with usefulness as a hair growth modulating agent.

7. The method of claim 4, wherein the nuclear hormone receptor is selected from the group consisting of an androgen nuclear hormone receptor, vitamin D nuclear hormone receptor, retinoic acid nuclear hormone receptor, aryl hydrocarbon nuclear hormone receptor, and thyroid stimulating nuclear hormone receptor.

8. A method for identifying a compound capable of promoting hair growth comprising:

(i) contacting a sample containing a *hairless* gene product with a test compound;

(ii) determining whether the test compound binds to the *hairless* gene product; and

(iii) determining whether the test compound inhibits complex formation between the *hairless* gene product and a binding partner selected from the group consisting of FKBP51/52, CyP40, a nuclear hormone receptor, a hsp90 protein, and a combination thereof;

wherein the ability of a test compound to both bind to the *hairless* gene product and inhibit complex formation has a positive correlation with hair growth promoting activity.

9. A method for identifying a compound capable of modulating hair growth comprising:

(i) contacting a cell that expresses FKBP51/52 or CyP40, and hsp90, a nuclear hormone receptor, and the *hairless* gene product, with a test compound in the presence of a nuclear hormone receptor ligand;

(ii) determining the level of nuclear translocation of the *hairless* gene product into the nucleus of the cell;

(iii) determining the level of nuclear translocation in the absence of the test compound; and

(iv) comparing the level of nuclear translocation measured in (ii) and (iii);

wherein a difference in the level of nuclear translocation measured in steps (ii) and (iii) has a positive correlation with hair growth modulating activity of the test compound.

10. The method of claim 8 or 9, further comprising the step of determining whether the test compound has immunosuppressive activity.

11. A method for identifying a compound capable of promoting hair growth comprising:

(i) contacting a sample containing an AFX-1 gene product with a test compound;

(ii) determining whether the test compound binds to the AFX-1 gene product; and

(iii) determining whether the test compound inhibits complex formation between the AFX-1 gene product and a binding partner selected from the group consisting of FKBP51/52, CyP40, a nuclear hormone receptor, a hsp90 protein, and a combination thereof;

wherein the ability of a test compound to both bind to the AFX-1 gene product and inhibit complex formation has a positive correlation with hair growth promoting activity.

12. A method for identifying a compound capable of modulating hair growth comprising:

(i) contacting a cell that expresses FKBP51/52 or CyP40, and hsp90, a nuclear hormone receptor, and the AFX-1 gene product, with a test compound in the presence of a nuclear hormone receptor ligand;

(ii) determining the level of nuclear translocation of the AFX-1 gene product into the nucleus of the cell;

(iii) determining the level of nuclear translocation in the absence of the test compound; and

(iv) comparing the level of nuclear translocation measured in (ii) and (iii);

wherein a difference in the level of nuclear translocation measured in steps (ii) and (iii) has a positive correlation with hair growth modulating activity of the test compound.

13. The method of claim 11 or 12, further comprising the step of determining whether the test compound has immunosuppressive activity.

14. A method for identifying a compound capable of promoting hair growth comprising:

(i) contacting a sample containing a gli3 gene product with a test compound;

(ii) determining whether the test compound binds to the gli3 gene product; and

(iii) determining whether the test compound inhibits complex formation between the gli3 gene product and a binding partner selected from the group consisting of FKBP51/52, CyP40, a nuclear hormone receptor, a hsp90 protein, and a combination thereof;

wherein the ability of a test compound to both bind to the gli3 gene product and inhibit complex formation has a positive correlation with hair growth promoting activity.

15. A method for identifying a compound capable of modulating hair growth comprising:

(i) contacting a cell that expresses FKBP51/52 or CyP40, and hsp90, a nuclear hormone receptor, and the gli3 gene product, with a test compound in the presence of a nuclear hormone receptor ligand;

(ii) determining the level of nuclear translocation of the gli3 gene product into the nucleus of the cell;

(iii) determining the level of nuclear translocation in the absence of the test compound; and

(iv) comparing the level of nuclear translocation measured in (ii) and (iii);

wherein a difference in the level of nuclear translocation measured in steps (ii) and (iii) has a positive correlation with hair growth modulating activity of the test compound.

16. The method of claim 14 or 15, further comprising the step of determining whether the test compound has immunosuppressive activity.

17. A method for modulating hair growth in a mammal, comprising administering to the mammal a compound that modulates complex formation between FKBP51/52, CyP40, or hsp90 and at least one protein selected from the group consisting of hairless, gli3, and AFX-1.

18. A method for modulating hair growth in a mammal comprising administering to the mammal a compound that modulates the nuclear translocation of a protein selected from the group consisting of the hairless, AFX-1, and gli3 protein.

19. A method for modulating hair growth in a mammal comprising administering to the mammal a compound that modulates AFX-1-or gli-3-mediated gene expression.

20. A method for modulating hair growth in a mammal, comprising administering to the mammal a compound identified by the assay of claim 1.

21. A method for modulating hair growth in a mammal, comprising administering to the mammal a compound identified by the assay of claim 4.

22. A method for modulating hair growth in a mammal, comprising administering to the mammal a compound identified by the assay of claim 8.

23. A method for modulating hair growth in a mammal, comprising administering to the mammal a compound identified by the assay of claim 9.

24. A method for modulating hair growth in a mammal, comprising administering to the mammal a compound identified by the assay of claim 11.

25. A method for modulating hair growth in a mammal, comprising administering to the mammal a compound identified by the assay of claim 12.

26. A method for modulating hair growth in a mammal, comprising administering to the mammal a compound identified by the assay of claim 14.

27. A method for modulating hair growth in a mammal, comprising administering to the mammal a compound identified by the assay of claim 15.

5

Figure 1

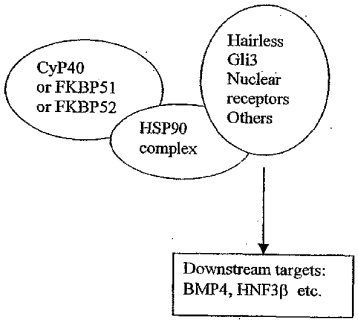
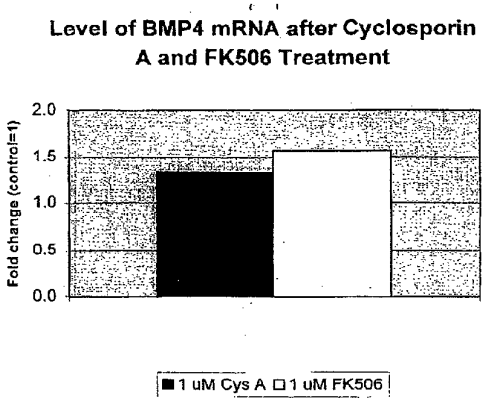

CyP40
or FKBP51
or FKBP52
HSP90
complex
Hairless
Gli3
Nuclear
receptors
Others
Downstream targets:
BMP4, HNF3β etc.

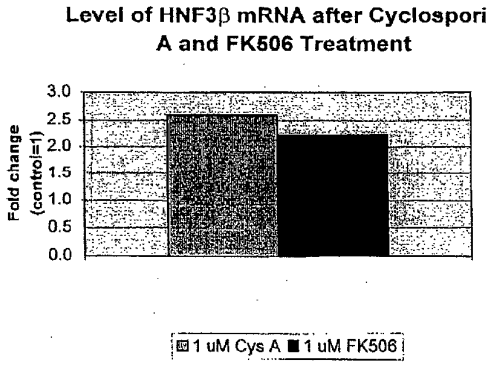
Figure 2

Genes	Tissues		
	Dermal Fibroblast	Dermal Papilla	Keratinocyte
FKBP52	+	+	+
FKBP51	+	+	+
Cyp 40	+	+	+

3A


Level of BMP4 mRNA after Cyclosporin A and FK506 Treatment
Fold change (control=1)
2.0
1.5
1.0
0.5
0.0
1 uM Cys A
1 uM FK506

3B


Level of HNF3β mRNA after Cyclosporin A and FK506 Treatment
Fold change (control=1)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
1 uM Cys A
1 uM FK506

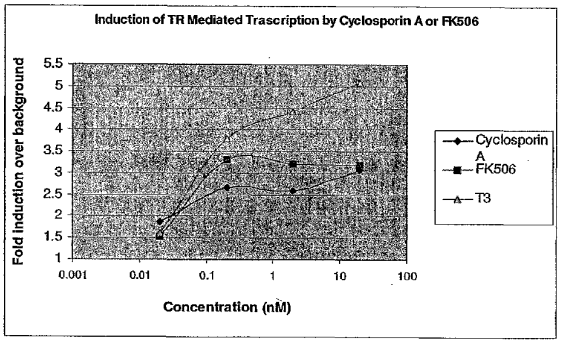

Induction of TR Mediated Trascription by Cyclosporin A or FK506
Fold Induction over background
5.5
5
4.5
4
3.5
3
2.5
2
1.5
1
0.001
0.01
0.1
1
10
100
Concentration (nM)
Cyclosporin A
FK506
T3

Figure 4

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(54) Title: FKBP51/52 AND CYP40-MEDIATED MAMMALIAN HAIR GROWTH

(57) Abstract: The present invention related to drug screening assays designed to identify non-immunosuppressive agents that modulate hair growth and the use of such agents for modulation of hair growth.

FKBP51/52 AND CYP40-MEDIATED MAMMALIAN HAIR GROWTH

BACKGROUND OF THE INVENTION

Field of the Invention

The invention relates to the fields of dermatology, cell biology, and molecular biology. More specifically, the present invention relates to drug screening assays designed to identify non-immunosuppressive agents that modulate hair growth and the use of such agents for modulation of hair growth.

Summary of the Related Art

The immunosuppressant drugs FK506, rapamycin and cyclosporin A are well known T-cell specific immunosuppressants that are routinely used to prevent graft rejection in organ transplant patients. In T cells, FK506 and cyclosporin A prevent calcineurin from dephosphorylating the transcription factor NF/AT (nuclear factor of activated T-cells), thereby blocking its translocation into the nucleus and preventing the receptor-mediated increase in synthesis and secretion of cytokines, such as interleukin-2 and, hence, T-cell proliferation (Heitman, J. et al., 1992, *The New Biologist* 4:448-460).

FK506 and cyclosporin A act by binding to endogenous intracellular receptor proteins termed immunophilins. Based on their structure and binding affinity for specific drugs, immunophilins have been divided into two classes of proteins; those proteins having an affinity for FK506 are referred to as FK506-binding proteins (FKBPs), while those having an affinity for cyclosporin are referred to as cyclophilins. Both FKBPs and cyclophilins possess a similar peptidyl-prolyl isomerase activity resulting in cis-trans-isomerization of proteins, which is believed to be important for protein folding and trafficking. In addition, both FKBPs and cyclophilins are characterized by their ability to interact with a variety of different proteins involved in signal transduction.

Several members of the FKBP family have been identified and named according to their calculated molecular mass (Lane, W.S. et al, 1991, *J. Protein*

Chem. 10:151-160; U.S. Patent No. 5,763,590). Cyclophilin A and FKBP12 were originally isolated as cyclosporin A and FK506 binding proteins, respectively, and were shown to exert immunosuppressive activity through inhibition of calcineurin. FKBP-51 was found to be expressed in T-cells where it inhibits calcineurin with much weaker potency, suggesting that multiple immunophilins may participate in mediating FK506 immunosuppressant activity. FKBP-51 has also been shown to be a component of the progesterone receptor complex (Nair, S.C. et al., 1997, *Mol. Cell Biol.* 17:594-603). FKBP52 was initially discovered as a component of the inactive steroid receptor complex (Smith, D.F. et al., 1993, *J. Biol. Chem.* 268:18365-71). The N-terminal domain, residues 1-149 of FKBP52, shares 55% homology with FKBP12, however, it does not have immunosuppressant activity when complexed with FK506. FKBP52 is phosphorylated by casein kinase II and has been found to have chaperone activity independent of isomerase activity (Miyata, Y. et al., 1997, *Proc. Natl. Acad. Sci. USA* 94:14500-14505). CyP40 has only a low affinity for cyclosporin A and is therefore capable of only slight reduction of the immunosuppressive effect of cyclosporin A.

Hsp90 is the most abundant of the heat shock proteins. A number of transcription factors and protein kinases involved in signal transduction are found complexed with hsp90 (Pratt, W.B. et al., 1999, *Cell Signal* 11:839-851; Pratt and Toft, 1997, *Endocrine Rev.* 18:306-360). When complexed with transcription factors, the hsp90 complexes are found to contain high molecular weight immunophilins with tetratricopeptide repeat (TPR) motifs (Duina, A.A. et al., 1996, *Science* 274:1713-1715; Bose, S. et al., 1996, *Science* 274:1715-1717). Such immunophilins include FKBP52 and CyP40 (Owens-Grillo, J.K., 1995, *J. Biol. Chem.* 270:20479-20484; Miyata, Y. et al., 1997, *Proc. Natl. Acad. Sci. USA* 94:14500-14505; Silverstein, A.M. et al., 1999, *J. Biol. Chem.* 274:36980-36986).

Causes of hair loss include aging, the action of male hormones, the loss of blood supply to hair follicles, and scalp abnormalities. In addition, genetic disposition may account for hair loss. For example, androgenic alopecia is considered to be genetically determined. Recently, a rare autosomal recessive form of hereditary alopecia, referred to as atrichia with papular lesions, was found to result from mutations in the human "*hairless*" gene (Ahmad, W. et al., 1998, *Science* 279:720-724). In individuals affected with this form of hair loss, hairs are typically absent from the scalp, and patients have very sparse eyebrows and eyelashes. Mutations in

the human homolog of the mouse *hairless* gene lead to congenital alopecia universalis and atrichia with papular lesions. In mice bearing a mutation in the *hairless* gene, the hair matrix cells appear to undergo premature and massive apoptosis together with a concomitant decline in Bcl-2 expression indicating that the *hairless* gene product may play a role in regulating cell proliferation, differentiation and apoptosis in the hair follicle. The human *hairless* gene has recently been isolated and is described in WO 99/38965.

It has been reported that topical application of FK506 and cyclosporin A stimulate hair growth in a dose dependent manner (Sainsbury, T.S.L. et al., 1991, *Transplant. Proc.* 23:3332-3334). For example, FK506 and cyclosporin A have been shown to stimulate hair growth in experimental animals, such as mice and rats (WO 98/55090; Maurer, M., 1997, *Am. J. Path.* 150:1433; Yamamoto, S. et al., 1993, *J. Invest. Dermatol.* 102:160). The effects of FK506 and cyclosporin A and related agents have been described (Tsuji Y. et al., 1999, *Exp. Dermatol.* 8:366-7; McElwee, K.J. et al., 1997, *Br. J. Dermatol.* 137:491-7; Iwabuchi T. et al., 1995, *J. Dermatol. Sci.* 9:64-9; Yamamoto S. and Kato R., 1994, *J. Dermatol. Sci. 7 Supp.* 1:547-54; and Yamamoto S. et al., 1994, *J. Invest. Dermatol*102:160-4).

In addition, Japanese patent application No. 11-174041 describes methods for identifying hair stimulating agents that can bind to immunosuppressive agent-bound proteins that can form a complex with steroid receptors, *i.e.*, FKBP 52 or cyclophilin 40, but do not bind to FKBPs that cannot form complexes with steroid receptors, *e.g.*, FKBP12.

The mechanism of mammalian hair growth stimulated by FK506 and cyclosporin A remains unknown. Despite their potential use as hair stimulating agents, immunosuppressive agents such as FK506 and cyclosporin A also exhibit toxic side effects such as immunosuppression. Thus, there is the need to identify and develop non-immunosuppressive agents that are useful as modulators of hair growth. The present invention is based on the discovery of the signaling pathway by which the immunosuppressive agents FK506 and cyclosporin A modulate hair growth. This discovery provides drug screening assays for identification of non-immunosuppressive agents capable of modulating hair growth.

Summary of the Invention

The present invention relates to drug screening assays designed to identify non-immunosuppressive agents that modulate hair growth and the use of such agents for modulation of hair growth. The invention is based on the discovery of the signal transduction pathway by which specific immunophilins, namely FKBP51 and FKBP52, and CyP40, modulate hair growth. As disclosed herein, the FKBP51/52 proteins are found to be expressed in hair follicle dermal papillae. In addition, FKBP51/52 are found complexed with the hsp90, Gli3, AFX-1 and hairless protein within the cell. Contact of dermal papillae cells with FK506 or cyclosporin A was also found to stimulate expression of the Gli3 target gene, BMP4 and HNF3β. The pathway of the invention serves as a basis for methods designed to identify non-immunosuppressive agents which can be used to modulate hair growth.

The invention relates to assays designed to screen for agents that modulate the components of the FKBP51/52 and CyP40 signal transduction pathway, *i.e.*, agents that act as agonists or antagonists of such components, including FKBP51/52, CyP40, hsp90, Gli3, AFX-1 and/or the hairless protein. In an embodiment of the invention, a method is provided for rational drug design of agents which specifically modulate activity and/or the association of FKBP51/52 or CyP40 with the hsp90, hairless, AFX-1 and/or Gli3 protein. The present invention further provides cell based and non-cell based assays for identifying agents which modulate the interaction and/or activity of the components of the inventive pathway, *i.e.*, the FKBP51/52, CyP40, hsp90, Gli3 AFX-1 and hairless protein.

Specifically, the invention provides a method for identifying a compound capable of modulating hair growth comprising:

(i) contacting a cell that expresses, or a preparation containing, FKBP51/52 or CyP40, hsp90 and a protein selected from the group consisting of hairless, AFX-1, and gli3, with a test compound;

(ii) determining the level of complex formation between FKBP51/52 or CyP40, hsp90 and at least one protein selected from the group consisting of the hairless, AFX-1, and gli3, in the cell, or preparation, contacted with the test compound; and

(iii) comparing the level of complex formation obtained in (ii) to the level of complex formation between FKBP51/52 or CyP40, hsp90, and at

least one protein selected from the group consisting of the hairless, AFX-1, and gli3, in the absence of test compound;

wherein a difference in the level of complex formation in the presence versus in the absence of test compound has a positive correlation with hair growth modulating activity.

As used herein, the term "preparation" refers to a composition comprising at least one cellular component that has been isolated, extracted or partially purified either from a cell in which it is naturally expressed or from a cell which has been genetically engineered to express the component, or a component that has been synthetically prepared, which composition can be used to carry out the recited method. Such preparations include, but are not limited to, cell fractions prepared by standard techniques, as well as aqueous, buffered solutions of cellular components prepared by combining together previously synthesized or purified components.

As used herein, the phrase "positive correlation with hair growth modulating activity" refers to an observation of the biological activity of a test compound wherein the activity indicates that the test agent is capable of either stimulating or inhibiting hair growth.

In another embodiment of the invention, a method is provided for identifying a compound capable of modulating hair growth comprising:

(i) contacting a cell that expresses a nuclear hormone receptor and a reporter gene under the transcriptional control of a *hairless*, AFX-1, or gli3 gene responsive element with a test compound and measuring the level of reporter gene expression in the cell;

(ii) measuring the level of reporter gene expression in the absence of the test compound; and

(iii) comparing the levels of reporter gene expression measured in (i) and (ii);

wherein a difference in the levels of reporter gene expression measured in steps (i) and (ii) has a positive correlation with hair growth modulating activity of the test compound.

The invention further provides a method for identifying a compound capable of promoting hair growth comprising:

(i) contacting a sample containing a *hairless*, AFX-1 or gli3 gene product with a test compound;

(ii) determining whether the test compound binds to the *hairless*, AFX-1 or gli3 gene product; and

(iii) determining whether the test compound inhibits complex formation between the *hairless*, AFX-1 or gli3 gene product and a binding partner selected from the group consisting of FKBP51/52, CyP40, a nuclear hormone receptor, a hsp90 protein, and a combination thereof;

wherein the ability of a test compound to both bind to the *hairless*, AFX-1 or gli3 gene product and inhibit complex formation has a positive correlation with hair growth promoting activity.

The invention further relates to a method for identifying a compound capable of modulating hair growth comprising:

(i) contacting a cell that expresses FKBP51/52 or CyP40, and hsp90, a nuclear hormone receptor, and the *hairless*, AFX-1 or gli3 gene product with a test compound in the presence of a nuclear hormone receptor ligand;

(ii) determining the level of nuclear translocation of the *hairless*, AFX-1 or gli3 gene product into the nucleus of the cell;

(iii) determining the level of nuclear translocation in the absence of the test compound; and

(iv) comparing the level of nuclear translocation measured in (ii) and (iii);

wherein a difference in the level of nuclear translocation measured in steps (ii) and (iii) has a positive correlation with hair growth modulating activity of the test compound.

Identified agents can be used to modulate hair growth. Such agents are particularly useful for treating baldness resulting from genetic factors, aging, local skin conditions and diseases that affect the body generally, *i.e.*, systemic diseases. Such disorders include, but are not limited to, male pattern baldness, female pattern

baldness, toxic baldness, alopecia areata and scarring alopecia. In addition, the agents can be used to treat subjects with hair loss associated with radiation or chemotherapy.

Thus, the invention encompasses a method for modulating hair growth in a mammal comprising administering to the mammal a compound that modulates complex formation between FKBP51/52, CyP40 or hsp90 and at least one protein selected from the group consisting of hairless, gli3, AFX-1, and hsp90.

The invention provides a method for modulating hair growth in a mammal comprising administering to the mammal a compound that modulates the nuclear translocation of a protein selected from the group consisting of the hairless, AFX-1 and gli3 protein.

In yet another embodiment of the invention, a method is provided for modulating hair growth in a mammal comprising administering to the mammal a compound that modulates *hairless*, AFX-1-or gli-3-mediated gene expression.

Description of the Figures

The foregoing and other objects of the present invention, the various features thereof, as well as the invention itself may be more fully understood from the following description, when read together with the accompanying drawings in which:

Figure 1 is a schematic representation depicting the proposed model of interactions between FKBP51/52, CyP40, steroid receptors, hsp 90 and other binding partners.

Figure 2 is a representation of RT-PCR analysis of human dermal papilla RNA, demonstrating that in cultured human dermal papilla cells, expression of FKBP51, FKBP52 and CyP40 is detected using RT-PCR.

Figure 3A is a graphic representation demonstrating stimulation of BMP4 transcription four days after FK506 and cyclosporin A treatment.

Figure 3B is a graphic representation demonstrating stimulation of HNF3β transcription four days after FK506 and cyclosporin A treatment.

Figure 4 is a graphic presentation of the induction of TR mediated transcription by cyclosporin A or FK506.

Detailed Description

The present invention is based on the discovery of the signal transduction pathway by which specific immunophilins, namely FKBP51, FKBP52 and CyP40, modulate hair growth. The discovery of the pathway by which agents such as FK506 and cyclosporin A modulate hair growth provides screening targets for agents that can be used to promote hair growth while having a reduced effect on the immune system.

The present invention encompasses assays designed to identify agents that modulate the interaction and/or activity of the components of the signal transduction pathway. Such components include, for example, the FKBP51/52, CyP40, hsp90, Gli3, AFX-1 and hairless proteins. Both cell based and non-cell based assays can be used to identify agents that either increase or decrease the activity of the FKBP51/52 and CyP40 signal transduction pathway. The present invention further provides for rational drug design of agents that specifically promote or inhibit association of FKBP51/52 or CyP40 with the hsp90, hairless, AFX-1 and/or Gli3 proteins.

In other embodiments, the present invention provides for agents designed or identified by the foregoing methods. Further, the present invention provides for the use of said agents for enhancement of hair growth. In particular, such agents can be used to treat baldness which can result from genetic factors, aging, and/or local skin conditions.

SCREENING ASSAYS FOR AGENTS USEFUL IN MODULATING THE ACTIVITY OF FKBP51/52/CYP40

The present invention relates to screening assay systems designed to identify agents or compositions that modulate FKBP51/52 or CyP40 activity or FKBP51/52 or CyP40 gene expression, and thus, may be useful for modulation of hair growth.

RECOMBINANT EXPRESSION OF PROTEINS INVOLVED IN HAIR GROWTH

For purposes of developing screening assays designed to identify agents or compositions that modulate hair growth, it may be necessary to recombinantly express the FKBP51/52 or CyP40 proteins and/or the proteins that

interact with FKBP51/52, and CyP40, *i.e.*, hsp90, Gli3, AFX-1 and hairless proteins. The cDNA sequences and deduced amino acid sequences of FKBP51 and FKBP52 have been characterized (PubMed Accession Nos.U71321 and M88279) and are incorporated herein by reference. The term FKB51/52 as used herein refers to either or both the FKBP51 and FKBP52 proteins. The cDNA sequence and deduced amino acid sequences of CyP40 have been characterized (Accession No. D63861) and is incorporated herein by reference. The cDNA sequences and deduced amino acid sequences of hsp90, Gli3, AFX-1 and *hairless* have also been characterized (PubMed Accession Nos. NMO18411 (hairless); NM000168 (Gli3) and NM005348 (hsp90); Accession No. U10072 and are incorporated herein by reference. For simplicity, recombinant expression is described below for FKBP51/52; however, the methods can also be utilized for recombinant expression of CyP40, hsp90, Gli3, AFX-1 and/or the hairless protein.

FKBP51/52 nucleotide sequences can be isolated using a variety of different methods known to those skilled in the art. For example, a cDNA library constructed using RNA from a tissue known to express FKBP51/52 can be screened using a labeled FKBP51/52 specific probe. Alternatively, a genomic library can be screened to derive nucleic acid molecules encoding the FKBP51 or FKBP52 protein. Further, FKBP51/52 nucleic acid sequences can be derived by performing a polymerase chain reaction (PCR) using two oligonucleotide primers designed on the basis of known FKBP51/52 nucleotide sequences. The template for the reaction can be cDNA obtained by reverse transcription of mRNA prepared from cell lines or tissue known to express FKBP51/52.

FKBP51/52 protein, polypeptides and peptide fragments, mutated, truncated or deleted forms of FKBP51/52 and/or FKBP51/52 fusion proteins can be prepared for a variety of uses, including, but not limited to, the identification of other cellular gene products involved in the regulation of FKBP51/52-mediated hair growth, and the screening for agents that can be used to modulate hair growth. FKBP51/52 fusion proteins include fusions to an enzyme, fluorescent protein, and a polypeptide tag or luminescent protein, all of which provide a marker function.

While the FKBP51/52 polypeptides and peptides can be chemically synthesized (*e.g.*, see Creighton, 1983, Proteins: Structures and Molecular Principles, W.H. Freeman & Co., N.Y.), large polypeptides derived from FKBP51/52 and the full

length FKBP51/52 proteins can be advantageously produced by recombinant DNA technology using techniques well known in the art for expressing nucleic acids containing FKBP51/52 gene sequences and/or other coding sequences. Such methods can be used to construct expression vectors containing the FKBP51/52 nucleotide sequences and appropriate transcriptional and translational control signals. These methods include, for example, *in vitro* recombinant DNA techniques, synthetic techniques, and *in vivo* genetic recombination. (See, for example, the techniques described in Sambrook et al., 1989, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Press, N.Y.; and Ausubel et al., 1989, Current Protocols in Molecular Biology, Green Publishing Associates and Wiley Interscience, N.Y.).

A variety of host-expression vector systems can be utilized to express the FKBP51/52 nucleotide sequences (See, *e.g.*, U.S. Patent No. 5,763,590 for expression of FKBP 52). Where the FKBP51/52 peptide or polypeptide is expressed as a soluble derivative and is not secreted, the peptide or polypeptide can be recovered from the host cell. Alternatively, where the FKBP51/52 peptide or polypeptide is secreted, the peptide or polypeptides can be recovered from the culture media.

The expression systems that can be used for purposes of the invention include, but are not limited to, microorganisms such as bacteria transformed with recombinant bacteriophage, plasmid or cosmid DNA expression vectors containing FKBP51/52 encoding nucleotide sequences, yeast transformed with recombinant yeast expression vectors containing FKBP51/52 encoding nucleotide sequences or mammalian cell systems, or insect cell systems containing FKBP51/52 recombinant expression constructs containing promoters derived from the genome of mammalian or insect cells or from mammalian or insect viruses.

Appropriate expression systems can be chosen to ensure that the correct modification, processing, and sub-cellular localization of the FKBP51/52 protein occurs. To this end, eukaryotic host cells that possess the ability to properly modify and process the FKBP51/52 protein are preferred. For long-term, high yield production of recombinant FKBP51/52 protein, such as that desired for development of cell lines for screening purposes, stable expression is preferred. Rather than using expression vectors which contain origins of replication, host cells can be transformed with DNA controlled by appropriate expression control elements and a selectable marker gene, *e.g.*, *tk*, *hgprt*, *dhfr*, *neo*, and *hygro* genes, to name a few. Following the

introduction of the foreign DNA, engineered cells may be allowed to grow for 1-2 days in enriched media, and then switched to a selective media. Such engineered cell lines can be particularly useful in screening and evaluating agents that modulate the endogenous activity of the FKBP51/52 gene product.

In addition, in some instances it may be necessary to co-express interactive binding proteins such as the CyP40, hsp90, Gli3, AFX-1 and hairless proteins for use in the screening assays of the invention. Methods described above for expression of FKBP51/52 can be similarly used to co-express such binding proteins.

NON-CELL BASED ASSAYS

In accordance with the invention, non-cell based assay systems can be used to identify agents that interact with, *i.e.*, bind to, FKBP51/52 or CyP40, and regulate the activity of such proteins. Such agents may act as antagonists or agonists of FKBP51/52 or CyP40 activity and can be used to regulate hair growth. In particular, such agents may function to disrupt or prevent the formation of a complex between FKBP51/52 or CyP40 and their binding partners, *i.e.*, the hsp90, Gli3, AFX-1 and/or hairless protein. For simplicity, the non-cell based assays are described below for FKBP51/52; however, they can be similarly utilized for CyP40 as well.

Recombinant FKBP51/52, including peptides corresponding to different functional domains, or FKBP51/52 fusion proteins, can be expressed and used in assays to identify agents that interact with FKBP51/52.

To this end, soluble FKBP51/52 can be recombinantly expressed and utilized in non-cell based assays to identify agents that bind to FKBP51/52. Recombinantly expressed FKBP51/52 polypeptides or fusion proteins containing one or more of the FKBP51/52 functional domains can be prepared as described above, and used in the non-cell based screening assays. One such functional domain is the tetratricopeptide repeat (TPR) which is important for protein/protein interactions. For example, the full length FKBP51/52, or a soluble truncated FKBP51/52, *e.g.*, in which one or more domains is deleted from the molecule but the TPR is retained, a peptide corresponding to the TPR motifs, or a fusion protein containing the FKBP51/52 TPR motif fused to a protein or polypeptide that affords advantages in the assay system (*e.g.*, for labeling or isolating the resulting complex) can be utilized. Where agents that interact with the TPR motif are sought to be identified, peptides corresponding to

the FKBP51/52 TPR motif and fusion proteins containing the FKBP51/52 TPR motif can be used. The FKBP51/52 protein can also be present as part of a crude or semi-purified extract.

The principle of the assays used to identify agents that bind to FKBP51/52 involves preparing a reaction mixture of FKBP51/52 and the test agent under conditions and for time sufficient to allow the two components to interact and bind, thus forming a complex which can be removed and/or detected in the reaction mixture. The identity of the bound test agent is then determined.

The screening assays are accomplished by any of a variety of commonly known methods. For example, one method to conduct such an assay involves anchoring the FKBP51/52 protein, polypeptide, peptide, fusion protein, or the test substance onto a solid phase and detecting FKBP51/52/test agent complexes adhered to the solid phase at the end of the reaction. In one embodiment of such a method, the FKBP51/52 reactant is anchored onto a solid surface, and the test agent, which is not anchored, may be labeled, either directly or indirectly.

In practice, microtitre plates conveniently can be utilized as the solid surface. The anchored component is immobilized to the solid surface by non-covalent or covalent attachment. The solid surfaces may be prepared in advance and stored. In order to conduct the assay, the non-immobilized component is added to the solid surface coated with the anchored component. After the reaction is completed, unreacted components are removed (*e.g.*, by washing) under conditions such that any complexes formed will remain immobilized on the solid surface. The detection of complexes anchored on the solid surface can be accomplished in a number of ways. Where the previously non-immobilized component is pre-labeled, the detection of label immobilized on the surface indicates that complexes have been formed. Where the previously non-immobilized component is not pre-labeled, an indirect label can be used to detect complexes on the solid surface, *e.g.*, using a labeled antibody specific for the previously non-immobilized component.

Alternatively, a reaction is conducted in a liquid phase, the reaction products are separated from unreacted components using an immobilized antibody specific for FKBP51/52 protein, fusion protein or the test agent, and complexes are detected using a labeled antibody specific for the other component of the complex.

In accordance with the invention, non-cell based assay systems can be used to identify agents that directly interfere with the interaction between FKBP 51/52 and one or more other proteins within the cell. The proteins that interact with the FKBP51/52 are referred to, for purposes of this discussion, as "binding partners." These binding partners are likely to be involved in the FKBP51/52 signal transduction pathway. Such binding partners include, but are not limited to, the hsp90, Gli3, AFX-1 and hairless protein. Therefore, it is desirable to identify agents that modulate the interaction of one or more of such binding partners with FKBP51/52. Such agents may interfere with or disrupt the interaction of one or more such binding partners with FKBP51/52 and may be useful in modulating hair growth. Alternatively, agents can be identified that increase the affinity or improve the interaction between the one or more binding partners and FKBP51/52.

The basic principle of the assay systems used to identify agents that interfere with the interaction between a FKBP51/52 moiety and one or more of its binding partners involves preparing a reaction mixture containing FKBP51/52 protein, polypeptide, peptide or fusion protein, and the one or more binding partner, and incubating the reaction mixture under conditions and for a time sufficient to allow the components to interact and bind, thus forming a complex. In order to test an agent for inhibitory activity, the reaction mixture is prepared both in the presence and absence of the test agent. The test agent may be initially included in the reaction mixture, or may be added at a time subsequent to the addition of the FKBP51/52 moiety with its binding partner(s). Control reaction mixtures are incubated without the test agent or with a placebo. The formation of any complexes between the FKBP51/52 moiety and the binding partner(s) is then detected. The formation of a complex in the control reaction, but not in the reaction mixture containing the test agent, indicates that the test agent interferes with the interaction of the FKBP51/52 and the interactive binding partner(s).

The assay for agents that interfere with the interaction of FKBP51/52 and binding partners can be conducted in a heterogeneous or homogeneous format. Heterogeneous assays involve anchoring either the FKBP51/52 moiety product or the binding partner onto a solid surface and detecting complexes attached to the solid surface at the end of the reaction. In homogeneous assays, the entire reaction is carried out in a liquid phase. In either approach, the order of addition of reactants can be varied to obtain different information about the agents being tested. For example,

test agents that interfere with the interaction by competition can be identified by conducting the reaction in the presence of the test substance, *i.e.*, by adding the test substance to the reaction mixture prior to or simultaneously with FKBP51/52 moiety and interactive binding partner. Alternatively, test agents that disrupt preformed complexes, *e.g.* agents with higher binding constants that displace one of the components from the complex, can be tested by adding the test agent to the reaction mixture after complexes have been formed. The various formats are described briefly below.

In a particular embodiment, an FKBP51/52 fusion can be prepared for immobilization. For example, the FKBP51/52 or a peptide fragment, *e.g.*, corresponding to the TPR motif, can be fused to a glutathione-S-transferase (GST) gene using a fusion vector, such as pGEX-5X-1, in such a manner that its binding activity is maintained in the resulting fusion protein. The interactive binding partner, *i.e.*, the hsp90, Gli3, AFX-1 or hairless protein, can be purified and used to raise a monoclonal antibody, using methods routinely practiced in the art. The generation of monoclonal antibodies can be omitted if such antibodies exist and are publicly available. This antibody can be labeled with the radioactive isotope ^{125}I, for example, by methods routinely practiced in the art. In a heterogeneous assay, for example, the GST-FKBP51/52 fusion protein can be anchored to glutathione-agarose beads. The interactive binding partner can then be added in the presence or absence of the test agent in a manner that allows interaction and binding to occur. At the end of the reaction period, unbound material can be washed away, and the labeled monoclonal antibody can be added to the system and allowed to bind to the complexed components. The interaction between the FKBP51/52 gene product and the interactive binding partner can be detected by measuring the amount of radioactivity that remains associated with the glutathione-agarose beads. A successful inhibition of the interaction by the test agent will result in a decrease in measured radioactivity.

Alternatively, the GST-FKBP51/52 fusion protein and the interactive binding partner can be mixed together in liquid in the absence of the solid glutathione-agarose beads. The test agent can be added either during or after the species are allowed to interact. This mixture can then be added to the glutathione-agarose beads and unbound material is washed away. Again, the extent of inhibition of the FKBP51/52 binding partner interaction can be detected by adding the labeled antibody and measuring the radioactivity associated with the beads.

In alternate embodiments of the invention, a homogeneous assay can be used. In this approach, a preformed complex of the FKBP51/52 moiety and the interactive binding partner is prepared in which either the FKBP51/52 or its binding partner is labeled, but the signal generated by the label is quenched due to formation of the complex. The addition of a test substance that competes with and displaces one of the species from the preformed complex will result in the generation of a signal above background. In this way, test substances which disrupt FKBP51/52 binding partner interaction can be identified.

In accordance with the invention, non-cell based assays can also be used to screen for agents that directly inhibit enzymatic activities associated with FKBP51/52. Such activities include, but are not limited to, proline isomerase activity. For example, a peptidyl-prolyl cis-trans isomerase assay performed according to Harrison and Stein (1980, *Biochem.* 29:3813-3816) with modifications described by Park et al. (1992, *Biol. Chem.* 267:3316-3324) can be used to measure the level of FKBP51/52 activity. To this end, a reaction mixture of FKBP51/52 and a test agent is prepared in the presence of substrate, and the enzymatic activity of FKBP51/52 is compared to the activity observed in the absence of test agent.

In non-limiting embodiments of the invention, a reaction mixture of FKBP51/52, a test agent and substrate is prepared and the enzymatic activity of FKBP51/52 is compared to the activity observed in the absence of the test agent, wherein a decrease in the level of FKBP51/52 enzymatic activity in the presence of the test agent indicates that an inhibitor of FKBP51/52 has been identified. Alternatively, a reaction mixture of FKBP51/52, a test agent and substrate is prepared and the enzymatic activity of FKBP51/52 is compared to the activity observed in the absence of the test agent, wherein an increase in the level of FKBP51/52 enzymatic activity in the presence of the test agent indicates that a FKBP51/52 agonist has been identified.

CELL BASED ASSAYS

In accordance with the invention, cell based assay systems can be used to identify agents that regulate the activity of FKBP51/52 or CyP40. In addition, it is believed that the FKBP51/52/hsp90/hairless/AFX-1/gli3 complex or CyP40/hsp90/hairless/AFX-1/gli3 is bound to nuclear hormone receptors within the cell. Thus, the cell based assays may be performed using cells expressing a nuclear

hormone receptor. Such nuclear hormone receptors include, but are not limited to, androgen, vitamin D, retinoic acid, aryl hydrocarbon and thyroid hormone receptors. When using such cells, the activity of a test agent can be tested in the presence or absence of a nuclear hormone receptor ligand. Cell based assays are described below for identification of agents that regulate the activity of FKBP51/52 proteins; however, such cell based assays may be used to similarly identify agents that regulate the activity of CyP40.

The present invention provides methods for identifying an agent that activates FKBP51/52 enzymatic activity comprising (i) contacting a cell expressing FKBP51/52 with a test agent and measuring the level of FKBP51/52 enzymatic activity; (ii) in a separate experiment, contacting a cell expressing FKBP51/52 protein with a vehicle control and measuring the level of FKBP51/52 enzymatic activity where the conditions are essentially the same as in part (i), and then (iii) comparing the level of FKBP51/52 activity measured in part (i) with the level of FKBP51/52 activity in part (ii), wherein an increased level of FKBP51/52 enzymatic activity in the presence of the test agent compared to the level of FKBP51/52 enzymatic activity in the presence of vehicle control indicates that the test agent is a FKBP51/52 enzyme activator.

The present invention also provides methods for identifying an agent that inhibits FKBP51/52 enzymatic activity comprising (i) contacting a cell expressing FKBP51/52 with a test agent in the presence of FK506 and measuring the level of FKBP51/52 enzymatic activity; (ii) in a separate experiment, contacting a cell expressing FKBP51/52 in the presence of FK506 and measuring the level of FKBP51/52 enzymatic activity, where the conditions are essentially the same as in part (i); and then (iii) comparing the level of FKBP51/52 enzymatic activity measured in part (i) with the level of FKBP51/52 enzymatic activity in part (ii), wherein a decrease in the level of FKBP51/52 enzymatic activity in the presence of the test agent compared to the level of FKBP51/52 enzymatic activity in the presence of vehicle control indicates that the test agent is a FKBP51/52 enzyme inhibitor.

In utilizing such cell systems, the cells expressing the FKBP51/52 protein are exposed to a test agent or to a vehicle control (*e.g.*, placebo). After or during exposure, the cells can be assayed to measure the enzymatic activity of

FKBP51/52 or the activity of the FKBP51/52 dependent signal transduction pathway itself.

The ability of a test molecule to modulate the enzymatic activity of FKBP51/52 can be measured using standard biochemical and physiological techniques, *e.g.*, as measured by a chemical, physiological, biological or phenotypic change, induction of a host cell gene or reporter gene, change in host cell kinase activity, etc. For example, FKBP51/52 associated peptidyl-prolyl isomerase activity can be measured. Assays for such activity include those described in Harrison and Stein (1980, *Biochem*, 29:3813-3816); Park, S.T. et al., (1992, *J. Biol. Chem.* 267:3316-3324); and U.S. Patent No. 5,763,590. Alternatively, the expression of genes known to be modulated by activation of the FKBP51/52 signal transduction pathway, such as BMP4 or HNF3β, can be assayed to identify modulators of FKBP51/52 or activity.

In addition, animal models can be utilized to identify agents capable of ameliorating hair loss. Such animal models can be used as test substrates for the identification of drugs, pharmaceuticals, therapies and interventions that can be effective in treating such disorders. For example, animal models can be exposed to an agent suspected of exhibiting an ability to modulate hair growth at a sufficient concentration and for a time sufficient to elicit such hair growth in the exposed animals. The response of the animals to the exposure can be monitored by assessing the modulation of hair growth. In a specific embodiment of the invention, a C3H mouse model can be used to measure the capacity of a test compound to initiate hair growth. Typically, approximately seven-week-old female C3H mice are used for experiments. The lower back hair of mice are sheared with an electrical clipper, followed by administration of the test agent. Visual observation of the test animals' hair growth will result in a determination regarding the ability of a test agent to modulate hair growth. In addition, the Dundee Bald rat model animal or chemotherapy treated mice can be used. With regard to intervention, any treatments which reverse any aspect of disorder-like symptoms should be considered as candidates for human therapeutic intervention. Dosages of test agents may be determined by deriving dose-response curves, as discussed below.

RATIONAL DRUG DESIGN

In an embodiment of the invention, computer modeling and searching technologies can be used for identification of agents that can modulate the protein interactions between FKBP51/52, CyP40, hsp90, Gli3, AFX-1 and/or hairless protein. For example, based on the knowledge of the FKBP51/52 or CyP40 binding sites and the study of complexes between FKBP51/52 or CyP40 and proteins such as hsp90, Gli3, AFX-1 and hairless, potential modulators of the FKBP51/52 or CyP40 signal transduction pathway can be identified.

The three dimensional geometric structure of binding sites can be determined using known methods, including X-ray crystallography, which can determine a complete molecular structure. On the other hand, solid or liquid phase NMR can be used to determine certain intra-molecular distances. Any other experimental method of structure determination can be used to obtain partial or complete geometric structures. The geometric structures may be measured with a complexed protein or agent, which may increase the accuracy of the active site structure determined.

If an incomplete or insufficiently accurate structure is determined, the methods of computer based numerical modeling can be used to complete the structure or improve its accuracy. Any recognized modeling method may be used, including parameterized models specific to particular biopolymers such as proteins, molecular dynamic models based on computing molecular motions, statistical mechanics models based on thermal ensembles, or combined models. For most types of models, standard molecular force fields, representing the forces between constituent atoms and groups, are necessary, and can be selected from force fields known in physical chemistry. The incomplete or less accurate experimental structures can serve as constraints on the complete and more accurate structures computed by these modeling methods.

Having determined the structure of the binding site, either experimentally, by modeling, or by a combination of such methods, candidate modulating agents can be identified by searching databases containing agents along with information on their molecular structure. Such a search seeks agents having structures that match the determined binding site structure and that interact with the groups defining the active site. Such a search can be manual, but is preferably

computer assisted. Agents found from this search are potential hair growth modulating agents.

Alternatively, these methods can be used to modify known hair growth modulating agents to improve their activity. A known agent can be modified and the structural effects of modification can be determined using the experimental and computer modeling methods described above. The altered structure can then be compared to the active site structure of the agent to determine if an improved fit or interaction results. In this manner, systematic variations in composition, such as by varying side groups, can be quickly evaluated to obtain modified modulating agents or ligands of improved specificity or activity, *e.g.*, modifying cyclosporin A to increase its affinity to CyP40 while reducing its affinity to cyclosporin A or B.

Further experimental and computer modeling methods useful to identify modulating agents based upon identification of the binding sites of FKBP51/52 or FKBP51/52 binding proteins will be apparent to those of skill in the art. In addition, experimental and computer modeling methods useful to identify modulating agents based upon identification of the binding sites of CyP40 or CyP40 binding proteins will be apparent to those of skill in the art.

Examples of molecular modeling systems are the CHARMm and QUANTA programs (Polygen Corporation, Waltham, MA). CHARMm performs the energy minimization and molecular dynamics functions. QUANTA performs the construction, graphic modeling and analysis of molecular structure. QUANTA allows interactive construction, modification, visualization, and analysis of the behavior of molecules with each other.

A number of articles review computer modeling of drugs interactive with specific proteins, such as Rotivinen, et al., 1988, *Acta Pharmaceut. Fennica* 97:159-166; Ripka, 1988, *New Scientist* 54-57; McKinaly and Rossmann, 1989, *Ann. Rev. Pharmacol. Toxiciol.* 29:111-122; Perry and Davies, 1989, QSAR: Quantitative Structure-Activity Relationships in Drug Design, pp. 189-193 (Alan R. Liss, Inc.); Lewis and Dean, 1989 *Proc. R. Soc. Lond.* 236:125-140 and 141-162; and, with respect to a model receptor for nucleic acid components, Askew, et al., 1989, *J. Am. Chem. Soc.* 111:1082-1090. Other computer programs that screen and graphically depict chemicals are available from companies such as BioDesign, Inc. (Pasadena, CA.), Allelix, Inc. (Mississauga, Ontario, Canada), and Hypercube, Inc. (Cambridge,

Ontario). As described herein, FKBP51/52 bind to a number of known transcription factors, including, but not limited to, AFX-1, gli3 and hairless protein. Thus, although the modeling described above is primarily designed for application to drugs specific to particular proteins, they can be adapted to design of drugs specific to regions of DNA or RNA, once that region is identified.

ASSAY FOR AGENTS THAT REGULATE THE EXPRESSION OF FKBP51/52 OR CYP40

In accordance with the invention, a cell based assay system can be used to screen for agents that modulate the expression of FKBP51/52 or CyP40 within a cell. Assays can be designed to screen for agents that regulate FKBP51/52 or CyP40 expression at either the transcriptional or translational level. The assays described below are designed for identification of agents capable of regulating FKBP51/52 gene expression; however, such assays can be similarly used to identify agents that regulate CyP40 gene expression.

In one embodiment, DNA encoding a reporter molecule can be linked to a regulatory element of the FKBP51/52 gene and used in appropriate intact cells, cell extracts or lysates to identify agents that modulate FKBP51/52 gene expression. Such reporter molecules include, but are not limited to, chloramphenicol acetyltransferase (CAT), luciferase, β-glucuronidase (GUS), growth hormone, or placental alkaline phosphatase. Such constructs are introduced into cells, thereby providing a recombinant cell useful for screening assays designed to identify modulators of FKBP51/52 gene expression.

Following exposure of the cells to the test agent, the level of reporter gene expression can be quantitated to determine the test agent's ability to regulate FKBP51/52 expression. Alkaline phosphatase assays are particularly useful in the practice of the invention where the enzyme is secreted from the cell, and tissue culture supernatant can then be assayed for secreted alkaline phosphatase. In addition, alkaline phosphatase activity can be measured by calorimetric, bioluminescent or chemiluminescent assays such as those described in Bronstein, I. et al., 1994, *Biotechniques* 17:172-177. Such assays provide a simple, sensitive, easily automatable detection system for pharmaceutical screening.

To identify agents that regulate FKBP51/52 translation, cells or *in vitro* cell lysates containing FKBP51/52 transcripts can be tested for modulation of

FKBP51/52 mRNA translation. To assay for inhibitors of FKBP51/52 translation, test agents are assayed for their ability to modulate the translation of FKBP51/52 mRNA in *in vitro* translation extracts.

In an embodiment of the invention, the level of FKBP51/52 expression can be modulated using antisense or ribozyme approaches to inhibit or prevent translation of FKBP51/52 mRNA transcripts, or triple helix approaches to inhibit transcription of the FKBP51/52 gene. Such approaches can be utilized to modulate hair growth.

Antisense approaches involve the design of oligonucleotides (either DNA or RNA) that are complementary to at least a portion of FKBP51/52 mRNA. The antisense oligonucleotides bind to the complementary mRNA transcripts and prevent translation. Absolute complementarity, although preferred, is not required. One skilled in the art can ascertain a tolerable degree of mismatch by use of standard procedures to determine the melting point of the hybridized complex.

In yet another embodiment of the invention, ribozyme molecules designed to catalytically cleave FKBP51/52 mRNA transcripts can be used to prevent translation of FKBP51/52 mRNA and expression of FKBP51/52. (See, *e.g.*, PCT International Publication WO 90/11364, published October 4, 1990; Sarver et al., 1990, *Science* 247:1222-1225).

Alternatively, endogenous FKBP51/52 gene expression can be reduced by targeting deoxyribonucleotide sequences complementary to the regulatory region of the FKBP51/52 gene (*i.e.*, the FKBP51/52 promoter and/or enhancers) to form triple helical structures that prevent transcription of the FKBP51/52 gene in targeted cells in the body. (See generally, Helene, C. et al., 1991, *Anticancer Drug Des.* 6:569-584; and Maher, LJ, 1992, *Bioassays* 14:807-815).

The oligonucleotides of the invention, *i.e.*, antisense, ribozyme, and triple helix forming oligonucleotides, can be synthesized by standard methods known in the art, *e.g.*, by use of an automated DNA synthesizer (such as are commercially available from Biosearch, Applied Biosystems, etc.). Alternatively, recombinant expression vectors can be constructed to direct the expression of the oligonucleotides of the invention. Such vectors can be constructed by recombinant DNA technology methods standard in the art. In a specific embodiment, vectors such as viral vectors

can be designed for gene therapy applications where the goal is *in vivo* expression of inhibitory oligonucleotides in targeted cells

ASSAY FOR AGENTS THAT REGULATE THE TRANSCRIPTIONAL ACTIVITY OF THE GLI3 AFX-1 AND/OR *HAIRLESS* TRANSCRIPTION FACTORS

In accordance with the invention, assays can be developed to identify agents that modulate transcriptional activation mediated by FKBP51/52, CyP40, Gli3, AFX-1 and the hairless protein. While not being bound to any one particular theory, it is believed that the binding of FK506 to the FKBP51/52/hsp90 complex, or cyclosporin A to the CyP40/hsp90 complex, promotes the activation and/or release of the zinc finger transcription factors *hairless* and/or Gli3 from the complex. Nuclear translocation of the hairless and/or Gli3 proteins results in transactivation of target genes and stimulation of hair production.

In accordance with the invention, an assay can be used to identify agents that modulate translocation of the Gli-3, AFX-1 and/or hairless protein into the nucleus. For purposes of the assay, the hairless, AFX-1 and/or Gli3 protein can be tagged with an easily detectable peptide tag such as GFP. Such an assay involves contacting a cell expressing a tagged hairless, AFX-1 or Gli3 protein with a test agent in the presence of FK506 or cyclosporin A. Alternatively, the assay can be performed using a cell expressing a nuclear hormone receptor in the presence of nuclear hormone receptor ligand. Following exposure to the test agent, the amount of tagged hairless, AFX-1 or Gli3 protein located within the nucleus is measured, *e.g.*, by measuring the amount of tagged protein present in the nucleus. If the amount of tagged protein detected in the nucleus is decreased in the presence of the test agent, as compared to the same assay conducted in the presence of a vehicle control, a modulator of hairless and/or Gli3 nuclear translocation has been identified.

In addition, cells expressing Gli3, AFX-1 or hairless tagged proteins can be used to assay for agents that modulate the dissociation of hairless, AFX-1 and/or Gli3 from the FKBP51/52/hsp90 or CyP40/hsp90 complexes. Such assays can be done in the presence of FK506 or cyclosporin A to identify agents that inhibit the FK506 – or cyclosporin A – mediated dissociation of hairless, AFX-1 and/or Gli3 from said complexes. For example, a cell expressing a tagged hairless, AFX-1 or Gli3 protein is contacted with a test agent in the presence of FK506. Following

contact with the test agent, a cell lysate can be prepared followed by immunoprecipitation of the FKBP51/52 or CyP40 protein complex. The immunoprecipitated complex is then analyzed to determine the presence or absence of the tagged Gli3, AFX-1 or hairless protein.

Downstream target genes of the Gli3 transcription factor are regulated by FK506 and cyclosporin A treatment. For example, BMP4 is a downstream target gene of the Gli3 pathway and expression of BMP4 is stimulated in the presence of FK506 and cyclosporin A. Thus, in a specific embodiment of the invention, constructs containing a Gli3 responsive element, *e.g.*, 5'TGGGTGGTC-3', can be linked to any of a variety of different reporter genes and introduced into cells expressing FKBP51/52. Such reporter genes, as set forth above, can include, but are not limited to, those encoding chloramphenicol acetyltransferase (CAT), luciferase, GUS, growth hormone, or placental alkaline phosphatase. Following exposure of the cells to the test agent, the level of reporter gene expression can be quantitated to determine the test agent's ability to regulate transcription of the reporter gene. In instances where identification of antagonists of FK506 induced transcription is desired, the cells are contacted with both FK506 and the test agent. Alkaline phosphatase assays are particularly useful in the practice of the invention because the enzyme is secreted from the cell. Therefore, tissue culture supernatant can be assayed for secreted alkaline phosphatase. In addition, alkaline phosphatase activity can be measured by calorimetric, bioluminescent or chemilumenscent assays such as those described above.

IMMUNOSUPPRESSIVE ACTIVITY OF TEST AGENTS

The present invention relates to the identification of agents capable of modulating hair growth without the side effect of immunosuppression. Thus, in accordance with the invention, any agents identified as possible modulators of hair growth are also tested for their ability to immunosuppress.

Assays designed to measure the immunosuppressive effect of a test agent include, for example, lymphocyte stimulation assays and assays designed to measure cytokine production, *i.e.*, IL-2 production may be performed. One such assay is conducted as follows.

Spleens are excised from euthanized (CO_2 asphyxiation) adult male C3H mice ranging in age from seven to sixteen weeks old (live mice commercially available from Harlan Sprague Dawley, Inc., Indianapolis, IN). The spleens are placed immediately in cold Hanks Balanced Salt Solution (HBSS, commercially available from Gibco-BRL, Gaithersburg, MD). The spleens are then ground up between frosted glass slides and filtered through a sterile screen to remove tissue debris. The resulting cell suspension is underlayed with an equal volume of Ficoll-Paque Plus (commercially available from Pharmacia Biotech, Piscataway, NJ) and centrifuged at 400 x g for approximately forty minutes at 20°C in order to collect the splenocytes. The splenocytes are collected from the interface using a disposable pipet and are washed twice with HBSS, followed by centrifugation at 100 x g for ten minutes at 20°C. Splenocytes are resuspended in five to ten mL of cell culture media consisting of phenol red-free RPMI 1640 (culture media commercially available from Gibco-BRL) containing 10% heat-inactivated fetal bovine serum (Gibco-BRL), penicillin (50 U/mL), streptomycin (100 µg/mL), L-glutamine (2 mM), 2-mercaptoethanol (10^{-5} M), and N-2 hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) (10 mM). The cells are counted and checked for viability using, for example, trypan blue. Splenocytes are resuspended in medium at 10^6 cells/mL and pipetted into 96 well round bottom plates at 10^5 cells/well. Splenocytes are activated by addition of 50 µL/well of conconavalin A (final assay concentration = 5 µg/ml) in the presence or absence of a test compound. Test compounds are made up as stock solutions in dimethyl sulfoxide (DMSO), then diluted in medium and 50 µL/well added, so that the final concentration of DMSO in the assay is below 0.05%. The plates are incubated at 37°C with 5% CO_2 for 48 hours. The cells are pulsed with 1 µCi/well of methy-^{3}H-thymidine (commercially available from Amersham, Buckinghamshire, England) and incubated an additional 24 hours. The cells are then harvested onto GF/C filter plates (commercially available from Packard, Downers Grove, IL), solubilized in Microscint 20 (Packard), and counted on a TopCount microplate scintillation and luminescence plate counter (Packard). Activity is measured as a percentage of control activity in the absence of test compound and plotted *versus* test compound concentration. The data are fit to a 4-parameter curve fit (Sigmaplot) and IC_{50} values are calculated. As used herein, test compounds are considered non-immunosuppressive if, by using this method, the ratio of (cyclosporin A IC_{50}/test compound IC_{50}) x 100 is less than or equal to 0.02, *i.e.*, as defined herein,

a non-immunosuppressive test compound has # 2% of the immunosuppressive activity of cyclosporin A.

Cell viability is assessed using the MTT (3-[4,5-dimethyl-thiazoyl-2-yl]2,5-diphenyl-tetrazolium bromide) dye assay as described by Nelson et al., *J. Immunol.*, 1993, 150(6):2139 –2147, with the exception that the assay is carried out in serum-free, phenol red-free RPMI 1640 and the dye is solubilized in 100 μL/well DMSO and read at an OD of 540 nm with a background correction at 650 nm on a SpectraMax Plus microplate reader (Molecular Devices, Menlo Park, CA).

Alternatively, animal studies can be performed to determine whether a test agent has an immunosuppressive effect.

AGENTS THAT CAN BE SCREENED IN ACCORDANCE WITH THE INVENTION

The assays described above can identify agents that modulate FKBP51/52 activity. For example, agents that affect FKBP51/52 activity include, but are not limited to, agents that bind to FKBP51/52 and modulate the activity of FKBP51/52. Alternatively, agents can be identified that do not bind directly to FKBP51/52, but are capable of altering FKBP51/52 activity by altering the activity of a protein involved in FKBP51/52 signal transduction. Further, agents that affect FKBP51/52 gene activity (by affecting FKBP51/52 gene expression, including molecules, *e.g.*, proteins or small organic molecules, that affect transcription or interfere with splicing events so that expression of the full length or the truncated form of the FKBP51/52 can be modulated) can be identified using the screens of the invention.

The agents which may be screened in accordance with the invention can include, but are not limited to, small organic or inorganic agents, peptides, antibodies and fragments thereof, and other organic agents (*e.g.*, peptidomimetics) that bind to FKBP51/52 and either mimic the activity triggered by any of the known or unknown substrates of FKBP51/52 (*i.e.*, agonists) or inhibit the activity triggered by any of the known or unknown substrates of FKBP51/52 (*i.e.*, antagonists). Agents that bind to FKBP51/52 and either enhance FKBP51/52 activities (*i.e.*, agonists) or inhibit FKBP51/52 activities (*i.e.*, antagonists), will be identified. Agents that bind to proteins that alter/modulate the activity of FKBP51/52 will be identified.

Agents can include, but are not limited to, peptides such as, for example, soluble peptides, such as members of random peptide libraries (see, *e.g.*, Lam, K.S. et al., 1991, *Nature* 354:82-84; Houghten, R. et al., 1991, *Nature* 354:84-86); and combinatorial chemistry-derived molecular libraries made of D- and/or L-configuration amino acids, phosphopeptides (such as members of random or partially degenerate, directed phosphopeptide libraries); (see, *e.g.*, Songyang, Z. et al., 1993, *Cell* 72:767-778), antibodies (such as polyclonal, monoclonal, humanized, anti-idiotypic, chimeric or single chain antibodies, and FAb, $F(ab')_2$ FV, and FAb expression library fragments, and epitope binding fragments thereof), and small organic or inorganic molecules.

Other agents that can be screened in accordance with the invention include, but are not limited to, small organic molecules that affect the expression of the FKBP51/52 gene or some other gene involved in the FKBP51/52 signal transduction pathway (*e.g.*, by interacting with the regulatory region or transcription factors involved in gene expression); or such agents that affect the activities of the FKBP51/52 or the activity of some other factor involved in modulating FKBP51/52 activity, such as for example, a protein that modifies FKBP51/52 and thereby inactivates FKBP51/52 enzyme activities.

COMPOSITIONS CONTAINING MODULATORS
OF HAIR GROWTH AND THEIR USES

The present invention provides methods of modulating hair growth comprising contacting a cell with an effective amount of a FKBP51/52 or CyP40 modulating agent, such as an FKBP51/52 or CyP40 agonist or antagonist identified using the assays as set forth above. An "effective amount" of the FKBP51/52 or CyP40 inhibitor, *i.e.*, antagonist, is an amount that detectably decreases hair growth. An "effective amount" of the FKBP51/52 or CyP40 activator, *i.e.*, agonist, is an amount that detectably increases hair growth.

The present invention further provides methods of modulating hair growth in a subject in need of such treatment, comprising administering to the subject an effective amount of an agent that modulates FKBP51/52 or CyP40 activity identified as set forth above.

The present invention further provides compositions comprising one or more activators or inhibitors of FKBP51/52 and CyP40 activity. The composition may act directly on FKBP51/52 or CyP40, or alternatively may act on proteins involved in the FKBP51/52 and CyP40 signal transduction pathway.

The present invention further provides pharmaceutical compositions comprising an effective amount of an agent capable of modulating the activity of FKBP51/52, CyP40-, or FKBP51/52, CyP40-mediated signal transduction and/or the expression of FKBP51/52 or CyP40, thereby regulating hair growth, and a pharmaceutically acceptable carrier. In a specific embodiment, the term "pharmaceutically acceptable" means approved by a regulatory agency of the federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopoeia for use in animals, and more particularly in humans. The term

"carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the therapeutic is administered. Examples of suitable pharmaceutical carriers are described in Remington: The Science and Practice of Pharmacy, Gennaro et al. (eds), 20th Edition, Lippincott Williams & Wilkins, Philadelphia, PA (ISBN 0-683-306472).

The invention further provides for the treatment of various disorders associated with hair growth by administration of an agent that regulates the expression or activity of FKBP51/52 or CyP40. Such agents include, but are not limited to, FKBP51/52 or CyP40 agonists and antagonists. Such disorders include, but are not limited to, male pattern baldness, female pattern baldness, toxic baldness, alopecia areata and scarring alopecia. In addition, the agent can be used to treat subjects with hair loss associated with exposure to radiation or chemotherapy.

The agents of the invention are preferably tested *in vitro*, and then *in vivo* in an animal system for a desired therapeutic or prophylactic activity, prior to testing and use in humans. For example, *in vitro* assays that can be used to determine whether administration of a specific therapeutic is indicated include *in vitro* cell culture assays in which cells expressing FKBP51/52 or CyP40 are exposed to or otherwise administered a therapeutic agent, where the effect of such a therapeutic agent on FKBP51/52 or CyP40 is then observed upon FKBP51/52 or CyP40 activity is then observed. In a specific embodiment of the invention the ability of an agent to regulate the signal transduction pathway mediated by CyP40 or FKBP51/52 is assayed.

The invention provides methods of treatment and/or prophylaxis comprising administering to a subject in need thereof an effective amount of a hair growth modulating agent of the invention. In a preferred aspect, the agent is substantially purified. The subject is preferably an animal, more preferably a mammal, and most preferably a human.

Various delivery systems are known and can be used to administer an agent capable of regulating hair growth, *e.g.*, encapsulation in liposomes, microparticles, microcapsules. Methods of introduction include, but are not limited to, intradermal, topical, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal, epidural, and oral routes. The agents may be administered by any convenient route, for example, by infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (*e.g.*, oral mucosa, rectal and intestinal mucosa,

etc.), and may be administered together with other biologically active agents. Administration can be systemic or local, and is preferably adopted for topical applications.

In a specific embodiment, it may be desirable to administer the compositions of the invention locally to a specific area of the body. This may be achieved, for example, and not by way of limitation, by topical application. The active compounds identified according to the methods of the present invention are generally administered in the form of pharmaceutical compositions comprising at least one of such compounds together with a pharmaceutically acceptable vehicle or diluent. Such compositions are generally formulated in a conventional manner utilizing solid or liquid vehicles or diluents as appropriate for topical administration, in the form of solutions, oils, gels, creams, jellies, pastes, lotions, ointments, salves, leave-on and rinse-out hair conditioners, shampoos, aerosols and the like.

Examples of vehicles for application of an active compound identified according to a method of the present invention include an aqueous or water-alcohol solution, an emulsion of the oil-in-water or water-in-oil type, an emulsified gel, or a two-phase system. Preferably, the compositions according to the invention are in the form of lotions, creams, milks, gels, masks, microspheres or nanospheres, or vesicular dispersions. In the case of vesicular dispersions, the lipids of which the vesicles are made can be of the ionic or nonionic type, or a mixture thereof.

Topical compositions containing the active compound can be admixed with a variety of carrier materials well known in the art, such as, for example, water alcohols, aloe vera gel, allantoin, glycerin, vitamin A and E oils, mineral oil, propylene glycol, PPG-2 myristyle propionate, and the like, as well as any of various types of penetration enhancers, viscosity enhancing agents, pH stabilizers, anti-oxidants, preservatives, perfumes, coloring agents, etc.

Other materials suitable for use in topical carriers include, for example, emollients, solvents, humectants, thickeners and powers, as known in the art.

The compositions of the present invention may also optionally comprise other hair growth modulating agents such as potassium channel openers, anti-androgens, thyroid hormones and derivatives and analogs thereof, prostaglandin agonists or antagonists, retinoids, triterpenes, and others known in the art or to be identified.

The amount of the agent of the invention which will be effective in the treatment of a particular disorder will depend on the nature of the disorder and can be determined by standard clinical techniques. *In vitro* assays may optionally be employed to help identify optimal dosage ranges. The precise dose to be employed in the formulation will depend on the route of administration and the nature of the disorder, and should be decided according to the judgment of the medical practitioner and in view of each patient's circumstances. Effective doses can be extrapolated from dose response curves derived from *in vitro* or animal model test systems.

The invention also provides a pharmaceutical pack or kit comprising one or more containers comprising one or more of the ingredients of the pharmaceutical compositions of the invention. Optionally associated with such container(s) can be a notice in a form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration. The kit may further comprise printed instructions or a printed label directing the use of the composition to modulate, *i.e.*, to stimulate or inhibit, hair growth.

The following examples illustrate the preferred modes of making and practicing the present invention but are not meant to limit the scope of the invention since alternative methods may be utilized to obtain similar

Example: FKBP51/52 Mediated Hair Growth

The examples presented below demonstrate that (i) FKBP51 and FKBP52 are selectively expressed in dermal papillae; (ii) FKBP51/52 co-precipitate with hsp90, AFX-1 Gli3 and the hairless protein; and (iii) expression of the BMP4 and HNF3β gene, a Gli3 responsive gene is activated by FK506.

MATERIALS AND METHODS

REVERSE TRANSCRIPTION

A commercial RT-PCR kit (Promega Access RT-PCR kit) was used for detection of FKBP51, FKBP52, and CyP40 mRNA in different human cells. The sequence of primers used for human FKBP51 is: TGAAGAAAGCCCCACAGC (SEQ ID NO:1) (forward primer) and CTCCAAAACCATATCTTGGTCC (SEQ ID NO:2) (reverse primers). Primer sequence for human FKBP52 is: ACATTGCCATAGCCACCA (SEQ ID NO:3) (forward primer) and AGCCAAGACACGATCTTC (SEQ ID NO:4) (reverse primer). Primer sequence for human CyP40 is: TGAAGGAAGGAGATGACGGG (SEQ ID NO:5) (forward primer) and TCCTCAGGGAAATCTGGATGA (SEQ ID NO:6) (reverse primer).

Total RNA was extracted from cultured cells using TRIZOL reagent (Life Technologies) according to manufacturer's instruction. The RNA was treated with DNase to remove potential contamination by genomic DNA and then used in RT-PCR reaction. The PCR reaction product was run on a 2% agarose gel to visualize the amplified product and digested with appropriate restriction enzymes to confirm the products.

TISSUE EXTRACTION

Human skin tissue was extracted using TPER reagent (Pierce, Rockford, IL) at a ratio of 10:1 (extraction buffer:tissue; vol:wt) on ice using a Polytron homogenizer in the presence of protease inhibitors (Protease Inhibitor Cocktail, 1:50 dilution; Sigma, St. Louis, MO) to form a lysate.

COUPLING OF ANTIBODIES TO MAGNETIC RESIN PROTEIN G SEPHAROSE

Antibodies used included anti-hsp90 (monoclonal IgG1 with 1 mg/ml BSA; TL) and anti-FKBP52 (anti-peptide polyclonal N17 and C19 Abs; Santa Cruz Biotechnologies). Antibodies were concentrated and washed 3x with PBS using Amicon Microcon-30. Antibodies were coupled to tosyl-activated Dyna M-450 beads in neutral pH PBS buffer overnight at 37°C. Resins were blocked with Tris-HCl, pH 8 for 4 hours, 37°C.

IMMUNOPRECIPITATION

FK506 was added to the lysate to a final concentration of 1 μM. Antibody complexes were immunoprecipitated in TPER buffer at 6°C overnight. Complexes were washed 10x with Tris/saline (4°C) and sequentially eluted with IM NH_4CO_3 or 20 mM Tris, pH 7.4, 0.3 M NaCl: (5 x 100 μl) and 0.1% TFA, 5% MeOH (5 x 100 μl). Complexes were concentrated and buffer was exchanged (mw 3500) for salt elutions or reduced in volume under vacuum for TFA/MeOH elutions. Samples were stored at -20°C.

PROTEASE DIGESTION

Proteins were suspended in 1 M GnHCl, 100 mM NH_4CO_3, 0.5 mM DTT and subject to digestion with trypsin (Promega) or gluC (Roche) for about 18 hours at 37°C. Digests were desalted using C18 ZipTips (Millipore) eluted with 60% acetonitrile, 0.1% TFA.

MASS SPECTROMETRY ANALYSIS

The peptide digests were analyzed by matrix-assisted laser disorption ionization (MALDI) time of flight (TOF) spectrometry. Essentially, peptides were mixed with matrix (1:1 sample: α-cyano-4-hydroxycinnamic acid; 20 mg/ml-HCCA, 30% acetonitrile, 0.1% TFA) in which the dried droplet method was used. Peptides were analyzed using a Voyager DE-Pro spectrometer (PE BioSystems) in reflector mode (2 m flight length) with a positive ion accelerating voltage of 20 kV, a grid voltage of 12.8 kV, guide wire voltage of 1400 V, using 100 ns delayed extraction. Greater than 64 scans were averaged per spectra. 30 - 50 fmols each of bradykinin and ACTH (amino acid residues 18-39) were used as internal mass standards.

DATABASE SEARCHING

Peptide spectra were compared using ProFound [http://nt2/prowl/prowl.html] or RADARS (OSI internal). Mass error tolerance was typically 30 ppm. One missed cleavage was allowed. Modifications were not initially considered. Both SWISSPROT and GENBANK NR databases were searched.

INDUCTION OF BMP4 AND HNF3β
mRNA BY CYCLOSPORIN A AND FK506 TREATMENT

Primary human dermal fibroblasts (for detectin of BMP4) and skin keratinocytes (for detection of HNF3β) were cultured and treated with 1 uM of cyclosporin A or FK506. Total RNA was harvested at days 2 and 4 of treatment. Real time PCR was used to quantify the BMP4 and HNF3β mRNA level. ABI PRISM 7700 sequence detector, TaqMan PCR kit, and PCR primers designed with commercial software labeled with fluorescence dyes were used for RNA quantification. The results as presented in Figure 3 show that cyclosporin A and FK506 both up-regulate the level of BMP4 and HNF3β mRNA.

In addition, downstream target genes of Gli3 were found to be up regulated by cyclosporin A and FK506 treatment in skin cells such as dermal fibroblasts and skin keratinocytes. As indicated in Figure 3, BMP4 and HNF3β mRNA level were induced by cyclosporin A or FK506 treatment.

INDUCTION OF THYROID HORMONE RECEPTOR (TR)
MEDIATED TRANSCRIPTION BY Fk506 AND CYCLOSPORIN A

Four tandem thyroid hormone response elements (TRE) (AGGTCA CAGG AGGTCA) (underlined sequence is repeated) (SEQ ID NO:7) were synthesized in a single oligonucleotide and ligated 5' of thymidine kinase (TK) promoter using standard procedures. A plasmid (TRE-TK/pUV120puro) was constructed by linking the resulting TRE/TK promoter 5' of the luciferase reporter gene from *H. pyralis*, de Wet Jr. et al, 1986, *Methods Enzymol* 133:3-14, together with a gene encoding a protein conferring resistance to the antibiotic puromycin expressed under the control of the SV40 promoter. In this plasmid the expression of the luciferase gene is under the direct control of the TRE-TK promoter and is inducible by agonists of the thyroid hormone nuclear receptor (TR). HeLa cells (ATCC, Manassas, VA 20108, #CCL-2)

were transfected with plasmid DNA by electroporation using standard procedures, and drug resistant cell lines were selected using puromycin Signa-Aldrich Corp., St. Louis, MI. Drug resistant cell lines (HeLa/TRE) were selected for responsiveness to thyroid hormone, and a single, stably transfected, clonal line was used in subsequent experiments.

Mass spectrometry experiments indicated that hair growth was likely mediated by a protein complex comprising in part hsp90, either cyclophillin 40, FKBP 51 or 52, the zinc finger transcription factor hairless, and a nuclear hormone receptor (thyroid hormone receptor, androgen receptor, vitamin D receptor or glucocorticoid receptor).

In order to establish the role of the thyroid hormone nuclear receptor in immunophillin signaling, cyclosporine A, FK506 and thyroid hormone T3 were evaluated for their ability to increase thyroid hormone nuclear receptor signaling. HeLa/TRE cells were seeded at a density of 10,000 cells per well in 96 well microtiter plates in DMEM culture media containing 1% charcoal stripped FCS, 2mM glutamine and antibiotics (penicillin and streptomycin). The cells were treated with increasing concentrations (20 nM, 2 nM, 200 pM, and 20 pM) of cyclosporin A or FK506) Sigma-Aldrich Corp., St. Louis, MI; (Calbiochem-Nova biocheryl Corp., San Diego, CA) for 16 hours and the activity of the luciferase reporter gene was measured) de Wet Jr. et al, 1986, *Methods Enzymol* 133:3-14.

RESULTS

Dermal Papillar (DP) cells are critical for hair growth. In cultured human dermal papillar cells, CyP40 and FKBP51/52 were detected using RT-PCR (Figure 2). The levels of FKBP51/52 are much greater than those of FKBP 12/13. The levels of FKBP12/13, which mediate the immunosuppressive effect of FK506, was close to the detection limit, indicating that their levels of expression were very low. The expression patterns in human dermal fibroblasts and keratinocytes have also been studied and were found to be identical.

Mass spectrometry analysis of hsp90 and FKBP52 immunoprecipitated complexes derived from human scalp skin reveal the presence of three transcription factors. The factors include the zinc finger protein hairless, the sonic hedgehog stimulated factor Gli-3, and AFX-1.

Data also suggest that in human skin FK506 might alter hairless interaction with hsp90 protein complexes. Hairless may then translocate to the nucleus and stimulate transcription of genes regulating hair growth.

In addition, downstream target genes of Gli3 such as BMP4 and HNF3 were found to be upregulated by FK506 treatment (Figure 3).

Hsp90 and FKBP52 complexes relevant to immunophillin action were identified from human scalp skin extracts using monoclonal antibodies, protease digestion, mass spectrometric measurement of peptide masses and protein database searching. The following proteins were identified from trypsin and/or GluC digests subject to mass spectrometric analysis:

Table 1

Anti-hsp90 and anti-FKBP52 *human skin extract + FK506* 30ppm (SWISSPROT)	Trypsin	GluC
Protein folding, immunophilins, stress-response		
hsp90-beta	x	x
hsp90-alpha	x	x
hsp70s	x	x
FKBP-51	x	x
hsp27	x	x
FKBP-52	x	x
FRAP	x	x
hsp110	x	x
hsp40 protein-3 (DNAJ homolog 1)	x	x
prolyl 4-hydroxylase (alpha and beta subunits)	x	
protein disulfide isomerases	x	x
osmotic stress protein 94 (hsp70 related)	x	x
cyclophilin 40	x	
Steroid-like receptors		
estrogen receptors	x	x
glucocorticoid receptor	x	x
androgen receptor	x	
RXR-beta		x
TRIP-12		x
thyroid hormone receptor	x	x
Protein degradation		
cullin-2	x	x
cullin-3	x	x
ubiquitin carboxy terminal hydrolases	x	x
26S proteosome subunits	x	x
ubiquitin activating enzyme E1		x
Transcription and chromatin remodeling		
human hairless (zfp)	x	x
heat shock factor protein-2 (HSF-2)	x	
GLI-3	x	x
AFX1 putative whn factor (forkhead domain)	x	

The data show that cyclosporin A or FK506 induces thyroid hormone receptor mediated transcription in a dose dependent manner (Figure 4). Binding of ligand to cyclosphilin 40 (cyclosporin A) or FKBP51/52 (FK506) proteins in the hsp90

complex clearly activates the transcriptional activity of thyroid hormone receptor. The hairless protein, an accessory protein of thyroid hormone receptor, also can modulate cyclosporin A or FK506 action and thereby regulate hair growth.

Equivalents

The present invention is not to be limited in scope by the specific embodiments described herein which are intended as single illustrations of individual aspects of the invention, and functionally equivalent methods and components are within the scope of the invention. Indeed, various modifications of the invention, in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Various publications are cited herein, the contents of which are hereby incorporated, by reference, in their entireties.

WE CLAIM:

1. A method for identifying a compound capable of modulating hair growth comprising:

(v) contacting a cell that expresses, or a preparation containing, FKBP51/52 or CyP40, hsp90 and a protein selected from the group consisting of hairless, AFX-1, and gli3, with a test compound;

(vi) determining the level of complex formation between FKBP51/52 or CyP40, hsp90 and at least one protein selected from the group consisting of the hairless, AFX-1, and gli3 in the cell or preparation, contacted with the test compound; and

(vii) comparing the level of complex formation obtained in (ii) to the level of complex formation between FKBP51/52 or CyP40, hsp90, and at least one protein selected from the group consisting of the hairless, AFX-1, and gli3, in the absence of test compound;

wherein a difference in the level of complex formation in the presence versus in the absence of test compound has a positive correlation with hair growth modulating activity.

2. The method of claim 1, wherein the level of complex formation is detected using an immunoassay.

3. The method of claim 1, further comprising the step of determining whether the test compound has immunosuppressive activity, where the absence of such activity, in the presence of a test compound that alters the level of complex formation has a positive correlation with usefulness as a hair growth modulating agent.

4. A method for identifying a compound capable of modulating hair growth comprising:

(i) contacting a cell that expresses a nuclear hormone receptor and a reporter gene under the transcriptional control of a *hairless*, AFX-1, or gli3 gene responsive element with a test compound and measuring the level of reporter gene expression in the cell;

(ii) measuring the level of reporter gene expression in the absence of the test compound; and

(iii) comparing the levels of reporter gene expression measured in (i) and (ii);

wherein a difference in the levels of reporter gene expression measured in steps (i) and (ii) has a positive correlation with hair growth modulating activity of the test compound.

5. The method of claim 4, wherein step (i) is performed in the presence of a nuclear hormone receptor ligand.

6. The method of claim 4, further comprising the step of determining whether the test compound has immunosuppressive activity where the absence of such activity, in the presence of a test compound that alters the level of reporter gene expression, has a positive correlation with usefulness as a hair growth modulating agent.

7. The method of claim 4, wherein the nuclear hormone receptor is selected from the group consisting of an androgen nuclear hormone receptor, vitamin D nuclear hormone receptor, retinoic acid nuclear hormone receptor, aryl hydrocarbon nuclear hormone receptor, and thyroid stimulating nuclear hormone receptor.

8. A method for identifying a compound capable of promoting hair growth comprising:

(i) contacting a sample containing a *hairless* gene product with a test compound;

(ii) determining whether the test compound binds to the *hairless* gene product; and

(iii) determining whether the test compound inhibits complex formation between the *hairless* gene product and a binding partner selected from the group consisting of FKBP51/52, CyP40, a nuclear hormone receptor, a hsp90 protein, and a combination thereof;

wherein the ability of a test compound to both bind to the *hairless* gene product and inhibit complex formation has a positive correlation with hair growth promoting activity.

9. A method for identifying a compound capable of modulating hair growth comprising:

(i) contacting a cell that expresses FKBP51/52 or CyP40, and hsp90, a nuclear hormone receptor, and the *hairless* gene product, with a test compound in the presence of a nuclear hormone receptor ligand;

(ii) determining the level of nuclear translocation of the *hairless* gene product into the nucleus of the cell;

(iii) determining the level of nuclear translocation in the absence of the test compound; and

(iv) comparing the level of nuclear translocation measured in (ii) and (iii);

wherein a difference in the level of nuclear translocation measured in steps (ii) and (iii) has a positive correlation with hair growth modulating activity of the test compound.

10. The method of claim 8 or 9, further comprising the step of determining whether the test compound has immunosuppressive activity.

11. A method for identifying a compound capable of promoting hair growth comprising:

(i) contacting a sample containing an AFX-1 gene product with a test compound;

(ii) determining whether the test compound binds to the AFX-1 gene product; and

(iii) determining whether the test compound inhibits complex formation between the AFX-1 gene product and a binding partner selected from the group consisting of FKBP51/52, CyP40, a nuclear hormone receptor, a hsp90 protein, and a combination thereof;

wherein the ability of a test compound to both bind to the AFX-1 gene product and inhibit complex formation has a positive correlation with hair growth promoting activity.

12. A method for identifying a compound capable of modulating hair growth comprising:

(i) contacting a cell that expresses FKBP51/52 or CyP40, and hsp90, a nuclear hormone receptor, and the AFX-1 gene product, with a test compound in the presence of a nuclear hormone receptor ligand;

(ii) determining the level of nuclear translocation of the AFX-1 gene product into the nucleus of the cell;

(iii) determining the level of nuclear translocation in the absence of the test compound; and

(iv) comparing the level of nuclear translocation measured in (ii) and (iii);

wherein a difference in the level of nuclear translocation measured in steps (ii) and (iii) has a positive correlation with hair growth modulating activity of the test compound.

13. The method of claim 11 or 12, further comprising the step of determining whether the test compound has immunosuppressive activity.

14. A method for identifying a compound capable of promoting hair growth comprising:

(i) contacting a sample containing a gli3 gene product with a test compound;

(ii) determining whether the test compound binds to the gli3 gene product; and

(iii) determining whether the test compound inhibits complex formation between the gli3 gene product and a binding partner selected from the group consisting of FKBP51/52, CyP40, a nuclear hormone receptor, a hsp90 protein, and a combination thereof;

wherein the ability of a test compound to both bind to the gli3 gene product and inhibit complex formation has a positive correlation with hair growth promoting activity.

15. A method for identifying a compound capable of modulating hair growth comprising:

(i) contacting a cell that expresses FKBP51/52 or CyP40, and hsp90, a nuclear hormone receptor, and the gli3 gene product, with a test compound in the presence of a nuclear hormone receptor ligand;

(ii) determining the level of nuclear translocation of the gli3 gene product into the nucleus of the cell;

(iii) determining the level of nuclear translocation in the absence of the test compound; and

(iv) comparing the level of nuclear translocation measured in (ii) and (iii);

wherein a difference in the level of nuclear translocation measured in steps (ii) and (iii) has a positive correlation with hair growth modulating activity of the test compound.

16. The method of claim 14 or 15, further comprising the step of determining whether the test compound has immunosuppressive activity.

17. A method for modulating hair growth in a mammal, comprising administering to the mammal a compound that modulates complex formation between FKBP51/52, CyP40, or hsp90 and at least one protein selected from the group consisting of hairless, gli3, and AFX-1.

18. A method for modulating hair growth in a mammal comprising administering to the mammal a compound that modulates the nuclear translocation of a protein selected from the group consisting of the hairless, AFX-1, and gli3 protein.

19. A method for modulating hair growth in a mammal comprising administering to the mammal a compound that modulates AFX-1-or gli-3-mediated gene expression.

20. A method for modulating hair growth in a mammal, comprising administering to the mammal a compound identified by the assay of claim 1.

21. A method for modulating hair growth in a mammal, comprising administering to the mammal a compound identified by the assay of claim 4.

22. A method for modulating hair growth in a mammal, comprising administering to the mammal a compound identified by the assay of claim 8.

23. A method for modulating hair growth in a mammal, comprising administering to the mammal a compound identified by the assay of claim 9.

24. A method for modulating hair growth in a mammal, comprising administering to the mammal a compound identified by the assay of claim 11.

25. A method for modulating hair growth in a mammal, comprising administering to the mammal a compound identified by the assay of claim 12.

26. A method for modulating hair growth in a mammal, comprising administering to the mammal a compound identified by the assay of claim 14.

27. A method for modulating hair growth in a mammal, comprising administering to the mammal a compound identified by the assay of claim 15.

5

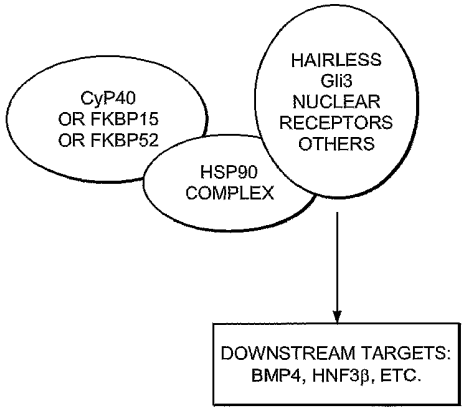

FIG. 1

GENES	TISSUES		
	DERMAL FIBROBLAST	DERMAL PAPILLA	KERATINOCYTE
FKBP52	+	+	+
FKBP51	+	+	+
Cyp 40	+	+	+

FIG. 2

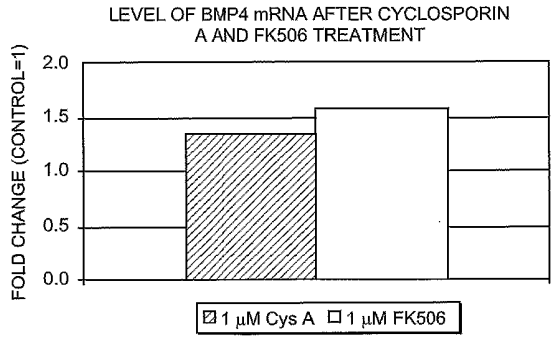
LEVEL OF BMP4 mRNA AFTER CYCLOSPORIN A AND FK506 TREATMENT
FOLD CHANGE (CONTROL=1)
2.0
1.5
1.0
0.5
0.0
1 μM Cys A
1 μM FK506

FIG. 3A

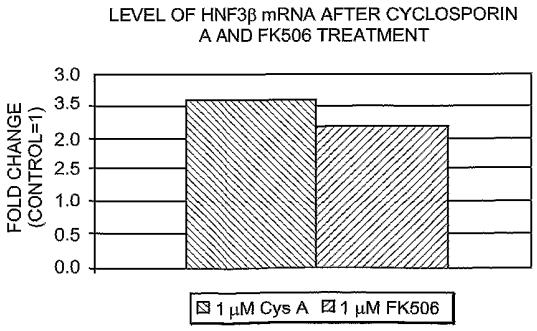
LEVEL OF HNF3β mRNA AFTER CYCLOSPORIN A AND FK506 TREATMENT
FOLD CHANGE (CONTROL=1)
3.0
3.5
2.0
2.5
1.0
0.5
0.0
1 μM Cys A
1 μM FK506

FIG. 3B

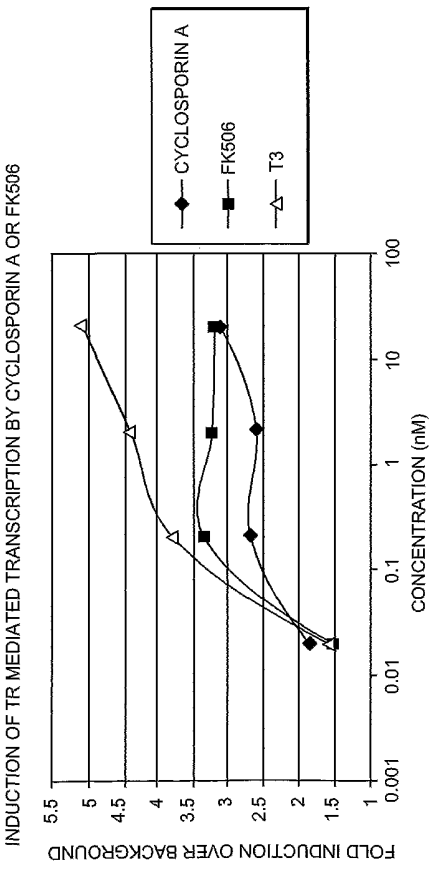
INDUCTION OF TR MEDIATED TRANSCRIPTION BY CYCLOSPORIN A OR FK506
CYCLOSPORIN A
FK506
T3
FOLD INDUCTION OVER BACKGROUND
5.5
5
4.5
4
3.5
3
2.5
2
1.5
1
0.001
0.01
0.1
1
10
100
CONCENTRATION (nM)

FIG. 4

【国際調査報告】

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US02/19948

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) : C12Q 1/00; A61K 39/00
US CL : 435/4; 424/184.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 435/4; 424/184.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
DIALOG EMBASE LIFESCI WPID MEDLINE BIOSIS WEST

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,763,590 A (PEATTIE ET AL) 9 June 1998 (09.06.1998), see entire document.	1-27

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 August 2002 (21.08.2002)	21 OCT 2002
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703)305-3230	Authorized officer Patrick J. Nolan Telephone No. 703-308-0196

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QS02 QS05 QS38 QX10
4C084 AA17 NA14 ZA922

专利名称(译)	由FKBP 51/52和CYP 40介导的哺乳动物毛发生长		
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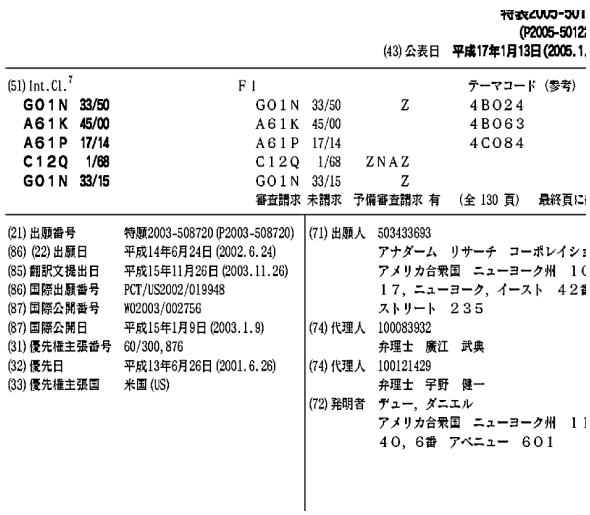
CPC分类号	A61P17/14 G01N33/5008 G01N33/502 G01N33/6881 G01N2800/20		
FI分类号	G01N33/50.Z A61K45/00 A61P17/14 C12Q1/68.ZNA.Z G01N33/15.Z G01N33/53.D G01N33/53.M G01N33/566 C12N15/00.A		
F-TERM分类号	4B024/AA11 4B024/BA08 4B024/BA63 4B024/BA80 4B024/DA03 4B024/EA04 4B024/GA14 4B024/GA27 4B063/QA08 4B063/QQ08 4B063/QQ20 4B063/QQ44 4B063/QQ79 4B063/QQ94 4B063/QR33 4B063/QR48 4B063/QR77 4B063/QR80 4B063/QS02 4B063/QS05 4B063/QS38 4B063/QX10 4C084/AA17 4C084/NA14 4C084/ZA922		
代理人(译)	宇野健一		
优先权	60/300876 2001-06-26 US		
外部链接	Espacenet		

摘要(译)

本发明涉及药物筛选试验，所述药物筛选试验设计为鉴定调节头发生长的非免疫抑制剂以及此类药物在调节头发生长中的用途。



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(43)公表日 平成17年1月13日(2005.1.13)

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A61K 45/00	A61K 45/00		4B063
A61P 17/14	A61P 17/14		4C084
C12Q 1/68	C12Q 1/68	ZNAZ	
G01N 33/15	G01N 33/15	Z	

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